Int. J. Dev. Biol. 42: 363-368 (1998)

Regulatory control of signal transduction during morphogenesis in *Drosophila*

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ABSTRACT Morphogenesis shapes pattern and size during development. The initiation and propagation of morphogenetic processes is led by the integrated activation of signaling cascades. Much is known about regulatory control of signaling cascades in cell culture systems. However, how this regulatory elements act when cells need to behave coordinately is still unknown territory. The morphogenetic process of dorsal closure proceeds through changes in cell shape and polarity under the control of JNK signaling. Amongst other regulatory elements, Puckered, a *Drosophila* MAPK phosphatase, is involved in a negative feedback loop that controls JNK signaling activity. *puckered* is expressed in many other tissues, could influence other developmental events and might regulate different signaling cascades. The negative regulatory control of signal transduction pathways could be a general mechanism regulating differentiation and morphogenesis.

KEY WORDS: *MAPK, dorsal closure, puckered, phosphatase, Drosophila, signal transduction*

Introduction

The generation of cell diversity depends on a series of events which occur during embryonic development. This set of events have to be coordinated in order to achieve the spatial organization which will allow normal morphogenesis. In most cases, the final size and shape of the organism are species-specific features, based in ordered cell proliferation and spatial cell differentiation. These two closely linked processes are under simultaneous control by signaling systems. The local activation of these signaling pathways might be controlled by cell-cell interactions through a global program of communication. "Entelechia" (see García-Bellido and de Celis, 1992 for a detailed description) is reached when this program of communication and the morphogenetic process are completed. In this sense, Driesch's "entelechia" as "a resultant action of many complicated elemental interactions" (Driesch, 1908), becomes implemented by discrete molecular and cellular operations. In a developmental landscape, as postulated by Antonio García-Bellido, local differences in the activity of "martial" genes, nuclear factors in charge of the developmental operations affecting spatial organization and differentiation, will drive the expression of control ligands or "emissors" which will affect neighboring cell proliferation. "Entelechia" would be attained when the level of activity of these "martial" genes turns equal in the total cell population and cell division stops.

Using this model, one would predict that the activity of signaling cascades required to attain "entelechia" should be under a tight temporal and quantitative control. This process could include the control by regulatory feedback loops (García-Bellido and de Celis, 1992). Indeed, the downregulation of signaling pathways which is dependent on their own signaling ability has been shown as a way of regulating their activity (Sun *et al.,* 1993). Negative feedback loops can generate very different behaviors, depending on the dampening parameters of the system. At one extreme they can provide homeostasis, while at the other extreme they may act as a mechanism by which a rapid and transient response to extracellular signals can occur.

In this paper, I would like to focus on the role that reversible phosphorylation could play in regulating differentiation and morphogenesis. Although the roles of kinases have been much more extensively studied, recent analyses have indicated that phosphatases are also critically important. We have been studying the product of the gene *puckered (puc),* which encodes a member of the VH1-like dual specificity phosphatase family (Guan *et al.,* 1991, Martín-Blanco *et al.,* 1998). Members of this family of phosphatases have been implicated in the downregulation of MAPK activity (reviewed in Keyse, 1995). In *puc* mutants, embryonic dorsal closure is affected and the dorsal cuticle shows

Abbreviations used in this paper: J-NK, Jun N-terminal kinase; JNKK, jnk kinase; MAPK, mitogen activated protein kinase; ERK, Extracellular-signal regulated kinase; dpp, decapentaplegic; hep, hemipterous; puc, puckered; rl, rolled; Sem, sevenmaker; TGFß, transforming growth factor ß; Fas III, fascyclin III; VH1, vaccina homology 1.

