

A temporal switch in DER signaling controls the specification and differentiation of veins and interveins in the *Drosophila* wing

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Summary

The *Drosophila* EGF receptor (DER) is required for the specification of diverse cell fates throughout development. We have examined how the activation of DER controls the development of vein and intervein cells in the *Drosophila* wing. The data presented here indicate that two distinct events are involved in the determination and differentiation of wing cells. (1) The establishment of a positive feedback amplification loop, which drives DER signaling in larval stages. At this time, *rhomboid* (*rho*), in combination with *vein*, initiates and amplifies the activity of DER in vein cells.

(2) The late downregulation of DER activity. At this point, the inactivation of MAPK in vein cells is necessary for the maintenance of the expression of *decapentaplegic* (*dpp*) and becomes essential for vein differentiation. Together, these temporal and spatial changes in the activity of DER constitute an autoregulatory network that controls the definition of vein and intervein cell types.

Key words: *Drosophila*, Signal transduction, DER, Raf, MAPK, Rhomboid

INTRODUCTION

The *Drosophila* EGF receptor (DER) belongs to a large family of transmembrane receptors with intrinsic kinase activity known as receptor tyrosine kinases (RTKs). Following ligand binding, these receptors undergo dimerization, which triggers transphosphorylation of specific tyrosine residues in their cytoplasmic domains. These phosphotyrosines define binding sites for proteins that interact with the receptor through their SH2 domains. One of these, Drk acts as an adapter for the guanine nucleotide releasing factor Son of sevenless (*Sos*), which facilitates the GDP-to-GTP exchange on Ras. Ras activates the Raf, MEK, MAPK phosphorylation cascade, which ultimately regulates the activities of specific transcription factors (see Perrimon, 1993).

One of the processes in which DER signaling has been extensively studied is cell fate determination in the *Drosophila* wing. The adult wing is made up of three basic components: the margin structures, the wing veins and the intervein regions. Wing veins are epidermal sclerotizations that arise at stereotypical positions on the wing blade and enclose trachea and nerves (García-Bellido and De Celis, 1992). Animals that 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 induced during larval stages of *DER* mutations and downstream components of RTK signaling (Díaz-Benjumea and García-Bellido, 1990; Díaz-

Benjumea and Hafen, 1994). Moreover, ectopic veins develop in wings after overexpression of downstream effectors of DER or from gain-of function alleles (Brunner et al., 1994; Martín-Blanco, 1998). Together, these results suggest that the activation of DER during larval development is responsible for the induction of vein cell fates.

Interestingly, the pattern of expression of DER during wing development is temporally regulated. DER expression, which during the larval period is uniform, is suppressed in vein territories from 8 hours after puparium formation (APF), while it is maintained in intervein cells (Sturtevant et al., 1994). This suggests the possibility that DER, and the Ras/Raf/MAPK cascade, serves a different function(s) during the pupal period.

Other genes involved in DER signaling and vein specification, such as *rhomboid*, *vein* or *argos*, are also dynamically regulated during both larval and pupal periods. Rhomboid (Rho), a seven transmembrane protein, is the earliest marker expressed along veins and *rho* hypomorphic alleles display a partial loss of wing veins (Sturtevant et al., 1993). Rho has been suggested to be involved in the processing of Spitz (Spi), a stimulatory DER ligand, and to participate in the localized activation of DER (Golembo et al., 1996). It also appears to be involved in DER downregulation from veins during the pupal period (Sturtevant et al., 1994). Vein (Vn) is a Neuregulin-like molecule (Simcox et al., 1996; Schnepf et al., 1996). In the wing disc, Vn is expressed in a stripe delimited by the veins 3 and 4 in larval periods and then it

expands to occupy all intervein territories in pupal stages (Simcox et al., 1996). *vn* mutants have a partial loss of vein phenotype (García-Bellido et al., 1994). Argos (Aos) has been demonstrated to be an inhibitory ligand of DER and appears to act similarly in vein formation (Schweitzer et al., 1995). Aos has been shown to be expressed in vein cells from the third larval instar and *aos* hypomorphic alleles show weak extra vein phenotypes (Sawamoto et al., 1994).

