

puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*

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The activation of MAPKs is controlled by the balance between MAPK kinase and MAPK phosphatase activities. The latter is mediated by a subset of phosphatases with dual specificity (VH-1 family). Here, we describe a new member of this family encoded by the *puckered* gene of *Drosophila*. Mutations in this gene lead to cytoskeletal defects that result in a failure in dorsal closure related to those associated with mutations in *basket*, the *Drosophila* JNK homolog. We show that *puckered* mutations result in the hyperactivation of DJNK, and that overexpression of *puc* mimics *basket* mutant phenotypes. We also show that *puckered* expression is itself a consequence of the activity of the JNK pathway and that during dorsal closure, JNK signaling has a dual role: to activate an effector, encoded by *decapentaplegic*, and an element of negative feedback regulation encoded by *puckered*.

[Key Words: *puckered*; JNK; *Drosophila*; dpp; Phosphatase; Signal transduction; dorsal closure]

Received September 26, 1997; revised version accepted November 18, 1997.

In many cases, cell differentiation represents a transition between two states of cellular activity—one in which cells proliferate and acquire information about their fates and identities, and another in which cells stop dividing and manifest the information gathered earlier. Many of the signaling pathways leading to cell differentiation depend on phosphorylation cascades. Mounting evidence points to signaling through MAP kinase (MAPK) pathways as a key component in this transition. Three distinct types of MAPK pathways have been identified: p42–p44 ERKs (extracellular signal-related kinases), p38 kinases, and p46–p54 JNKs (Jun N-terminal kinases). These major subfamilies transduce signals from different stimuli. The ERKs respond predominantly to growth factors and hormones and are activated in a Ras-dependent manner. The p38 and JNKs respond to different environmental stresses and are activated preferentially downstream of Rac1 and Cdc42 small G proteins (for review, see Canman and Kastan 1996). In most cases, MAPK activation is a transient event, even in the continuing presence of the stimulus that leads to its activation. MAPK activity is controlled

by the balance of MAPK kinase and MAPK phosphatase activities.

The dorsal closure of the *Drosophila* embryo provides an example of cell differentiation and how this is usually coupled to morphogenetic events and movements that shape late stages in development. Half way through embryogenesis, the dorsal surface of the embryo is covered by an extraembryonic membrane, the amnioserosa, which contacts the epidermis. After proliferation stops, the epidermis stretches dorsally and, as it encroaches the amnioserosa, closes the existing gap. Three phases lead to the successful completion of this event. The dorsalward movement of the epidermal cells, an anteroposterior stretching of the embryo and the seaming of the dorsal epidermis (Martinez-Arias 1993). The completion of this process takes several hours and is associated with specialized behavior of the dorsal-most epidermal cells. These cells display planar polarity reflected in the arrangement of the cytoskeleton, which is essential for the normal process of dorsal closure (Ring and Martinez-Arias 1993; Young et al. 1993).

There are several mutations that disrupt the process of dorsal closure. In *basket* (*bsk*) and *hemipterous* (*hep*) mutants, dorsal closure fails and the embryo exhibits a hole in the dorsal cuticle. *hep* encodes a *Drosophila* homolog of MKK7, a kinase that regulates JNK MAPKs (Holland et al. 1997; Tournier et al. 1997) and *bsk* en-

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codes a *Drosophila* homolog of JNK (Riesgo-Escovar et al. 1996; Sluss et al. 1996). The involvement of the JNK pathway in dorsal closure is further emphasized by the observation that mutants for *Djun*, a target of DJNK signaling, fail to close dorsally (Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997), and that ectopic expression of a dominant-negative form of Drac1 (DN-Drac1), the *Drosophila* homolog of Rac1, also leads to the same dorsal closure defects (Harden et al. 1995).

Here, we describe the molecular characterization of the *puckered* (*puc*) gene. In *puc* mutants, dorsal closure takes place, but an abnormal organization of the cells at the leading edge of the epidermis results in a defective process and puckering, which provides the name of the mutant (Ring and Martinez-Arias 1993). We show that *puc* encodes a member of the MAPK subfamily (MKPs) of the VH1 like dual specificity phosphatases. Our results indicate that *puc* regulates signaling through the JNK pathway and participates in a negative feedback loop leading to a transient activity of the JNK signal during dorsal closure.

Results

Molecular characterization of *puc*

The *puc* gene was identified through a P(*lacZ*) insertional mutation that highlights the most dorsal epidermal cells as they finish proliferation, and causes defects during dorsal closure (Ring and Martinez-Arias 1993). The *puc*^{E69} allele is caused by a single insertion at 84E. Genomic DNA from the region around the insertion point was isolated with a probe for *lacZ* against a λ library constructed with *puc*^{E69} genomic DNA. A fragment of the resultant λ clone, containing only genomic DNA sequences, was then used as a probe to screen a wild-type genomic library to isolate DNA from a larger genomic region. The isolated clones were aligned and mapped by restriction analysis (Fig. 1A; Materials and Methods).

A 3.7-kb genomic DNA fragment close to the P-element insertion site in *puc*^{E69} was used to screen a 12–24 hr embryonic cDNA library (Brown and Kafatos 1988). Three cDNA clones were isolated and mapped onto the genomic region (Fig. 1A; Material and Methods). The longest, cDNA12, is 2.3 kb long and contains an ORF capable of encoding a protein of 496 amino acids with a predicted molecular mass of 57.6 kD (Fig. 1B). Northern blot analysis of embryonic RNA detects a 2.9-kb RNA present throughout embryogenesis (see Fig. 4A, below).

The *puc*^{E69} insertion and three additional lethal P-elements insertions, *puc*³²⁰, *puc*^{A251.1}, and *puc*^{B48}, which do not complement *puc*^{E69}, have been identified. *puc*³²⁰, *puc*^{A251.1}, and *puc*^{E69} map to the second intron of the cDNA (Fig. 1A). The mutations caused by *puc*^{E69} and *puc*³²⁰ insertions have been reverted to wild type with loss of the resident P element, which suggests that these P elements are the cause of the *puc* mutation (Ring 1993). The *puc*^{B48} P-element insertion site has been sequenced and maps to the first intron of *puc*. RT-PCR of

single embryos detects *puc* transcripts in wild type, but not in extracts from homozygous *puc*^{B48} or *puc*^{B48R23}, a lethal revertant of *puc*^{B48} that removes the genomic region adjacent to the insertion (data not shown).

The *Puc* ORF encodes a protein tyrosine phosphatase, with a catalytic domain between amino acids 214 and 226 that includes the invariant cysteine known to be required for phosphatase activity (Guan et al. 1991). The protein contains eight putative sites for phosphorylation by MAPK, distributed throughout the carboxy-terminal part of the protein (Fig. 1B). The predicted protein contains no clear hydrophobic sequences indicative of either signal sequence or transmembrane domain, which suggests that the *Puc* protein is neither a secreted nor an integral membrane protein (Fig. 1B).

The phosphatase encoded by *puc* has high similarity to nonreceptor dual specificity phosphatases of the VH-1 subfamily (Fig. 2A). Phylogenetic analysis (see Materials and Methods), indicates that its closest relative is the protein encoded by the *Caenorhabditis elegans* CEL-F08B1 ORF (Wilson et al. 1994) that has 38% identical residues over 158 amino acid overlap. When conservative residues are taken into account, the comparison yields 59.5% similarity between the two sequences. Very high similarities with other proteins of this family (see Fig. 2) highlight the conservation of their catalytic sites, which are identical at 9–11 of 13 amino acids. Interestingly, *Puc*, like yeast MSG-5, lacks the amino-terminal domains with homology to the *cdc25* proteins that are present in all the mammalian MKPs (Keyse and Ginsburg 1993). A single copy of an internally repeated domain of unknown function (amino acids 238–312; amino acids 386–460) (Fig. 2B) is present in all VH-1 family phosphatases.

Puckered encodes a *Drosophila* JNK phosphatase

To establish whether *Puc* is a phosphatase, we first determined its enzymatic activity towards *p*-nitrophenyl phosphate (PNPP), a chromogenic substrate structurally related to phosphotyrosine (Keyse and Emslie 1992). The first 424 amino acids of *Puc*, including the phosphatase domain, were fused in-frame to GST. Variable amounts of the GST-*Puc* protein were added to reactions containing PNPP and cleavage was analyzed spectrophotometrically (Fig. 3A). PNPP cleavage was dependent on the addition of the GST-*Puc* fusion protein and increased linearly with the added protein. These results indicate that *Puc* functions as a protein phosphatase.