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0214-6282/98/$10.00

Printed in Spain

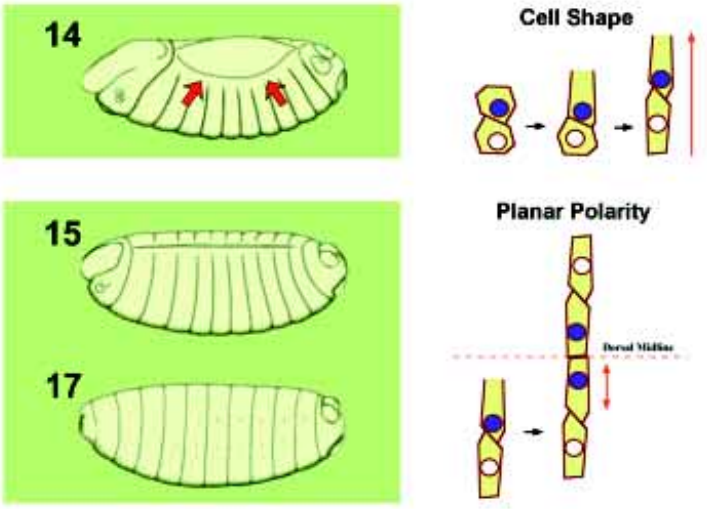

Fig. 1. The stages of dorsal closure. *Dorsal closure proceeds after the retraction of the germ band. The lateral epidermis stretches dorsally from stage 13 meeting the contralateral sheet at the dorsal midline by stage 15. The earliest cellular event in the process is the elongation along the D/V axis of the dorsalmost cells. This elongation proceeds in parallel to the acquisition of planar polarity. This is manifested by the ventral localization of nuclei (in blue) (Ring and Martinez-Arias, 1993) and the asymmetric membrane distribution of Fas III (in brown), excluded from the amnioserosa-epidermis interphase. Secondarily, ventral cells change shape as well, although they do not show planar polarity, and the epidermal layers stretch. When they reach the midline, the dorsalmost cells lose their polarity, nuclei migrate to a more central position and Fas III is distributed now homogeneously around the periphery of the cell.*

puckering, hence the name of the mutant (Ring and Martinez-Arias, 1993). Our results indicate that *puc* regulates signaling through the JNK pathway and participates in a negative feedback loop during dorsal closure (Martín-Blanco *et al.,* 1998).

Here, I discuss dorsal closure as a morphogenetic event, the role of the JNK signaling and *puc* in this process and I present some evidence suggesting that *puc* could have similar roles in other developmental processes. Finally, I would like to speculate on the existence of other molecules of the same class, that could act on other signaling devices to direct morphogenesis in other systems.

Results and Discussion

Morphogenesis in the absence of proliferation: embryonic dorsal closure

During *Drosophila* embryogenesis, one of the most striking examples of cellular coordination is the process of dorsal closure. Dorsal closure refers to the dorsalward movement of the lateral and ventral epidermis to enclose the embryo (Fig. 1). Halfway through embryogenesis, the epidermal layer covers the ventral surface of the embryo but dorsally is appended to an extraembryonic membrane, the amnioserosa. During the late stages of embryogenesis, the amnioserosa sinks into the embryo as the epidermis stretches dorsally to cover the dorsal surface (Martinez Arias, 1993). The dimensions of the epidermal layer are controlled not by proliferation, but by cell shape and polarity changes. First, the dorsalmost cells of the lateral epidermis acquire planar polarity and stretch along the dorso-ventral axis. Second, the cells of the lateral epidermis elongate coordinately starting from the most dorsal and proceeding towards the most ventral ones in a morphogenetic wave. Coupled with this dorsalward movement, the anterior/posterior (A/P) axis stretches along the dorsal edge. These two movements result in the dorsalmost cells of the epidermis forming two straight lines (Martinez Arias, 1993; Ring and Martinez Arias, 1993). Finally, the epidermal layers meet at the dorsal midline, the dorsalmost cells lose their polarity, cell stretching stops and the epithelial sheet seals the gap to form a continuous epidermis. We could say, by analogy, that epidermal morphogenesis reaches "entelechia" and epidermal cells initiate their final differentiation on completion of dorsal closure.