The differentiation of vein and intervein cells during the pupal period is less well understood. Amongst the genes involved in this process is *decapentaplegic* (*dpp*), a TGF β homologue, which appears to be necessary for vein differentiation. *dpp* is upregulated in vein cells during pupal period and its expression depends on DER activity (Yu et al., 1996; De Celis, 1997).

In this study, we have found that the level of DER/Ras/Raf/MAPK pathway activity is regulated in time and space during wing development. We show that during the larval period, DER signaling is required for the activation of *rho* expression in the future vein cells triggering a positive signal amplification loop. This early activation of DER signaling is necessary for the acquisition of 'vein competence'. We further show that DER signaling (MAPK activity) is downregulated in vein cells and restricted to intervein tissue during pupal development. As a consequence, *aos* expression is also limited to intervein cells. These changes in the activity of DER signaling control a cell specification switch. DER downregulation in vein cells is necessary for the maintenance of the expression of *dpp* and the implementation of vein differentiation. Conversely, late DER signaling during pupal stages specifies intervein cells differentiation.

MATERIALS AND METHODS

Drosophila strains

The *scaGAL-4*, *Gal-4⁶⁰⁴* and *Gal-4^{MS1096}* lines are insertions of a GAL-4 construct (Brand and Perrimon, 1993). *Gal-4⁶⁰⁴* is expressed in the dorsal and ventral wing blade regions. This expression is maintained in pupal stages. *Gal-4^{MS1096}* expression starts on the dorsal wing pouch early in third larval instar and expands later to the ventral surface (Capdevila and Guerrero, 1994). The *scaGAL-4* is a hypomorphic *sca* mutation whose expression initiates in second larval instar and progress to late pupal stages (see Fig. 6). The *UAS-Sem* and *UAS-Vn* lines have been already described (Martín-Blanco, 1998; Simcox et al., 1996). The *UAS-Aos* line was a gift of Mathew Freeman. The *hs-DN-DER²⁹⁻²⁹⁻¹* line was provided by Alan Michelson and encodes a kinase-dead DER.

DNA constructs

A truncation of the *D-Raf* coding sequence in which amino acids 2-431 are removed, which results in a constitutively activated protein (Stanton et al., 1989), was cloned into the vector pUAST (Brand and Perrimon, 1993) (*UAS- Δ D-Raf^{f20}*). The same *D-Raf* truncation was also subcloned into pCaSpeR-hs vector to make *hs- Δ D-Raf^{f22}* (Brand and Perrimon, 1994). *UAS-KM-Raf^{3.1}* and *UAS-KM-Raf^{2.1}* represent different UAS lines expressing a kinase dead protein. This protein acts as a dominant negative molecule (Sprenger et al., 1992).

Antibody staining and in situ hybridization to imaginal discs

Antibody staining with the anti-active MAPK antibody (Sigma) (1:200 dilution) was performed in third instar larval discs and 24-30 hours old pupal wings fixed during 20-30 minutes with 4%

paraformaldehyde in PBS. Fluorescein-conjugated anti-mouse antibodies (Jackson) were used at 1:500 dilution and staining visualized using a confocal microscope. *dpp*, *aos* and *rho* in situ hybridization to imaginal discs was performed as described in Sturtevant et al. (1993).

RESULTS

A positive feedback loop between *rho* and D-Raf mediates vein cell fate specification

In the third instar wing imaginal disc, the activated form of MAPK, detected with antibodies that recognize phosphorylated Rolled (dpERK) (Gabay et al., 1997), shows a prominent localization along the veins in the wing pouch (Fig. 1A; Gabay et al., 1997). This activated MAPK distribution reflects the activity of DER via the Ras signaling cascade.