Members of the VH-1 family of phosphatases have been implicated in the down regulation of MAPK activity (for review, see Keyse 1995). To study if *Puc* could have such function, we measured the endogenous MAPK activity of embryo extracts, prepared in the presence of phosphatase inhibitors (see Materials and Methods). Extracts prepared from *puc* mutant embryos showed a two-fold increase in JNK activity relative to wild type by use of (1–86) cJun-GST captured on glutathione-Sepharose beads, whereas ERK activity on myelin basic protein

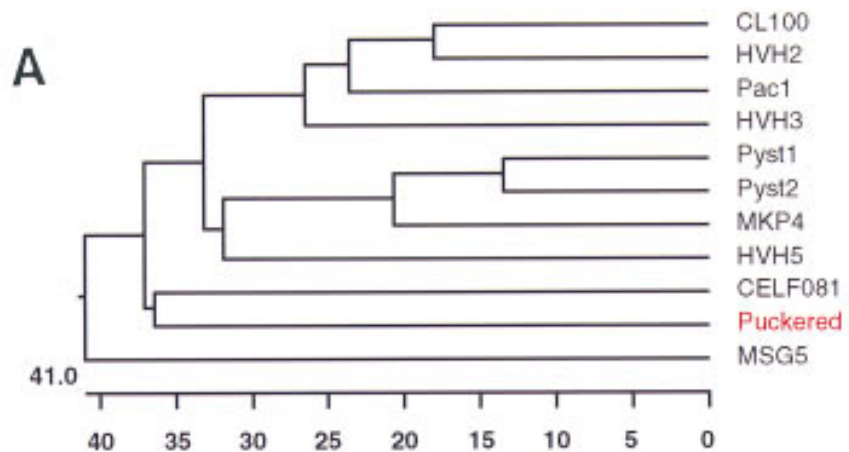
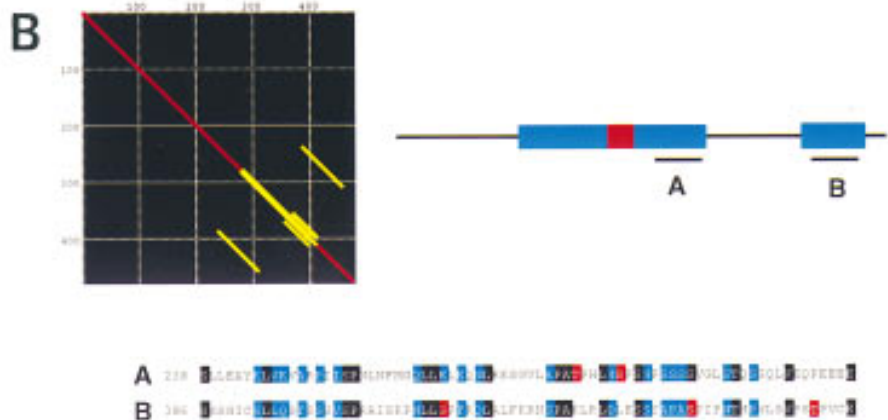
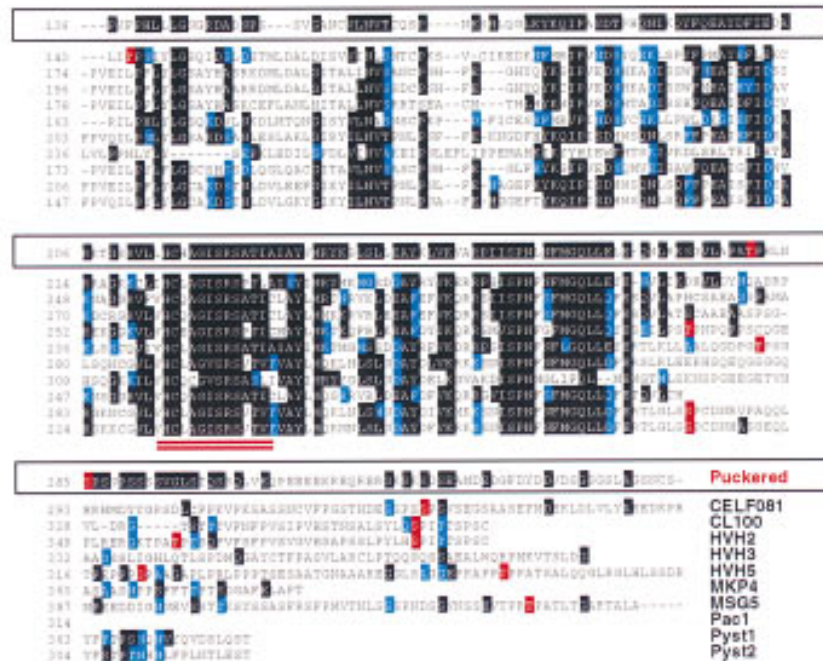


Figure 2. Homology of Puc to VH-1 family phosphatases. (A) Sequence alignment (ClustalV) of Puc and other VH-1 family phosphatases [*Drosophila* Puc; human CL100 (Keyse and Emslie 1992); human Pac1 (Rohan et al. 1993); human HVH-2 (Guan and Butch 1995); human HVH-3 (Ishibashi et al. 1994; Kwak and Dixon 1995); human HVH-5 (Martell et al. 1995); human Pyst1 (Groom et al. 1996); human Pyst2 (Groom et al. 1996); human MKP-4 (Muda et al. 1997); *C. elegans* CEL-F08B1 (Wilson et al. 1994) and *Saccharomyces cerevisiae* MSG5 (Doi et al. 1994)]. Identical residues are in black, conservative changes in blue. CEL-F08B1, Pyst1 and homologs, Pyst2, and HVH-5 gave the highest homology scores to Puc in BLAST/BEAUTY searches (BCM Launcher). The other enzymes complete the whole series of distinct human dual phosphatases isolated so far. Yeast MSG-5, which share some characteristics with Puc, is also included. Phylogenetic trees (DNASTar program) point to the *C. elegans* CEL-F08B1 as the closest relative of Puc in the databases. CEL-F08B1 has been identified recently in the *C. elegans* Genome Project, but its function is unknown. Residues in the alignment highlighted in red represent putative MAPK phosphorylation sites. Interestingly, they seem to cluster for almost every protein in a low homology region at the carboxy-terminal end of the catalytic domain (double underlined), which suggest a possible functional homology. (B) Matrix alignment of Puc with itself shows the existence of an internal repeat in the protein. These domains correspond to the putative MAPK phosphorylated region and a further sequence close to the carboxy-terminal end of the molecule. Again, in the second repeat, several tentative phosphorylation sites can be identified.