The activation of JNK signaling as a developmental operation triggering dorsal closure

The coordinated behavior of epidermal cells during dorsal closure strongly suggests that this cellular function depends on a signaling process which begins in the dorsalmost cells of the epidermis and propagates ventrally. Although no ligands or receptors which could trigger or relay such a signal have been identified, very recent analysis indicates that this process is dependent on the activity of the *Drosophila* homologs of the stress activated kinases, DJNKK and DJNK, which constitute the core of one of the related MAPK pathways present in *Drosophila* (see Fig. 2). These mol-

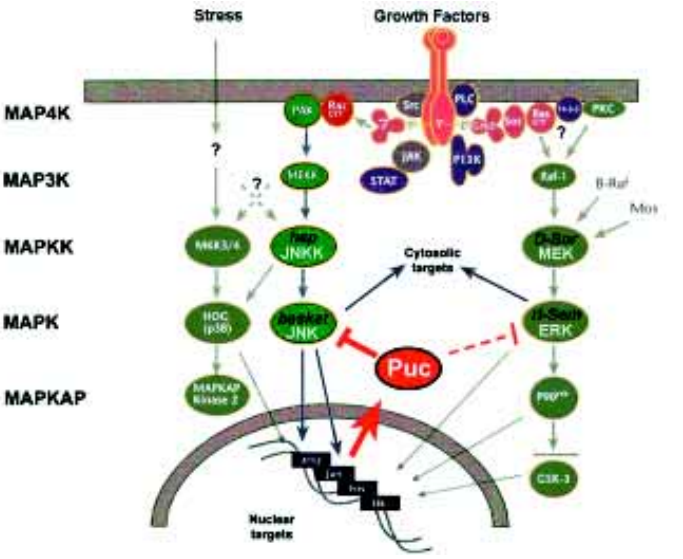

Fig. 2. The different MAPK pathways: the role of puckered. *MAPK pathways are organized as cascades of kinases (MAP4K, MAP3K, MAPKK, MAPK) that eventually impinge in cytosolic and nuclear targets. The latter regulate the expression of downstream genes. Three different kinds of cascades have been identified so far. In Drosophila, members for three of them have been isolated (from left to right: P38-DMPK2 (unpublished data); JNK-basket; and ERK-rolled). In cell culture, the ERK cascade is mainly activated via growth factors, receptor tyrosine kinases and Ras. The JNK and P38 pathways, however, are activated in response to stress. One of the nuclear factors which responds to JNK is Jun. puckered encodes for a MAPK (JNK) phosphatase that mediates a feedback loop. Its expression gets activated by JNK signaling (and probably by Jun) and in turn it dephosphorylates and inactivates JNK.*