Based primarily upon the enhanced loss of veins observed in mutant combinations of *DER* and *rho*, it has been suggested that Rho acts upstream of DER in the specification of vein cell fates (Sturtevant et al., 1993). Moreover, *rho* strongly interacts with *vein*, a putative DER ligand, as the double-mutant combination *rho^{ve}vn¹* results in a complete loss of veins. To directly analyze the role of these genes in DER activation, we examined the expression of activated MAPK in conditions where we altered the expression of Rho and Vn.

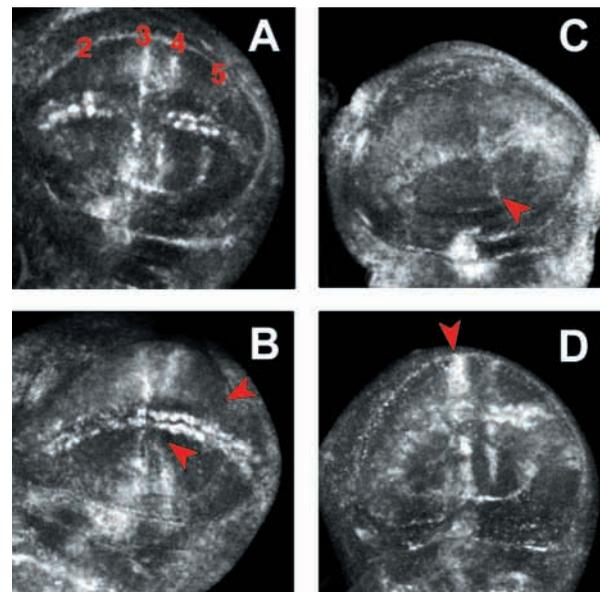


Fig. 1. *vein* and *rhomboid* cooperate in the activation of MAPK in vein territories. (A) Pattern of MAPK activation in a mature third larval instar wing disc. dpERK is readily detected in all veins and in the wing margin of wild-type discs. (B) MAPK activity in *rho^{ve}* mutant discs. Although *rho* expression in the wing disc is completely eliminated, dpERK expression is just reduced from the distal parts of L3 and L4 and the entire L5 (arrowheads). (C) MAPK activity in double mutants *rho^{ve}vn¹*. dpERK is eliminated from vein territories in correlation with the complete loss of veins in adult wings (arrowhead points to remnants of vein 4). (D) Rho overexpression leads to MAPK activation in the wing disc. dpERK expression domain expands in vein territories after 1 hour heat-shock induction of Rho (*Hs-Rho^{30A/+}*) (arrowhead).