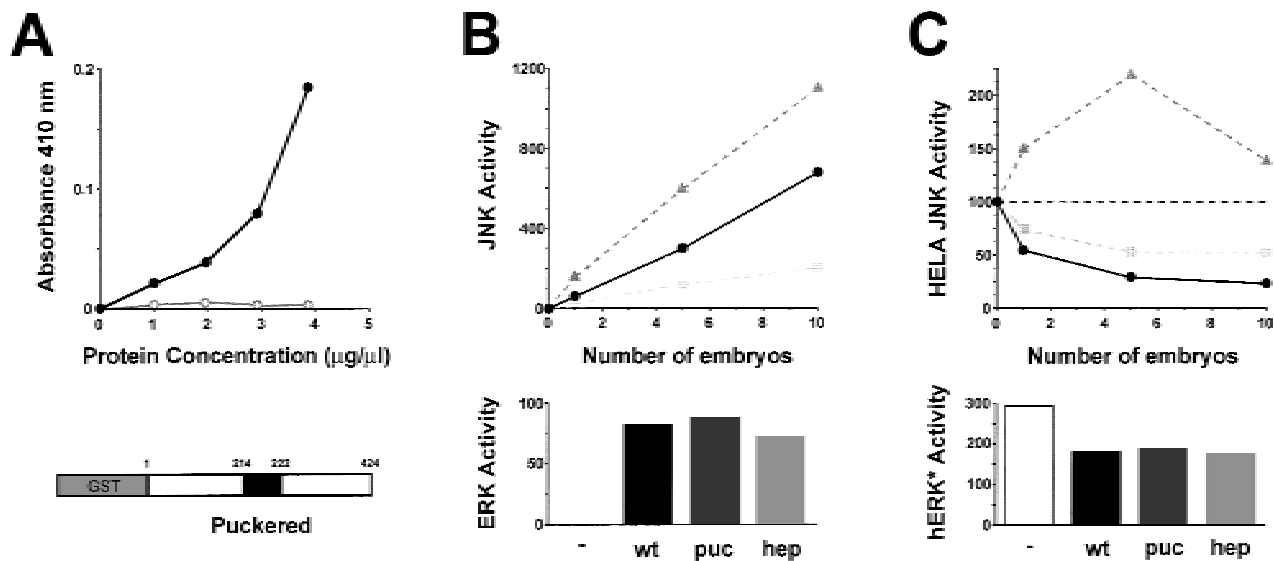


Figure 3. *puc* encodes a JNK phosphatase. (A) In vitro phosphatase activity of a GST-Puc fusion protein. Results of PNPP assay in which cleavage of PNPP was measured by change in absorbance at 410 nm as a function of added protein. (●) Purified GST-Puc protein; (○) control points from extracts obtained from untransformed bacteria following similar protocols. (Bottom) Schematic representation of the fusion protein. The phosphatase catalytic domain is highlighted in black (residues 214–222). (B) Endogenous JNK and ERK activity of wild-type (wt), *puc*^{E69}/*puc*^{E69} (*puc*) and *hep*¹/*hep*¹ (*hep*) embryos. (Top) JNK assays were performed with normalized amounts of embryo extracts (1 embryo/ μl) prepared in the presence of phosphatase inhibitors (as indicated in Materials and Methods). Kinase activity is measured in arbitrary units from imaging analysis. (Solid circles) Wild-type (wt) extracts; (shaded triangles) *puc* embryo extracts; (shaded squares) *hep* embryos. JNK activity increases twofold in *puc* mutants and reduced threefold in *hep*. (Bottom) ERK assays were performed by in-gel kinase assay with a normalized amount of extract, in the linear range for JNK, equivalent to five embryos. Histograms represent quantitation of kinase activity (arbitrary units). Wild-type, *puc*, and *hep* extracts have equivalent levels of ERK activity. (C) Puc phosphatase activity on heterologous JNK and ERK. (Top) JNK activity induced in HeLa cells was measured in the absence of any extract to deduce the basal level of activity (100% JNK activity—broken line). Equivalent amounts were incubated with normalized embryo extracts (1 embryo/ μl) prepared in the absence of phosphatase inhibitors. The results are expressed in percentage of JNK activity. (Solid circles) Wild-type extracts; (shaded triangles) *puc* embryo extracts; (shaded squares) *hep* embryos. Wild-type embryos have high levels of JNK phosphatase activity (HeLa JNK activity is reduced fivefold). *Puc* extracts do not show JNK phosphatase activity, indeed HeLa JNK activity gets increased because of the high levels of JNK activity of *puc* extracts (it can be brought back to basal levels by previous heat inactivation; see also Discussion). In *hep* extracts, JNK phosphatase activity is reduced to 50% of that of wild-type embryos. (Bottom) ERK activity of preactivated human ERK (hERK*) was assayed as indicated in Materials and Methods. Extracts (5 embryos) from wild-type, *puc*, and *hep* embryos display the same level of ERK phosphatase activity, reducing hERK* activity by 40%. Histograms represent quantitation of kinase activity (arbitrary units). Positive controls were performed with purified CL100 phosphatase (50 $\mu\text{g}/\text{ml}$) (data not shown).

Extracts derived from wild-type *Drosophila* embryos were able to inhibit HeLa JNK activity up to 80%. In contrast, extracts from *puc* mutants were unable to repress exogenous JNK and a gain of kinase activity was observed (Fig. 3C), probably caused by a feedback loop between JNK and *puc* (see below). ERK phosphatase activity measured in these extracts was unaffected in *puc* mutants (Fig. 3C). These results strongly suggest that *puc* encodes a protein capable of regulating JNK, but not ERK, activity through dephosphorylation.

If *puc* encodes a JNK phosphatase activity, then it should inactivate JNK in vivo. To test if this is the case, we placed the *puc* cDNA under the control of Gal4/UAS to target its expression during development (see Materials and Methods). Ubiquitous expression of Puc results in a dorsal hole during embryogenesis (see Fig. 5, below) reminiscent of the phenotype of mutations in *hep* and *bsk* that encode a JNKK and a JNK, respectively.

These results suggest that the phosphatase encoded by

Puc act as a JNK phosphatase in vivo and might play a role in the regulation of the activity of JNK signaling during dorsal closure.

The expression and activity of Puc during embryonic development are regulated by the Drosophila JNK pathway

The temporal pattern of *puc* expression during embryogenesis was determined by use of a *puc* cDNA probe. Northern analysis detects a 2.9-kb RNA present throughout embryogenesis (Fig. 4A). This RNA was apparent in early embryos (0–4 hr AEL) suggesting the presence of maternal transcripts. Minor transcripts of 2.6 and 2.4 kb are also detected.

The spatial distribution of *puc* was determined by whole mount in situ hybridization. *puc* expression was detected, by use of a variety of genomic and cDNA derived probes, only in a small number of the experiments,

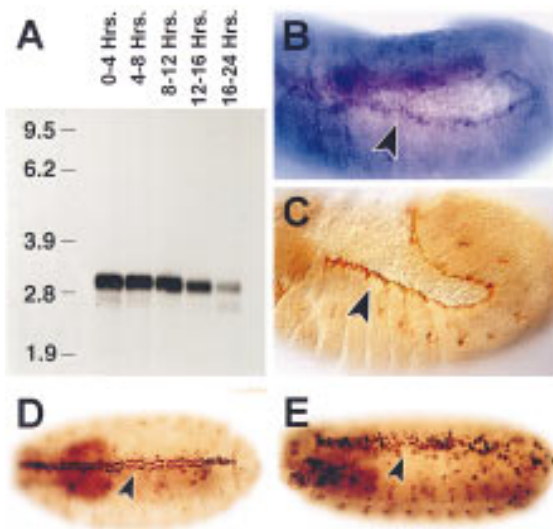


Figure 4. *puc* expression: Its modulation by *puc* activity. (A) Northern analysis of *puc* RNA expression in embryos at various times during development. (A) 0–4 hr; (B) 4–8 hr; (C) 8–12 hr; (D) 12–16 hr; and (E) 16–20 hr. The 2.9-kb *puc* transcript is apparent. (B) *puc* RNA detected in stage 13 by whole mount in situ hybridization. The expression in the dorsal-most epidermal cells is indicated. (C) stage 13 *puc^{E69}* heterozygous embryos stained with an antibody against β -galactosidase. The arrowhead points to the cells of the leading edge of the epidermis expressing β -gal. Notice that these are the same cells as in B. At early stages, evident *puc* expression is present in amnioserosa cells. (D) β -galactosidase expression of *puc³²⁰* heterozygous embryos (stage 14). (E) A considerably higher number of cells, and at higher levels, express β -galactosidase in *puc³²⁰/puc³²⁰* stage 15 embryos (arrowhead).

which suggests a very low level of expression or transcript instability. We were able to observe expression of *puc* in the dorsal-most cells at the leading edge of the epidermis (Fig. 4B). This pattern was identical to that described previously for the early expression of β -galactosidase in the insertional alleles (cf. Fig. 4B and C). After stage 11, *puc* mRNA expression slowly decays on the leading edge, whereas β -galactosidase is found up to completion of dorsal closure (Ring and Martínez-Arias 1993).

It has been shown previously that β -galactosidase expression by the *puc^{E69}* insertion is abolished in *hep* and *bsk* mutants (Glise et al. 1995; Riesgo-Escovar et al. 1996). Furthermore, β -galactosidase activity is enhanced after overexpression of activated forms of Drac1 (DRac1V12) and Dcdc42 (Dcdc42V12) (Glise and Noselli 1997) that activate JNK signaling. As *puc* encodes a JNK phosphatase that appears to down-regulate the JNK pathway, we studied the expression of *puc* (β -galactosidase) in *puc* mutants, in which we have shown that JNK activity is enhanced. The misregulation of *puc^{E69}* LacZ activity had been reported previously (Ring and Martínez-Arias 1993; Glise and Noselli 1997). We found that for all *puc* insertional alleles, mutant embryos show higher levels and more cells expressing β -galactosidase than wild type, for example, in the leading edge of the epidermis,

the amnioserosa, the ectoderm, and the nervous system (*puc³²⁰*; Fig. 4D,E).