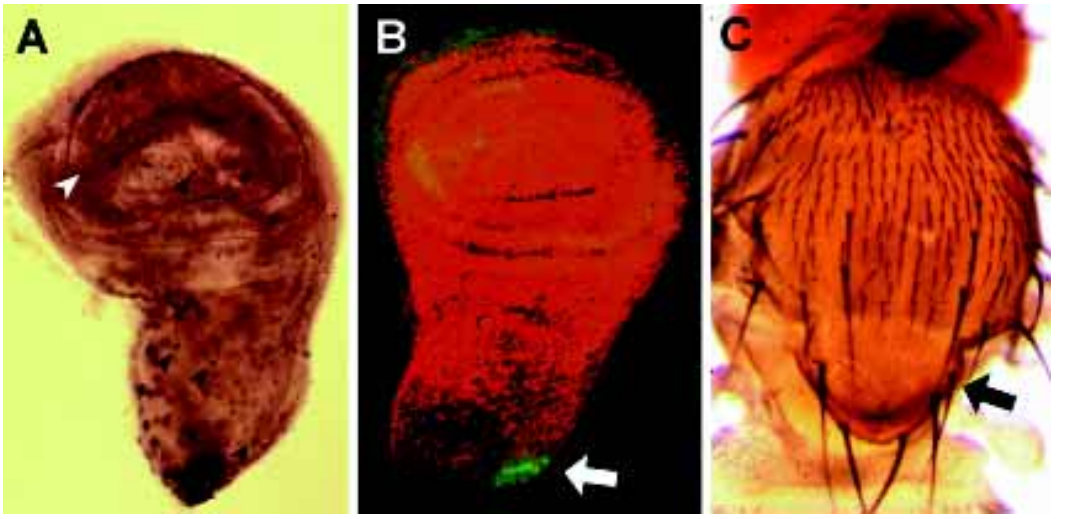

Fig. 3. Expression and ectopic activity of *puckered* in the wing disc. (A) *In third instar larvae, puc-LacZ is present in all larval cells and in specific patterns in imaginal cells. In wing discs, puckered is found in the peripodial membrane and also in two rows of cells along the wing margin (white arrowhead) and in group of cells that could correspond to proneural clusters. In some cases it is possible to observe single cells emerging from these clusters that could correspond to sensory mother cells (black arrowheads).* **(B)** *Pattern of expression of the 455.2-Gal4 line detected with a UAS-GFP construct. The expression of GFP in the scutellum area is indicated by an arrow.* **(C)** *Notum from an adult fly where puckered has been overexpressed in the scutellum with the 455.2-Gal4. The arrow points to an ectopic macrochetae. With this line, 80-90% of the emerging flies had extra macrochetaes (1-3) in the scutellum, compared to 5-10% in all controls.*

ecules are encoded by the genes he*mipterous (hep)* and *basket* (Glise *et al.,* 1995; Riesgo-Escovar *et al.,* 1996; Sluss *et al.,* 1996). Mutants on both of these genes show a dorsal open phenotype consequence of the lack of elongation of the cells of the lateral epidermis. Furthermore, this phenotype is also observed in mutants for DJun, a transcription factor whose activity is regulated through phosphorylation by the DJNK signal transduction chain (Hou *et al.,* 1997; Kockel *et al.,* 1997; Riesgo-Escovar and Hafen, 1997).

An interesting possibility is that the morphogenetic wave which runs from the dorsalmost cells of the epidermis could be caused by the sequential activation of DJNK signaling, and as a consequence by activation of DJun. Local differences in the activity of DJun, would be transmitted to neighboring cells, via a secondary ligand, and would result in the cell shape change. Activated DJun would hence behave as a nuclear "martial" gene and would activate a secondary "emissor" transmitting the signal to adjacent ventral cells. The best candidate for such a molecule is *decapentaplegic (dpp),* a secretable molecule of the TGFβ superfamily (St. Johnston *et al.,* 1990). DJNK signaling, and DJun, appear to control the expression of d*pp* and indeed, *dpp* is activated by DJNK signaling in the leading edge (Glise and Noselli, 1997; Hou *et al.,* 1997; Riesgo-Escovar and Hafen, 1997). To fit in the proposed model, *dpp* in turn should activate DJNK signaling in nearby cells, something that has not been tested yet.

In signaling terms, dorsal closure will be completed, or reach "entelechia" in García-Bellido's expression, when all epidermal cells reach the same low level of DJNK or DJun activity, and all have changed shape.

Puckered: exploring the mechanism of signaling inactivation and cell polarity

Expression of *puc* within the epithelium is initiated prior to dorsal closure in the cells located at the dorsalmost edge of the epidermis (Ring and Martinez Arias, 1993). These cells are first identifiable in the early extended germ band as a distinct group because they undergo cell division synchronously (mitotic domain 19, Foe, 1988). They show planar polarity, which is evident in the exclusion of fasciclin III from the dorsal-most side of the cells, from early stages of dorsal closure (Ring and Martinez Arias, 1993). Later, nonmuscle myosin accumulates on their dorsal edge, suggesting cytoskeletal specialization (Young *et al.,* 1993), and they become very elongated along the dorsal/ventral axis (Martinez Arias, 1993). Finally, their planar polarity is lost when the stretching of lateral epidermal cells is completed, once the dorsalmost cells meet at the midline.

Mutations in *puc* do not interfere with the first steps of dorsal closure: polarization and elongation of dorsalmost cells and D/V stretching, but result in a conspicuous puckering of the dorsal cuticle along the midline. Though some of the specializations of the dorsalmost cells do occur in *puc* mutant embryos, they fail to depolarize (unpublished results) and as a result dorsal fusion proceeds in a disorganized manner (Ring and Martinez Arias, 1993).