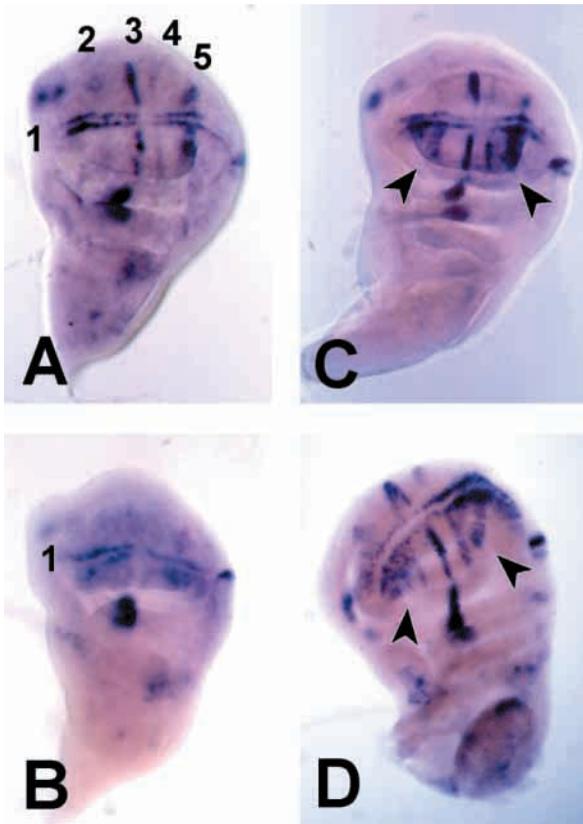


Fig. 2. *rhomboid* expression requires D-Raf and responds to the activation of RTK signaling. (A) *rho* in situ hybridization in wild-type third instar larvae imaginal wing disc. *rho* expression in the presumptive wing veins is indicated. (B) A third instar larvae wing disc from a cross between the *Gal-4^{MS1096}* line and the *UAS-KM-Raf^{3.1}* line. In the presence of a dominant negative form of D-Raf, *rho* expression is strongly reduced in the vein areas. (C) A third instar larvae wing disc from a cross between the *Gal-4^{MS1096}* line and the *UAS-Sem* line. *rho* expression is detected in the intervein areas in the dorsal wing pouch (arrowheads). Interestingly, *rho* expression is not induced in the intervein between veins 3 and 4. This area is refractory to the development of vein tissue in most vein affecting mutants (Sturtevant and Bier, 1995). (D) A third instar larvae wing disc from a cross between the *Gal-4^{MS1096}* line and the *UAS-Vn* line. Strong *rho* expression is evident in intervein areas upon Vn ectopic expression (arrowheads).

In wings from the *rho* regulatory mutation *rho^{ve}*, which eliminates all *rho* expression in the larval wing disc (Sturtevant et al., 1993), dpERK expression is eliminated from the distal parts of veins L3 and L4 and the entire L5 (Fig. 1B). These changes in the distribution of activated MAPK correlate with the absence of vein differentiation in equivalent positions in mutant adults (see Díaz-Benjumea and García-Bellido, 1990). In a more extreme condition, *rho^{ve}vn¹*, where all veins are eliminated, the expression of dpERK is abolished from all vein territories in the larval disc (Fig. 1C). In contrast, the overexpression of *rho* (Sturtevant et al., 1993) leads to a broadening of dpERK expression on vein territories (Fig. 1D). In this condition, the adult wing vein tissue is dramatically enlarged (data not shown). All these data suggest that *rho* acts upstream of MAPK activation and that *vn* and *rho* cooperate in the activation of Ras signaling.