The requirement for the DJNK pathway to activate *puc* expression was also mirrored in the reduction of JNK phosphatase activity in mutants for *hep*: JNK activity is reduced threefold in *hep¹* mutants extracts, as expected for a loss of function in a JNK activator (Fig. 3B). In addition, extracts from *hep¹* mutants show less phosphatase activity on exogenous JNK (a 50% reduction), but identical levels of ERK phosphatase function, compared with wild-type embryo extracts (Fig. 3C).

Altogether, these results support a role for the *Drosophila* JNK pathway in the control of *puc* expression.

Loss and overexpression of *puc* have different effects in dorsal closure

Embryos mutant for *puc* develop defects along the dorsal midline of the larval cuticle during dorsal closure. These defects manifest as misaligned segments in the weakest allele, *puc^{Ex}* (Ring and Martínez-Arias 1993) (Fig. 5A), and become more pronounced in stronger alleles. For example, in embryos carrying the *puc^{E69}* allele (Fig. 5B), dorsal hairs are absent along the midline, leaving a strip of naked cuticle along most of the midline; these embryos display strong puckering of the epidermis (Ring and Martínez-Arias 1993).

To study if *puc* restrains JNK activity during the different steps of dorsal closure, we analyzed the phenotype of embryos in which we had expressed *puc* ectopically using hsGal4 and a UASpuc line (Fig. 5). Embryos heat-shocked very early [between 4 and 5 hr after egg laying (AEL)] failed to achieve dorsal closure and exhibit a large dorsal opening (Fig. 5F). Most of the embryos heat-shocked between 5 and 7 hr AEL displayed dorsal holes (Fig. 5E) or phenotypes similar to *puc* loss of function alleles (Fig. 5D). Heat-shocking embryos after 7 hr AEL produced a *puc* loss-of-function phenotype, and occasionally a dorsal hole that, on average, was smaller than that of embryos heat-shocked earlier.

In these experiments, early overexpression of *Puc* mimics the phenotype of *bsk* mutants and the complete inactivation of DJNK signaling. Late expression of *Puc* affects the ability of the dorsal-most cells to differentiate properly and induces the same defects as *puc* loss-of-function alleles. Furthermore, *puc* in heterozygous condition can rescue the dorsal open characteristic of low JNK signaling: Hemizygous *hep¹* embryos develop a dorsal open phenotype (Fig. 5G), which is partially rescued by *puc^{E69}* (Fig. 5H) or *puc^{A251.1}* (data not shown) in heterozygous condition.

These results support a role of *puc* in limiting DJNK activity during dorsal closure and suggest the existence of a feedback loop through which JNK dependent expression of *puc* regulates JNK activity (see Discussion).

puckered *alters actin and nonmuscle myosin localization and affects epidermal morphogenesis*

During dorsal closure, the shape of the epidermal cells in

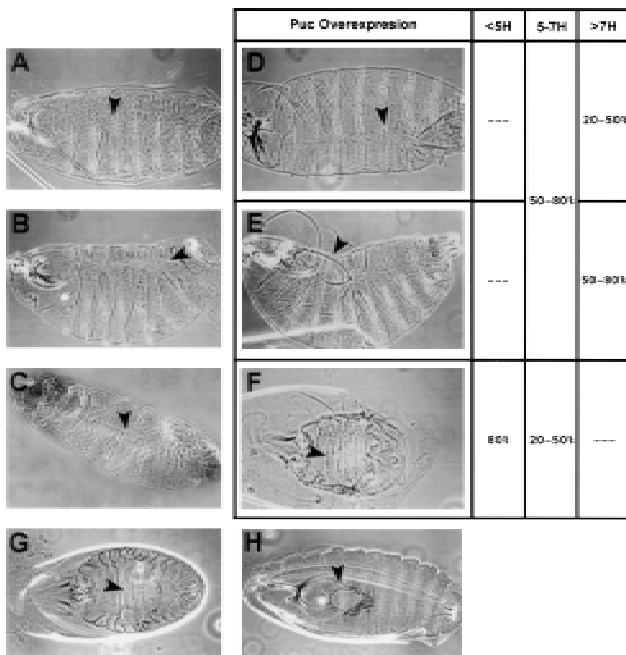


Figure 5. Cuticle phenotypes of *puc* mutants and those generated by ectopic expression of *puc* and *dpp*. Rescue of *hep* phenotype by *puc* in heterozygous condition. (A) Dorsal view of *puc^{Eh}/puc^{Eh}* embryo. (B) Dorsolateral view of *puc^{E69}/puc^{E69}* mutant embryos. (C) Dorsolateral view of ArmGal4/UASdpp embryo. Arrowheads point to defects, puckering, and naked cuticle, along the dorsal midline. (D-E) Embryos from a cross of flies carrying the UASpuc with flies carrying a hsGal4 insert that were exposed to a 30 min heat shock at various times during development. Cuticle preparations revealed three classes of phenotype depending on the age of the embryo at the time of heat shock. (D) *puc* loss of function-like; (E) dorsal hole; (F) dorsal open. Arrowheads indicate dorsal cuticle defects. Dorsal open embryos are observed more frequently after early heat shocks. Dorsal hole phenotypes appear at intermediate times and *puc* loss-of-function-like embryos are present mainly after late heat shocks (see columns at the right). (G) *hep¹* hemizygous embryo, note the dorsal open phenotype. (H) *hep¹* hemizygous, *puc^{E69}* heterozygous embryo. A partial rescue of the dorsal open phenotype leads to small dorsal holes (arrowhead).

the dorsal region of the embryo changes dramatically. Beginning with cells immediately flanking the amnioserosa, there is an elongation along the dorsoventral axis that gradually spreads ventrally through the epidermis. These cell shape changes stretch the opposing sides of the lateral epidermis until they meet along the dorsal midline.

Dorsal cuticle puckering and dorsal holes are indicative of a defect in dorsal closure, probably dependent on cell shape changes (for review, see Martinez-Arias 1993). In *bsk*, *hep*, and *Djun* mutants, cell shape changes are disrupted (Glise et al. 1995; Riesgo-Escovar et al. 1996; Riesgo-Escovar and Hafen 1997; Hou et al. 1997; Kockel et al. 1997). It is interesting that expression of DN-Drac1 displays phenotypes that are similar to that of *hep* and *bsk* mutants, suggesting that Drac1 might initiate signaling through this cascade. Drac1 seems to be involved

in the control of the cell cytoskeleton. In wild-type embryos, the onset of dorsal closure coincides with a specific subplasmalemmal accumulation of nonmuscle myosin at the leading edge of the dorsal-most epidermal cells (Fig. 6A,B; Young et al. 1993). It is likely that non-muscle myosin (NMM) probably contributes to the elongation of these cells by participating with actin in forming a dorsal constriction. In DN-Drac1 embryos, NMM and actin in epidermal cells are strongly reduced (Harden et al. 1995). These changes in the cytoskeleton are also evident in *Djun* mutant embryos (Hou et al. 1997).