In embryos mutant for the DJNKK encoded by *hep* or for the DJNK encoded by *basket*, there is no *puc* expression in the dorsalmost cells (Glise *et al.,* 1995; Riesgo-Escovar *et al.,* 1996). Moreover, when Puc is overexpressed in the epidermis, dorsal closure fails in a manner similar to that observed in *hep* or *basket* embryos showing an extreme lack of cell shape change (Martín-Blanco *et al.,* 1998). Accordingly, the polarized localization of nonmuscle myosin is lost and a strong reduction in both myosin and actin levels is observed. These results suggest a model in which signaling through Hep and Basket leads to the expression of a regulator encoded by *puc*. The function of the *puc* regulator is to exert a negative feedback on the signaling cascade (Fig. 2).

The control of DJNK activity by Puckered also affects the maintenance and modulation of *dpp*. In *hep* (Glise and Noselli, 1997) and *D-jun* (Hou *et al.,* 1997; Riesgo-Escovar and Hafen, 1997) mutants the expression of *dpp* is abolished in the dorsalmost cells and dorsal closure is never initiated. On the other hand, *dpp* expression along the leading edge expands in *puc* mutants and is abolished after Puckered overexpression. It is interesting that overexpression of Dpp leads to similar problems during dorsal

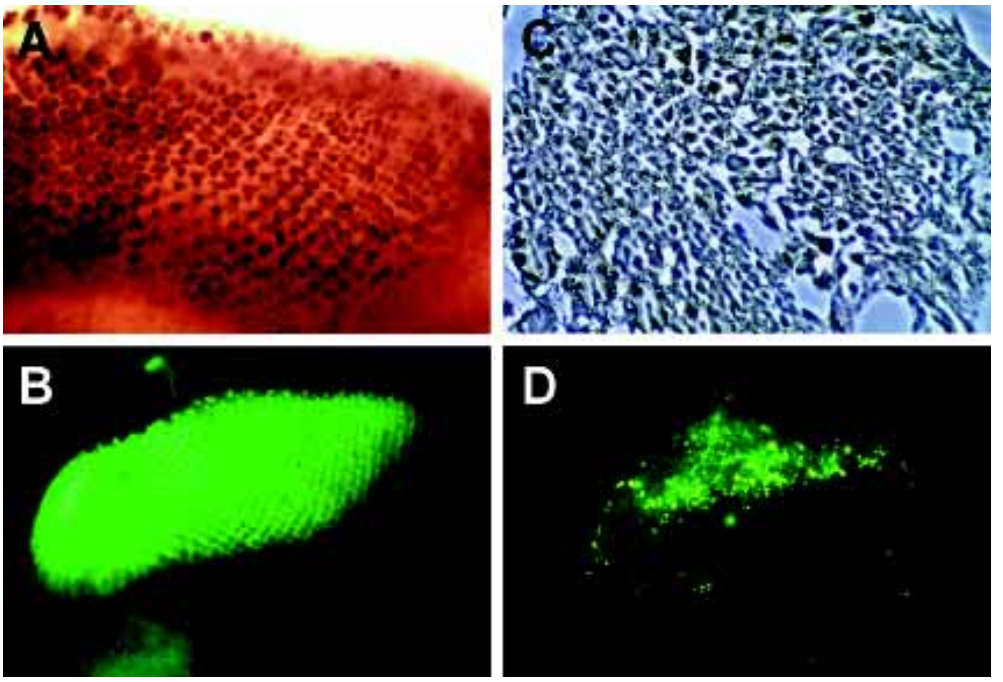

Fig. 4. Expression and ectopic activity of *puckered* in the eye disc. (A) *Pattern of expression of puc-LacZ in the eye discs. All photoreceptors express puckered sequentially as they progress from the morphogenetic furrow.* **(B)** *Pattern of expression of the Glass-Gal4 line detected with a UAS-GFP construct. All the cells posterior to the furrow express GFP.* **(C)** *Section through the eye of an adult fly expressing puckered under the control of the Glass-GAL4 construct. Most of the ommatiadia have the normal pattern of photoreceptors. However, many accessory cells are missing and big areas devoid of tissue can also be observed.* **(D)** *Cell death (detected with Acridine Orange) in an animal of the same genotype. Extensive death can be visualized in the same areas where the GAL4 is expressed.*