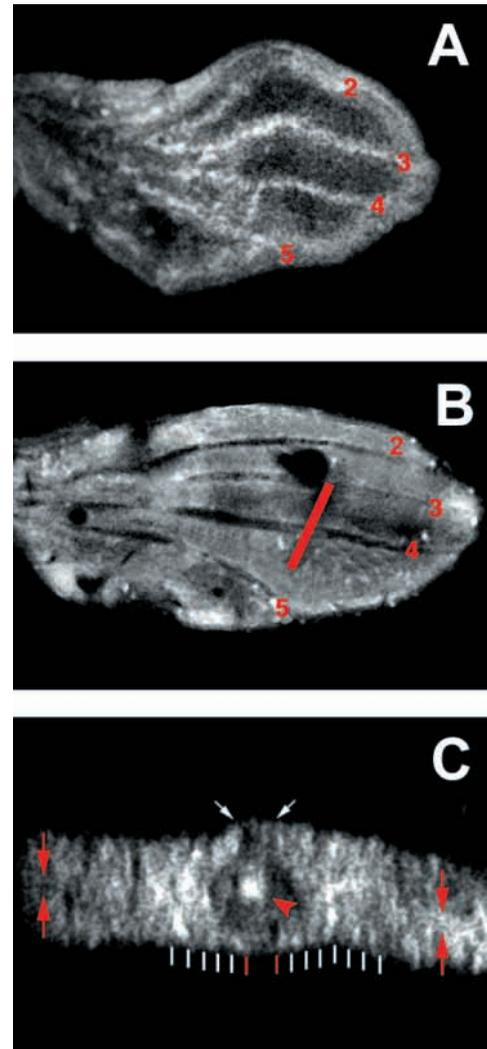


Fig. 3. Activated MAPK is detected in intervein cell during pupal development. (A) A pupal wing (20 hours APF) taken from a wild-type animal. Expression of activated MAPK is observed in vein primordia. dpERK expression initiates in intervein territories of the epithelia. (B) A pupal wing (30 hours APF) from a wild-type animal. Expression is restricted to intervein cells. Red bar indicates the position of the confocal Z section presented in C. (C) Z section through the blade of a 30 hours APF pupal wing. Intervein cells show high expression of dpERK (vertical lines). This expression appears to be mainly cytoplasmic and is not accumulated in the basal lamina (bracketed with red arrows). A positive haemocyte is shown in the vein cavity (arrowhead). Note that the presumptive vein cells are devoid of dpERK (bracketed with white arrows).

In wild-type wing discs, *rho* is expressed in the presumptive veins beginning in the mid-third instar larval stage and through pupal stages (Fig. 2A; Sturtevant et al., 1993). To examine the effects of reduced RTK signaling on *rho* expression in the wing, we ectopically expressed a dominant negative *UAS-KM-Raf^{3.1}* transgene from second larval instar, under the control of the *GAL-4^{MS1096}* line (Capdevila and Guerrero, 1994), and we found that this prevented the expression of *rho* mRNA in vein territories during third instar larval stages (compare Fig. 2A and B).

These results indicate that the maintenance of *rho* expression in the wing disc requires D-Raf activity.

In support of these observations, we also found that DER signaling can activate *rho* expression. *rho* mRNA is ectopically expressed in intervein territories after the expression of *UAS-Sem* (an activated form of the *rolled* MAPK; Brunner et al., 1994; Martín-Blanco, 1998) and *UAS-Vn* (Schnepp et al., 1996; Simcox et al., 1996). The highest levels of *rho* accumulate in dorsal territories in the wing pouch where the expression of the *GAL-4^{MS1096}* line is stronger (Fig. 2C,D). Under these conditions, the expression of *UAS-Sem* or *UAS-Vn* direct the formation of ectopic veins with the same pattern (data not shown).

MAPK activity is temporally and spatially regulated during pupal wing development

During the early pupal period, the expression of DER rapidly disappears from the vein territories as these territories are established (Sturtevant et al., 1994). This suggests that the downregulation of DER signaling during pupal stages could be a critical event in the differentiation of wing cells.

To determine the activity of the DER signaling cascade at different times during pupal development, we again took advantage of the anti-dpERK antibodies. As described above, activated MAPK was detected along the veins of third instar larval wing discs (Fig. 1A). From 20–24 hours after puparium formation (APF), high levels of activated MAPK expression in veins are accompanied by expression in intervein territories (Fig. 3A). At late stages, activated MAPK expression is eliminated from veins (Fig. 3B), which correlates with the downregulation of DER expression. By confocal analysis, we found that activated MAPK is maintained in the intervein territories, both in the dorsal and the ventral surfaces of the wing blade and in haemocytes that colonize the vein cavities (Fig. 3C). These data show that DER mRNA downregulation is followed by the downregulation of MAPK activity in the veins, while differentiating intervein cells have high levels of MAPK activity.

In pupal stages D-Raf activity represses wing veins, whereas suppression of DER and D-Raf signaling leads to ectopic wing vein differentiation

To test if DER activation has an instructive role during pupal stages in vein and/or intervein differentiation, we crossed flies that carried a

Fig. 4. Late expression of activated D-Raf in the wing disc results in MAPK activation and loss of veins. (A) Strong phenotype from an adult wing taken from a *UAS-ΔD-Raf^{F20/+}; GAL-4^{604/+}* animal. This wing exhibits an almost complete absence of veins as well as an overall reduction of size. (B) A pupal wing (30 hours APF) taken from a *UAS-ΔD-Raf^{F20/+}; GAL-4^{604/+}* animal. MAPK activity (dpERK staining) is present in the whole wing area. (C) Adult wing from a *hs-ΔD-Raf^{F22}* animal, heat shocked late in the larval third instar. (D) Adult wing from a *hs-ΔD-Raf^{F22}* animal, heat shocked at early-to-mid second larval stage. These animals developed ectopic vein tissue in proximity to vein territories (arrowheads).