We compared the effects of the absence of *puc* (*puc^{E69}*) and its overexpression (ArmGal4/UASpuc) in the levels and organization of the actin cytoskeleton and NMM. We find that in *puc^{E69}* both myosin and actin do not change dramatically their expression in the periphery of the cells in lateral regions of the embryo, but fail to accumulate along the leading edge of the epidermis (Fig. 6D-F). Cell shape changes proceed almost normal. In contrast, epidermal cells of ArmGal4/UASpuc embryos fail to change their shapes and accumulate low levels of

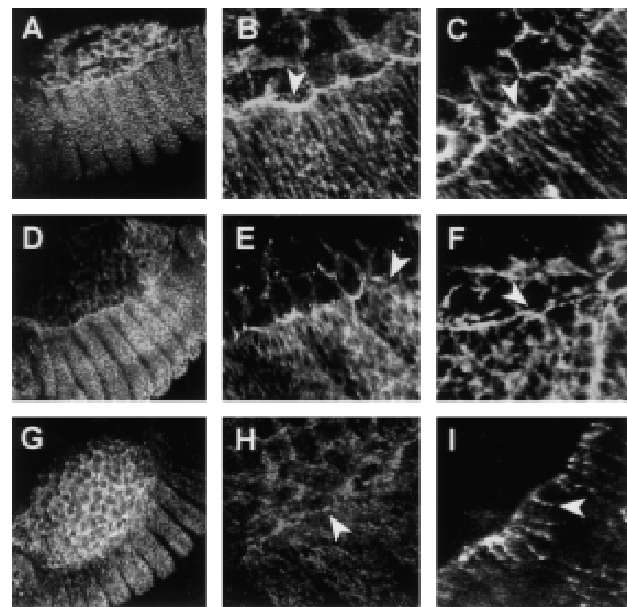


Figure 6. *puc* controls the accumulation of actin and myosin in the leading edge of the epidermis during dorsal closure. Confocal fluorescent micrographs of the boundary between the amnioserosa and the epidermis in stage 13 embryos. The distribution of nonmuscle myosin (A,B,D,E,G,H) and filamentous actin (C,F,I) are shown in wild-type embryos (A-C), *puc^{E69}* embryos (D-F) and ArmGal4/UASpuc embryos (G-I). Embryos were stained for filamentous actin with phalloidin or for nonmuscle myosin with antibodies. Whereas actin and NMM are accumulated along the leading edge in wild-type embryos (arrowheads in B and C), in *puc* mutants their level decreases and it is possible to observe gaps (arrowheads in E and F) between the amnioserosa and the epidermis. After Puc overexpression, NMM is maintained in the amnioserosa (arrowhead in H) but the level of expression in the epidermis is severely reduced. Actin ceases to be expressed in the amnioserosa and it appears on patchy spots in the epidermis (arrowhead in I).

spatially disorganized myosin at the leading edge (Fig. 6G,H). In these embryos, actin fails to be expressed in the amnioserosa and its levels are reduced in the epidermis (Fig. 6I). Actin and myosin tend to form clumps in these epidermal cells.

Our data indicate that *puc* is an essential component in the control of the different steps of dorsal closure progression by modulating the apical accumulation of actin and myosin at the leading edge and directing cell shape changes. These results correlate with those of the effects of overexpression of DN-Drac1 and *Djun* mutants, and further suggest a role for Puc in the control of JNK activity over the cytoskeleton.

dpp in the leading edge is regulated by *puc* and its overexpression disrupts completion of dorsal closure

The gene *dpp* encodes a member of the TGF β superfamily and has been identified as a secreted signaling molecule that mediates inductive interactions during *Drosophila* development (Wharton et al. 1993). During the second half of embryogenesis, *dpp* is expressed in specific regions in the embryo, particularly in the dorsal-most epidermal cells that express *puc* (Fig. 7B). It has been shown recently that the maintenance of *dpp* expression along the leading edge of the epidermis depends on the activity of the DJNK pathway (Glise and Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997).

In *puc*^{E69} mutant embryos, the expression of *dpp* in the dorsal-most epidermal cells is enhanced from stage 11 (cf. Fig. 7B with C; see also Glise and Noselli 1997). Furthermore, after germ-band shortening there are more

epidermal cells expressing *dpp* than in wild type, but this expression is still mainly limited to dorsal cells (cf. Fig. 7E and H with F and I). In contrast, ubiquitous expression of Puc leads to a decrease in the expression of *dpp* in those cells during stage 11 (Fig. 7A) and the complete absence at stages 12 and 13 (Fig. 7D,G). Puc overexpression does not affect the levels of *dpp* on the visceral mesoderm or the ventral epidermis. These results suggest a role for *puc* in the control of *dpp* expression, which could be mediated by Puc activity on JNK signaling (See Discussion).

The null phenotype for *dpp*, a completely ventralized embryo, reflects its initial role over the dorsal epidermis and might obscure the function of later patterns of expression (Wharton et al. 1993). Mutants for *thick veins*, however, which encodes a *dpp* receptor, display dorsal holes similar to those of *hep* or *bsk* mutants (Affolter et al. 1994). To test if the higher levels of *dpp* present in *puc* mutants could be involved in puckering phenotypes, we overexpressed *dpp* using ArmGal4 as the driving system. ArmGal4/UAS *dpp* embryos undergo an extreme dorsalization of the epidermis, but they still have a dorsal midline, in which, in many cases, we observed phenotypes (Fig. 5C) very similar to those observed in *puc*^{E69} mutants (cf. Fig. 5C with B). This indicates that, besides an early function of *dpp* in epidermal cell stretching, the downregulation of *dpp* in the dorsal-most epidermal cells is necessary for completion of dorsal closure.

Discussion

Two different MAPKs, encoded by the *bsk* and *rolled* (*rl*)

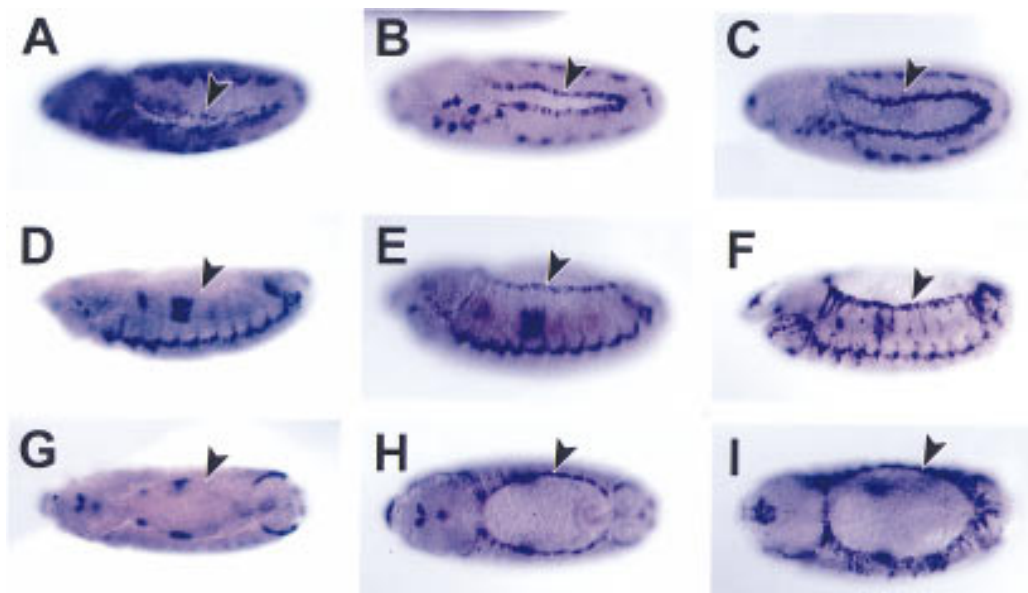


Figure 7. Effects of wild-type, mutant *puc*^{E69} and Puc expressed under the control of the *Armadillo* promoter on *dpp* transcription. (A,D,G) *dpp* RNA expression in ArmGal4/UASpuc embryos. (B,E,H) *dpp* RNA expression in wild-type embryos. (C,F,I) *dpp* RNA expression in *puc*^{E69} embryos. (A–C) lateral views of stage 11 embryos. Anterior is to the left; dorsal is up. At this early stage, it is possible to observe a reduction in *dpp* expression after ectopic expression of Puc and new cells expressing *dpp* along the epidermal border in *puc* mutants (arrowheads). (D–F) lateral views of stage 13 embryos. (G–I) dorsal views of stage 13 embryos. At this stage, *dpp* disappears from the dorsal-most epidermal cells after ectopic Puc expression and it is present in at least two rows of cells at the leading edge of each lateral hemisegment in *puc* mutants (arrowheads). The expression of *dpp* in the visceral mesoderm is unaffected.

genes, have been identified so far in *Drosophila*. The phenotype of mutations in these genes suggests that they play different roles during development. The MAPK encoded by *rl* has been implicated downstream of multiple RTK signal transduction pathways during cell fate determination (Diaz-Benjumea and Hafen 1994). On the other hand, the MAPK encoded by *bsk* behaves as a homolog of mammalian JNK and is involved in the process of dorsal closure (Riesgo-Escovar et al. 1996; Sluss et al. 1996).