closure as does loss of *puc* function mutants (Martín-Blanco *et al.,* 1998). *dpp* affects first the morphogenetic changes of the lateral epidermal cells (Riesgo-Escovar and Hafen, 1997), and second the polarity and adhesion of the leading edge cells at closure completion.

The activation of a negative feedback loop mediated by Puckered in the dorsalmost epidermal cells appears to be necessary for overcoming the effects of DJNK activity and indeed, DJNK signaling is hyperactive in *puc* mutants (Martín-Blanco *et al.,* 1998). DJNK activity seems to be necessary for polarity, whilst Puc is necessary to suppress planar polarity prior to dorsal closure completion.

The other roles of puckered

puckered is not only expressed in the dorsalmost cells of the epidermis. During embryogenesis *puc* appears to be expressed in the central and peripheral nervous system and in the endoderm (unpublished results) suggesting a possible role in these tissues. During larval development *puc* is also present. The spatial pattern of *puc* gene expression in larvae was analyzed in the puc^{E69} and $puc^{A251.1}$ P element inserts using anti β–gal antibodies (see Materials and Methods). Both lines gave indistinguishable results, though puc^{E69} is somehow stronger. β–gal is expressed in all polytenic larval cells, including muscles and cells from the peripodial membranes. It is also expressed in specific patterns in imaginal discs.

Puc is expressed in the eye-antenna disc in a dynamic pattern posterior to the morphogenetic furrow (Fig. 3A). β–gal is detected in photoreceptor cells (R8 founder cells first and in a fixed sequence R2 and R5, R3 and R4; and R1, R6 and R7) as they proceed towards neuronal differentiation (reviewed in Dickson and Hafen, 1993). In leg discs, puc^{E69} β–gal expression is observed in the seven sensory neurons, as well as in the leg chordotonal organ (data not shown) (*Jan et al.,* 1985; Tix *et al.,* 1989). In third larval instar wing discs, β–gal is expressed in two rows along the wing margin in presumptive precursors of sensory bristles. It appears also in patches reminiscents of the clusters of cells expressing *achaete* and *scute* genes. Its expression within these clusters accumulates in single cells that could correspond to SMCs (Fig. 4A) (Campuzano and Modolell, 1992).

No experiments addressing the effects of the loss of function of *puc* in larval derivatives have been performed yet. However, if *puc* function is to counteract the JNK pathway or other similar signaling device, a hint to *puc* activities could come from overexpression experiments. Accordingly, we used a UASpuc construct (Martín-Blanco *et al.,* 1998) to promote regulated high expression of Puckered with different GAL4 lines during adult development. This misexpression results in the induction of different kind of defects in diverse imaginal derivatives.

In the eye disc, the overexpression of Puc with a *Glass*-GAL4 driver (Fig. 3B) affects the development of the ommatidia. Using this driver, the accessory cells and most of the cells recruited at late stages (Dickson and Hafen, 1993) are missing (Fig. 3C). These defects occur as a consequence of an extreme induction of cell death after Puc overexpression (Fig. 3D). It was recently suggested that JNK and its target cJun are critical mediators of apoptosis in mammalian cells. However other reports have emphasized the ability of JNK to act as a survival factor in cultured neurons (see Xia *et al.,* 1995). In the context of the eye, the effects of Puckered overexpression suggest a role for DJNK as a survival factor during cell fate specification.

The ectopic expression of Puc in the wing causes mild effects, such as occasional lack of veins (data not shown). However, its overexpression with a scutellar GAL4 driver (see expression in Fig. 4B), and other lines expressing in the notum, consistently induces the appearance of ectopic macrochaetes (Fig. 4C). A role for the JNK pathway in bristle specification or differentiation remains to be explored. Alternatively, the overexpression of Puc could be interfering in a different unrelated pathway (see below) that could eventually affect bristle development.