UAS-ΔD-Raf^{F20} transgene, which produces a constitutively activated form of D-Raf, to flies with the *GAL-4⁶⁰⁴* insertion. Expression in this *GAL4* line is very strong in pupal stages. Transheterozygous animals had reduced viability and displayed dominant wing phenotypes: ectopic veins and sensilla and a loss of endogenous veins (Fig. 4A). Several other *GAL-4* lines active during pupal stages in combination with *UAS-ΔD-Raf* produce similar defects (data not shown). Examination of MAPK activation during pupal stages in these animals correlates with the suppression of vein fates. MAPK activity in *UAS-ΔD-Raf* animals was high all over the wing blade during pupal stages (Fig. 4B), ruling out possible antimorphic effects of the activated Raf construct.

We confirmed these results by analyzing the effects of elevation of D-Raf activity at different times. When 30 minute heat shocks were given to animals carrying a constitutively active D-Raf protein under the control of a heat-shock promoter (*hs-ΔD-Raf^{F22}*) during late third instar larval stages, they displayed a significant amount of vein loss (Fig. 4C). Flies of the same genotype, which were heat shocked at early-to-mid second instar stages, developed ectopic vein tissue, in the majority of cases in close proximity to a vein (Fig. 4D). We further verified these effects by using an activated form of Ras1. As described for *hs-ΔD-Raf^{F22}*, heat-shock induction of *hs-Ras1^{V12}* late in larval development also results in a loss of veins (data not shown). The observed inhibition of vein differentiation after ectopic expression of activated D-Raf or D-Ras at late developmental periods strongly suggests that the downregulation of DER RTK signaling is crucial for the differentiation of wing vein cells.

If vein specification can be reversed as a consequence of high levels of RTK signaling during late larval and pupal stages, then late downregulation of DER activity should be sufficient to trigger vein specification and differentiation. We analyzed this possibility by using a dominant negative receptor. Heat shocks were given very late in third instar and into pupal stages to animals carrying *hs-DN-DER²⁹⁻²⁹⁻¹*. These adults consistently displayed ectopic veins, often in close proximity to normal veins (see Fig. 5A), showing that late suppression of DER activity promotes vein differentiation.