Down-regulation of the activity of MAPK proteins through a feedback mechanism that is dependent on their own signaling ability has been suggested as a way of regulating the activity of MAPK pathways (Sun et al. 1993). Here, we have identified a novel member of the MKP subfamily of VH-1 protein tyrosine phosphatase (PTPs), which is involved in the regulation of JNK activities during dorsal closure in *Drosophila*. This phosphatase is associated with insertional mutations in the *puc* gene (Ring and Martinez-Arias 1993).

Three arguments lead us to the conclusion that the PTP that we have cloned is encoded by the *puc* gene. First, four independent P-element insertions cause *puc* mutant phenotypes and map to two different introns of the gene that we have cloned. Two of these mutations have been reverted to wild type with loss of the resident P element. Also, transcripts for this gene are not present in embryos of one of these mutants and one lethal revertant. Second, extracts from *puc* mutants have more JNK activity than wild type. And third, whereas loss of function of *puc* leads to ectopic expression of *dpp* in the dorsal epidermis, ubiquitous expression of the Puc protein leads to the loss of *dpp* expression.

We noticed the presence of maternally contributed RNA in early embryos. Consistent with a function of this RNA, the phenotype of *puc^{Eh}/Df(3R)dsx10* embryos derived from *Df(3R)dsx10* females was stronger than that of embryos derived from *puc^{Eh}* females (Ring 1993). Similar differences were also observed in reciprocal crosses between strong and weak alleles. The function of this maternal RNA during oogenesis and early embryogenesis is currently under study.

Evidence that Puc is a JNK-specific phosphatase is provided by biochemical assays. When extracts from wild-type and *puc* mutant embryos are assayed for their endogenous JNK activity, *puc* mutants show a significant increase in JNK activity compared with wild type. When similar extracts are tested for their ability to inactivate preactivated JNK, up to 50% inhibition was obtained after 30 min from wild-type embryos compared with abolition of activity in extracts from *puc* mutants. In similar assays, we never observed changes in ERK and ERK phosphatase activities.

A feedback loop between the Puc phosphatase and JNK signaling

The expression of most members of the VH-1 family of PTPs is subject to tight transcriptional regulation (i.e., Charles et al. 1993). The same is likely to be true for *puc* because it displays dynamic patterns of expression in the

embryo (Ring and Martinez-Arias 1993) and the adult (E. Martin-Blanco, unpubl.).

During and after germ band shortening, *puc* is expressed in the dorsal-most epidermal cells that play a leading role in the process of dorsal closure. In embryos mutant for the JNKK encoded by *hep* or for the JNK encoded by *bsk*, there is no *puc* expression in these cells and dorsal closure fails in a manner similar to that produced by overexpression of *puc* (Glise et al. 1995; Riesgo-Escovar et al. 1996). These results suggest a model in which signaling through Hep and Bsk leads to the expression of effectors of dorsal closure and a regulator encoded by *puc*. The function of the latter is to exert a negative feedback on the signaling cascade of *hep* and *bsk*. Interestingly, in mutants for *Djun*, a likely target of JNK activity, *puc* expression is absent at the leading edge of the epidermis (N. Perrimon, pers. comm.), suggesting a transcriptional link between the activity of the JNK encoded by *bsk* and the expression of *puc*. The phenotypes of gain and loss of function of *puc*, together with the levels of JNK and JNK phosphatase activities of extracts from *puc* embryos support this model.

Expression of a dominant-negative Drac1 protein results in defects in the dorsal epidermis that are similar to those of *hep* and *bsk* mutants and, occasionally, produces defects that resemble weak *puc* mutant phenotypes (Harden et al. 1995). A similar situation can be observed when Puc is overexpressed at different times in embryogenesis: Whereas early overexpression leads to a *hep/bsk* mutant phenotype, overexpression during dorsal closure leads to phenotypes that resemble *puc*. The function of *puc* in a negative feedback loop can account for these observations. Early overexpression of Puc or dominant-negative Drac1 would inactivate signaling through JNK and thus inhibit the expression not only of *puc*, but also of the effectors of the pathway. On the other hand, overexpression of *puc* later would have allowed for some expression of the effectors but would result in the late inhibition of the feedback loop by suppression of *puc* expression. Therefore, the phenotype of *puc* mutants represents a failure in the negative regulation of signaling after the process has been initiated.

A similar feedback has been proposed as a mechanism by which a rapid and transient response to extracellular signals may occur. Erp, another member of the VH-1 phosphatase family, is expressed as an early event in the proliferative response of fibroblasts to serum and yet, constitutive expression of Erp in fibroblasts suppresses proliferation, suggesting this same negative feedback mechanism (Noguchi et al. 1993).

puc, signaling, and dorsal closure

During the first half of embryogenesis, the dorsal side of the *Drosophila* embryo is covered by an extraembryonic membrane, the amnioserosa, which bridges the two edges of the open epidermal sheet. The process of dorsal closure closes the gap by bringing these two epidermal edges together and intruding the amnioserosa into the embryo (Ring and Martinez-Arias 1993; Young et al. 1993).

The cell shape changes that accompany this process suggest that cytoskeletal rearrangements play an important role. Consistent with this, *zipper* mutant embryos (Nüsslein-Volhard et al. 1984), which lack the cytoplasmic myosin heavy chain (Young et al. 1993), display defective cell shape changes that lead to an abnormal dorsal closure. On this basis, a model for dorsal closure has been suggested in which myosin plays an active role in producing force for cell shape change and as a mechanically contractile band at the leading edge that powers the epidermal movements during dorsal closure (Young et al. 1993).

In *puc* mutants, the dorsal-most epidermal cells retain nonmuscle myosin and actin expression, albeit at reduced levels. In contrast, after Puc overexpression, the polarized localization of nonmuscle myosin is lost and a strong reduction in both myosin and actin levels is observed, correlating with an extreme lack of cell shape change. These defects are very reminiscent of the effects of dominant-negative Drac and *Djun* mutations on these cytoskeletal elements (Harden et al. 1995; Hou et al. 1997). In all these cases, actin became excluded from the amnioserosa. This disruption of the cytoskeleton is probably the cause of the absence of dorsoventral elongation of the epidermal cells associated with dorsal holes.

It is possible that independently of its effects on *hep* and *bsk*, DRac1 could be controlling the process of dorsal closure by direct effects on the cytoskeleton. The fact that mutations in *hep* suppress the effects of the expression of an activated form of Drac1 (Drac1V12) (Glise and Noselli 1997), and that the inhibition of DJNK activity by *puc* seems to produce equivalent cytoskeletal and morphogenetic defects as the expression of DN-Drac, however, suggest that during dorsal closure, the outcome of Drac1 signaling is the activation of DJNK.

puc control of Dpp expression and cellular morphogenesis

The control of DJNK activity by Puc affects the maintenance and modulation of *dpp*, which might mediate many of the requirements for dorsal closure (Fig. 8). In *hep* (Glise and Noselli 1997) and *Djun* (Hou et al. 1997; Riesgo-Escovar and Hafen 1997) mutants, the expression of *dpp* is abolished in the dorsal epidermal cells and dorsal closure is never initiated. On the other hand, *dpp* expression along the leading edge is augmented in *puc* mutants and is abolished after Puc overexpression.

Mutants for the *dpp* receptor *thick veins* have a prominent dorsal hole similar to that of *hep* and *bsk* mutants (Affolter et al. 1994). It is therefore interesting that, as we have shown here, overexpression of Dpp leads to similar problems during dorsal closure as does loss of *puc* function. *dpp* would affect first the morphogenetic changes of the lateral epidermal cells during closure progression (Riesgo-Escovar and Hafen 1997), and second, the proper recognition and adhesion of the leading edge cells at closure completion.