Dual specificity phosphatases: multiple regulatory possibilities

Three different MAPK subfamilies have been identified: p42-p44 extracellular-signal regulated kinases (ERKs), p38 Kinases, and p46-p54 Jun N-terminal kinases (JNKs) (Canman and Kastan, 1996). These major subfamilies respond to different signals, exhibit a distinct substrate specificity and seem to have different roles (see also Fig. 2).

In *Drosophila*, distinct receptor tyrosine kinase (RTKs) exert their effects through a single ERK cascade (Fig. 2, right). This pathway is mediated sequentially by the genes *Son of Sevenless (Sos), Ras1*, *D-Raf*, *D-Sor* and *rolled (rl)* MAPK (reviewed in Perrimon, 1994).

The *Drosophila* EGF receptor (DER) plays crucial roles in a variety of cell fate decisions throughout development. During larval development, DER is necessary both for growth and for patterning of the imaginal discs and then is required during pupal development for continued viability (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990). One of the developmental processes in which DER signaling has been extensively studied is in cell fate determination in the wing. The adult wing is made up of three basic components: the margin structures (innervated and non-innervated), the wing veins and the intervein regions. Animals which carry viable combinations of *DER* alleles exhibit a partial loss of wing veins (Clifford and Schüpbach, 1989). Similar defects are also observed in somatic clones of *DER* mutations, and in somatic clones of mutations in the downstream components of RTK signaling (Díaz-Benjumea and García-Bellido, 1990; Díaz-Benjumea and Hafen, 1994). On the contrary, extra veins are observed in mutants for *Sevenmaker (Sem)*, a gain of function allele of *rolled* (Brunner *et al.,* 1994). Taking all these results together, it has been suggested that signaling through the *Drosophila* ERK *(rolled)* signaling cascade is involved in wing vein specification.

To determine if *puc* was able to interact with the *rolled* signaling pathway, we expressed an activated form of *rolled* MAPK *(Sem)* in the wing imaginal disc. The expression of UASSem with the 455.2 and 604-GAL4 drivers (see Material and methods) lead to production of extra veins at different degrees (compare Fig. 5A and B). Simultaneous expression of UASpuc (Martín-Blanco *et al.,* 1998) along with UASSem suppresses partially the wing vein phenotype induced under the 455.2-GAL4 driver (Fig. 5C). UASpuc expression with this GAL4 line does not induce any wing vein defect (Fig. 5D). Similar effects were observed in the eye disc (data not shown). These observations show that Puc counteracts *in vivo* the action of an activated form of ERK. Suppression of Sem overexpresion by Puc was always modest, indicating that Puckered activity on the Rolled product is somewhat weaker than on DJNK. This indicates that a feedback control could be presumably at work limiting the activity of the ERK kinase, and that other phosphatases, yet unidentified could be involved in the downregulation of the *rolled* cascade.

Most likely, each MAPK pathway has its own feedback control mediated by a Puc-like molecule, although Puc may act on two of them. This feedback can be a way of controlling the flow of information through the system, by turning it on and off depending on the circumstances.

Concluding remarks

In the context of dorsal closure, we can define DJun as a "martial" gene, whose level of activity is sensed throughout the epidermal layer. During dorsal closure, in contrast to the case of the wing (García-Bellido and de Celis, 1992), change of dimensions is attained by cell shape changes. Here, in the limited space of the embryonic epidermis, the morphogenetic information is transmitted, not through cell-cell contact but by the secreted "emissor" Dpp. The negative feedback mediated by *puc* limits the signaling of both DJNK and *dpp*, and promotes cell depolarization at the leading edge.

As we have seen for dorsal closure, the negative regulation of signaling cascades appears to have an important role controlling morphogenetic events. MAPK-phosphatases such as *puc* could have important inputs in other developmental events. However, their roles could be limited to more simple operations, such as the control of cell differentiation or, as in the eye, cell death. We must be aware that molecules as DJun, may have been co-opted as "martial" genes for specific morphogenetic events and have more pleiotropic functions. We need to learn more about genes acting as drivers during morphogenesis, about new signaling cascades involved in these kinds of events and about new molecules involved in the negative control of signaling.

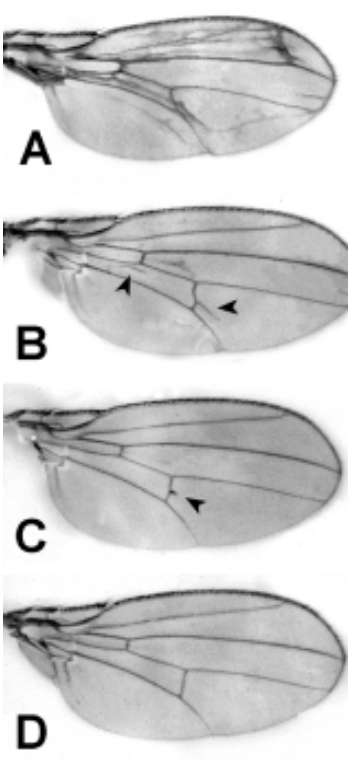

Fig. 5. Suppression of ERK signaling by *puckered*. (A) *Wing from a fly where Sevenmaker [a gain of function rolled (ERK) protein] has been overexpressed with the 604-GAL4 line. Extensive plexate is observed in many areas of the wing, as it would occur with the hyperactivation of the DER pathway (see text).* **(B)** *A weaker phenotype obtained with Sevenmaker is overexpressed in the wing with the 455.2-GAL4 line. Arrowheads point to extra pieces of vein tissue.* **(C)** *The simultaneous overexpression of puckered along with Sevenmaker supress almost completely the extra vein phenotype induced with the 455.2-GAL4. Stronger phenotypes are partially suppressed at most.* **(D)** *The overexpression of puckered with the 455.2-GAL4 in the same conditions has no effect in the vein pattern, and these flies resemble the wild type condition.*

Materials and Methods

Drosophila strains and culture

All flies were maintained at 25°C on standard medium. The puc^{E69} and $puc^{A251.1}$ lines have been previously described Ring and Martinez Arias, 1993; Martín-Blanco *et al.,* 1998). The 455.2-Gal4 line was a gift from J. Campos Ortega. The 604-Gal4 line was from J. Urban and G. Technau. The Glass-Gal4 is a gift from M. Freeman. The UAS-GFP is from T. Klein.

Drosophila wings were dissected in SH (1 part of glycerol:3 parts of ethanol) and mounted in Hoyer's medium.

Inmunocytochemistry

Antibody staining was performed using standard techniques (Ashburner, 1989). The primary antibodies were commercial rabbit anti-β–galactosidase. Biotinylated secondary antibodies and streptavidin conjugated to

horseradish peroxidase (Jackson laboratories) were used.

Cell death was analyzed by staining with Acridine Orange following standard procedures (White *et al.,* 1994).

Eye sections and counterstainings were performed following standard protocols (Ashburner, 1989).

Transgenic flies

The UASpuc construct has been already described (Martín-Blanco *et al.,* 1998). To construct UASSem, a modified *rolled* cDNA was cloned in the vector pUAST (Brand and Perrimon, 1993). Embryo injection and selection of recombinants were performed by standard procedures.

Flies which carried the UASSem, UASpuc or both transgenes were crossed to flies which carried GAL4 insertions expressed in the developing wing discs.

Acknowledgments

Fist of all, I would like to thank Antonio García-Bellido, with whom, after many years of smashing flies, I learned to look at them and to appreciate what they can tell us. Secondly, to our editor, for all his patience with endless delays. I would also like to thank Mathew Freeman for his help with eye sections, James Castelli-Gair and Beatriz San Martin for their help with the manuscript and Alfonso Martinez-Arias for his support. My work is funded by a Marie Curie fellowship from the European Union.

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