Taken together, the existing results suggest that the outcome of JNK signaling during dorsal closure is the

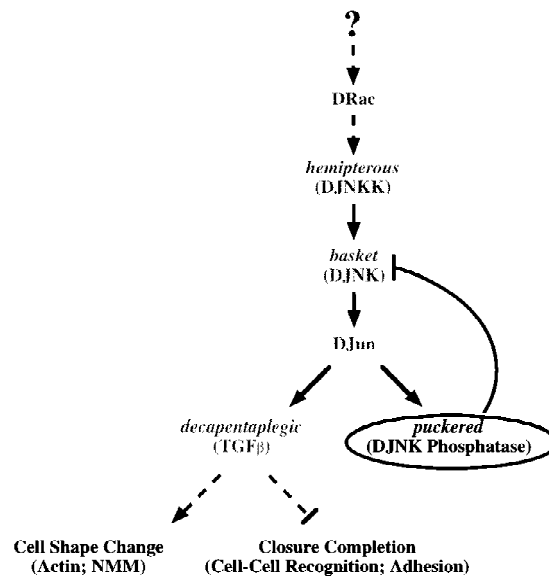


Figure 8. A model for the role of *puc* in JNK signaling during dorsal closure. Halfway during embryogenesis, in the cells at the leading edge of the epidermis, the *hep/bsk* pathway becomes activated, probably by Drac. As a consequence, DJun is itself activated and gets involved in the maintenance of *dpp* and *puc* expression. Puc will drive its own down regulation through inactivation of *bsk*, and it will control the level of expression of dorsal closure effectors as *dpp*. *dpp* might have two different roles: to induce the cellular events required for dorsal closure in the lateral cells and to participate in the specializations in the dorsal-most cells required for the last steps of closure. In *puc* mutants, JNK signaling becomes hyperactivated in the leading edge, the dorsal-ward stretching of the lateral cells proceeds normally, but the excess of *dpp* interferes with proper cell differentiation and affects midline alignment. When *puc* is ectopically expressed early throughout the epidermis, it blocks signaling through the *bsk* pathway leading to the disappearance of *dpp* from the dorsal-most cells and to a failure in dorsal closure. Late Puc overexpression it affects only the cellular differentiation of the leading edge cells.

expression via DJun of *puc* and *dpp* in the dorsal-most cells of the epidermis. Whereas *dpp* provides an effector of dorsal closure, *puc* encodes the regulatory element that controls the amount of signaling through the pathway. Experiments in tissue culture in vertebrates have suggested the possibility of such feedback loops on signaling cascades, and we have shown here one case in which it occurs in vivo. The existence of this feedback mechanism provides a sensitive way of regulating and controlling signaling through the pathway, something that might be important during morphogenetic events like the one of dorsal closure that requires coordinated fine tuning of cellular behavior.

Materials and methods

Drosophila strains and culture

All flies were maintained at 25°C on standard medium. The $P[ry^+, lacZ]E69$ line (renamed *puc^{E69}*) was generated from the

starter strain that carried the *ry*⁵⁰⁶ P[*ry*⁺, *lacZ*]C49 chromosome, kindly provided by C. O'Kane, as described in Ring and Martinez-Arias (1993). P[*w*⁺, *lacZ*]320c (renamed *puc*³²⁰) was a gift from J. Campos-Ortega (University of Koln, Germany). I(3)84EhK19 (renamed *puc*^{Eh}) was supplied by B. Baker (Stanford University, CA). P[*w*⁺, *lacZ*]A251.1 (renamed *puc*^{A251.1}) was a gift from W. Gehring (Biozentrum, University of Basel, Switzerland). The P[*w*⁺, *lacZ*]B48 (renamed *puc*^{B48}) and the revertant *puc*^{B48R23} were a gift from C. Ruhsloew and A. Pai (New York University, NY). The *hep*¹ mutant stock was a gift of S. Noselli (Centre de Biologie du Developpement, CNRS, Toulouse, France). The UASdpp line was a generous gift of F. M. Hoffmann (University of Wisconsin, Madison). The HsGal4 line was from A. Brand (Wellcome/CRC Institute, University of Cambridge, UK). The ArmGal4 is expressed ubiquitously during embryonic development and is a gift of J.P. Vincent (NIMR, Medical Research Council, London, UK).

Embryo cuticle preparations were done according to Wieschaus and Nüsslein-Volhard (1986) except that embryos were not fixed before mounting.

Immunocytochemistry and whole mount *in situ* hybridization

For all procedures, embryos were dechorionated, fixed for 20 min at room temperature in heptane/4% paraformaldehyde, and devitelinized with methanol. mRNAs were detected by *in situ* hybridizations with DIG-labeled DNA probes following standard protocols (Tautz and Pfeiffle 1989). Two *puc* probes were used, a probe generated by PCR with primers flanking the second exon of cDNA12 and a probe generated from a *Hind*II–*Bgl*III fragment of cDNA12 that includes 73 nucleotides from the pNB40 plasmid and 114 nucleotides from the 5' end of cDNA12 located within the 5' untranslated region. The *dpp* probe was generated from a 2-kb *Eco*RI fragment from a *dpp* cDNA clone (St. Johnston et al. 1990).

Antibody staining was performed by standard techniques (Ashburner 1989). The primary antibodies were commercial rabbit anti-β-galactosidase and rabbit anti-non muscle myosin antibody (Kiehart and Feghali 1986). Biotinylated secondary antibodies and streptavidin conjugated to horseradish peroxidase or Texas Red (Jackson laboratories) were used. F-actin staining was performed with rhodamine–phalloidin on embryos devitelinized with 80% ethanol as described previously (Harden et al. 1995).

Analysis of genomic DNA at the *puc* locus

Genomic DNA was isolated from *puc*^{E69}/TM3*sb* flies, partially digested with *Sau*3A and size selected on 0.75% low melting point agarose following the protocol described by Kaiser and Murray (1985). Fragments were subcloned into λEMBL3 with the Stratagene cloning kit. Recombinants were packaged into phage particles by use of the Stratagene Gigapack II Plus Packaging Extract and transformed into *Escherichia coli* strain P2392 (Stratagene). The *puc*^{E69} genomic library was screened with a probe against *lacZ* sequences. Genomic DNA from the resultant 12-kb clone was then used to probe a wild-type *Drosophila* genomic library (gift from J. Tamkin, University of Colorado, Boulder) to isolate wild-type genomic DNA from the *puc* locus. Library screening was done by standard techniques (Sambrook et al. 1989). DNA probes were radioactively labeled with [α-³²P]ATP (Amersham) by random priming with hexadeoxynucleotides (Pharmacia) according to the protocol described by Feinberg and Vogelstein (1984). The genomic map shown in Figure 2 was generated by restriction mapping of overlapping

genomic clones. Resident P elements in *puc*^{E69}, *puc*³²⁰, and *puc*^{A251.1} (Ring 1993) were localized by Southern hybridization by standard techniques (Sambrook et al. 1989). *puc*^{B48} was mapped by sequencing of a rescued plasmid.

Cloning and sequencing of *puc* cDNA

A 12- to 24-hr embryonic cDNA library in pNB40, kindly provided by N. Brown (Brown and Kafatos 1988) was screened by use of the 3.7-kb *Eco*RI *puc* genomic fragment. Filters for hybridization were prepared with the added step of washing nitrocellulose filters in CHCl₃ for 2 min before baking. Hybridization and probe preparation were performed by standard technique (Sambrook et al. 1989). Restriction fragments of cDNAs were subcloned and sequenced in both directions by automatic sequencing.

Northern hybridization and RT-PCR

RNA purification from *Drosophila* embryos was performed by the hot phenol/chloroform method (Jowett 1986). Northern blot analysis was done by standard techniques (Sambrook et al. 1989). The Northern was probed with the 1037-bp *Bgl*III–*Eag*I fragment of *puc* cDNA12 that had been labeled with ³²P by random priming.

RT-PCR was performed from single embryos as follows: Extracts from embryos (stage 13–14) were made in the cold in 10 μl of PCR buffer [10 mM Tris (pH 8.2), 1.2 mM EDTA, 25 mM NaCl, 0.1% Tween 20, 0.1% gelatin] supplemented with 5 units of RNasin/ml. The extracts were mixed with 10 μl of proteinase K (50 μg/ml) in RNasin-PCR buffer and incubated for 1 hr at 50°C, followed by denaturation at 94°C for 10 min. Five microliters was taken into a 20-μl reverse transcriptase reaction [1 unit of RT, 0.5 μM dNTPs, 5 units of RNasin, 0.1 μg of a reverse primer from the third exon of *puc* (3.3REV; GAGGTCAATC-TGGATGAGCAG)] at 42°C, for 30 min. After heat inactivation, the transcription reactions were subjected to PCR amplification after the addition of a forward primer from the first exon of *puc* (1.1ATG) (GTGCATATGTGTGTGAATCGAG) and *Taq* polymerase.

Construction of recombinant expression plasmids

The *Puc* fusion construct (pGST–*Puc*T1) contains the *Nde*I (blunt-ended)–*Eco*RI fragment of pNB40/cDNA12 ligated to the expression plasmid PGEX-2T (Smith and Johnson 1988) digested with *Sma*I and *Eco*RI. This recombinant plasmid encodes the first 424 amino acids of *Puc*, including the phosphatase catalytic domain, fused in frame to the carboxy-terminal end of GST.

The UAS*puc* construct was made by directional cloning of the *Bgl*III–*Not*I fragment of PNB40/cDNA12 in the vector pUAST (Brand and Perrimon 1993). Embryo injection and selection of recombinants were performed by standard procedures.

Protein purification and *in vitro* phosphatase activity assay

One-liter cultures grown to mid-log phase from freshly transformed cells were induced by IPTG (1 mM) and harvested after 3 hr. Harvested cells were frozen in dry ice for at least 30 min and thawed at 0°C. Two microliters of HKEDN buffer [25 mM HEPES (pH 7.6), 0.1 M KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 10 mg/ml of leupeptin, 0.1 mM benzamidine, 10 mg/ml of pepstatin A, 1 mM PMSF, 10 mg/ml of aprotinin, 1 mg/ml of phenanthroline] was added, and aliquots of 1 ml were sonicated. Recombinant protein was insoluble and went to the pellet by

centrifugation (TL100 rotor, 68,000 rpm for 45 min at 4°C). The pellet fraction was frozen in dry ice. The fusion protein was recovered in a denatured form, dissolved in 6 M guanidine-HCl in HKEDN and refolded by gradual removal of the denaturant by dialysis (Claasen et al. 1991).

Hydrolysis of PNPP was in a reaction volume of 200 μ l containing 50 mM imidazole at pH 7.5, 0.1% β -mercaptoethanol, 20 mM PNPP, and different concentrations of recombinant protein or control extracts at 30°C for 30 min. The reaction was stopped by addition of 800 μ l of 0.25 M NaOH, and the absorbance at 410 nm (A_{410}) was measured (Keyse and Emslie 1992).

Kinase assays and measurement of JNK/ERK-phosphatase activity

For assessment of endogenous ERK and JNK activity, 50 dechorionated embryos at stage 13, identified under the microscope by their mutant phenotypes, were homogenized in 50 μ l of ice-cold extraction buffer (25 mM HEPES at pH 7.7, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, 10 μ g/ml each of leupeptin and aprotinin). The extracts were clarified by centrifugation (12,000g, 4°C for 20 min).

For measurement of endogenous ERK activities, an in-gel kinase assay was performed according to the procedure of Kameshita and Fujisawa (1989) by use of myelin basic protein (MBP) as the substrate. Extracts prepared in the manner described above were fractionated on a SDS/10% polyacrylamide gel containing 500 μ g/ml of MBP. The gel was then washed twice in Tris-HCl buffer (50 mM at pH 8) containing 20% (vol/vol) isopropanol, followed by washes in Tris-HCl buffer supplemented with 5 mM β -mercaptoethanol (buffer A). The proteins were then denatured for 1 hr with two changes of buffer A containing 6 M guanidine-HCl and finally renatured for 16–20 hr at 4°C by incubating the gel in buffer A containing 0.04% (vol/vol) Tween 40. Renatured MBP kinase activity was detected by incubating the gel for 30–45 min at 30°C in kinase buffer (50 mM Tris-HCl at pH 8, 5 mM DTT, 5 mM MgCl₂, 1 mM EGTA, 50 μ M ATP, 20 μ Ci/ml [α -³²P]ATP). Unincorporated radioactivity was removed by extensive washing of the gel in 5% (wt/vol) TCA solution containing 1% (wt/vol) tetrasodium pyrophosphate, dried, and analyzed by phosphorimaging. Intensity of bands was measured by the ImageQuant (Molecular Dynamics, Kent, UK) software.

For measurement of endogenous JNK activity, an aliquot of the clarified total extract was diluted so that the final concentration of the buffer was modified to 20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, and 10 μ g/ml each of leupeptin and aprotinin. The diluted extracts were then precleared by mixing with 10 μ l of glutathione (GSH)-agarose for 3–4 hr at 4°C. After this time, GSH-agarose was pelleted and the supernatants were transferred into new tubes and tumbled overnight at 4°C in the presence of GST-cJun (1–86) coupled to glutathione-agarose beads. The beads were finally pelleted and washed extensively (5 \times 1 ml) in ice-cold HEPES-binding buffer (20 mM HEPES at pH 7.7, 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100). The beads were then resuspended in 35 μ l of kinase buffer (20 mM HEPES at pH 7.6, 20 mM MgCl₂, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, 2 mM DTT, 20 μ M ATP, 10 μ Ci [α -³²P]ATP), and the reaction was allowed to continue for 1 hr at 30°C. The reactions were terminated by washing the beads twice with ice-cold HEPES-binding buffer and the phosphorylated proteins were eluted by the addition of Laemmli buffer. The proteins were then resolved on a SDS/10% polyacrylamide gel. The gel

was Coomassie stained, dried, and exposed to PhosphorImager screens.

In experiments designed to measure ERK- and JNK-specific phosphatase activity, embryonic extracts were prepared in the manner described above but with the exclusion of phosphatase inhibitors in all buffers. ERK phosphatase activity was measured by the ability of clarified extracts to inhibit the phosphotransferase activity of activated human ERK toward myelin basic protein. Clarified extracts (10 μ l) were mixed with kinase buffer containing activated human ERK and MBP (10 μ g) in a final volume of 50 μ l. Kinase reactions were initiated by the addition of [α -³²P]ATP (2.5 μ Ci/reaction) and the reaction allowed to proceed at 35°C for 15 min before termination by EDTA (20 mM). The entire reaction volume was then spotted to P81 phosphocellulose paper. After several washes in phosphoric acid (1%) and a rinse in acetone, the P81 paper was air dried and exposed to PhosphorImager screens for quantitation.

To measure JNK-phosphatase activity, clarified extracts were preincubated (30 min at 35°C) with GST-cJun (1–86) coupled to glutathione-agarose beads with prebound activated JNK derived from anisomycin-treated HeLa cells. Kinase reactions were initiated by the addition of kinase buffer (20 mM HEPES at pH 7.6, 20 mM MgCl₂, 2 mM DTT, 20 mM ATP, 10 μ Ci [γ -³²P]ATP) and allowed to proceed for 1 hr at 35°C. Control reactions were performed in kinase buffer (lacking phosphatase inhibitors) either in the absence of any extract or kinase buffer containing CL100 (kindly provided by S. Keyse, ICRF, Ninewells Hospital, Dundee, UK). Reactions were terminated as described above and fractionated on a SDS/10% polyacrylamide gel. The intensity of phosphorylated GST-cJun (1–86) protein was quantitated by PhosphorImaging.

Loading controls for all the extracts (in the presence or absence of phosphatase inhibitors) were performed by Western blotting. Western blots were tested with an anti-*Drosophila* ERK (Rolled) antibody kindly provided by L. Zipursky (HHMI, University of California, Los Angeles). This antibody recognizes a single 45-kD band. The results were quantified by densitometric scanning of ECL films, and the results of kinase and phosphatase assays were normalized to these values.

Acknowledgments

We thank C. O’Kane, W. Gehring, B. Baker, J. Campos-Ortega, J.P. Vincent, F.M. Hoffman, J. Pradel, A. Brand, C. Ruhslow, A. Pia, and the Bloomington Stock center for generously providing *Drosophila* stocks; L. Zipursky for the anti-Rolled antibody; S. Keyse for the CL100 protein; C.M. Marshall for the activated human ERK; and E. Hafen, S. Noselli, and N. Perrimon for helpful discussions and communication of data prior to publication. We are grateful to S. Keyse, M. Freeman, and A. Prokop for comments on the manuscript, and S. Rolfe for technical assistance. E.M.-B. was supported by The Wellcome Trust and the European Union. A.G. and J.R. were supported by a Jane Coffin Child fellowship and a Commonwealth studentship respectively. K.V. and A.M.T. are supported by the Wellcome Trust. A.M.-A. is a Senior Fellow of The Wellcome Trust.

Note

The cDNA sequence data reported in this paper have been submitted to the GenBank/EMBL library under accession no. AJ223360.

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