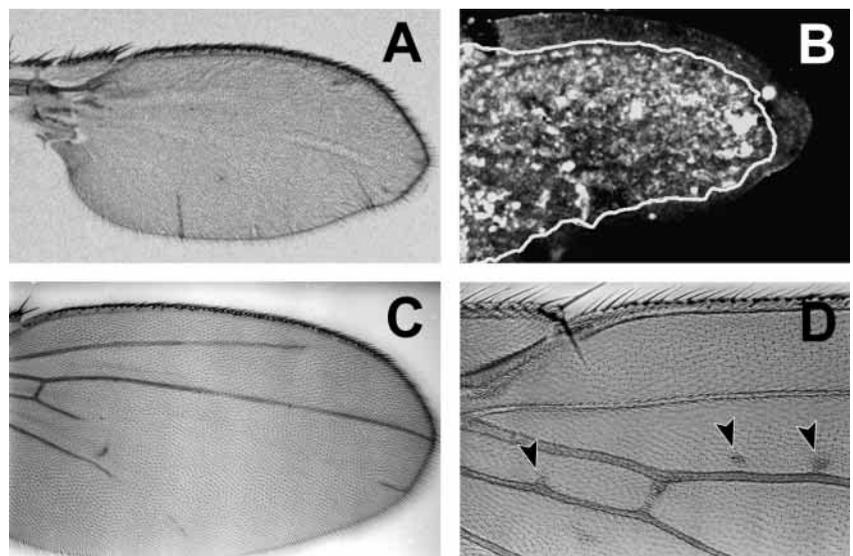
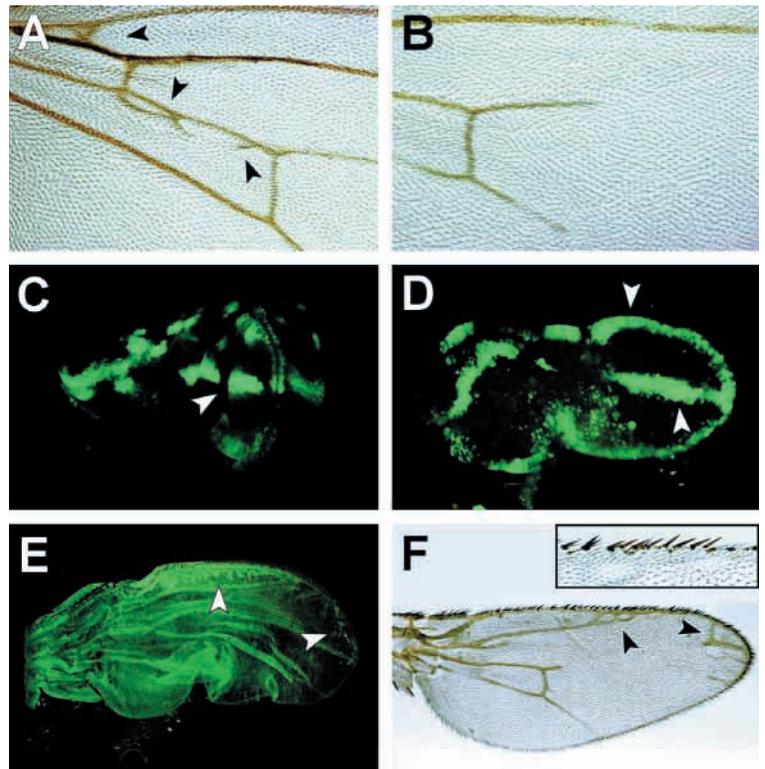


Fig. 5. Dominant negative DER and D-Raf promote wing veins late in imaginal development. (A) Extra vein tissue (arrowheads) in the wing blade, produced after *hs-DN-DER* overexpression in pupal stages. Ectopic vein cells developed in proximity to vein territories. (B) Overexpression of *hs-DN-DER* in late second instar stages results in an important reduction of vein tissue. (C) Pattern of expression of the *scaGAL-4* line in a third instar wing disc revealed by the expression of a *UAS-GFP* construct. The arrowhead points to the A/P border (L3/L4 intervein area) which shows strong GFP expression. (D) 20 hours APF pupal wing of the same genotype as C showing persistent expression of GFP in the A/P border and high levels of expression in and around the anterior wing margin (arrowheads). (E) Pupal wing from young pharates of the same flies showing GFP expression around the wing margin (arrowheads), but not in the A/P border. (F) Vein pattern from a cross *scaGal-4/ UAS-KM-Raf^{2.1}* rise at 29°C. Veins L3 and L4 are severely disrupted in the areas that correlate with early GAL-4 expression. Arrowheads point to the ectopic veins where GAL-4 expression is sustained until very late stages. The inset highlights the effects of the overexpression of *UAS-KM-Raf^{2.1}* in the wing margin.



Conversely, *hs-DN-DER²⁹⁻²⁹⁻¹* animals, heat shocked during early-to-mid second instar, displayed substantial vein losses (Fig. 5B).

To verify that the downregulation of RTK signaling during pupal stages is sufficient to induce vein tissue, a dominant negative Raf transgene *UAS-KM-Raf^{2.1}* was expressed using the *scabrous-GAL-4* line (*sca-GAL-4*). This line directs expression in third instar wing discs in most of the proneural clusters and along the anteroposterior boundary (Fig. 5C). At 24 hours APF, high levels of expression are detected near the wing margin (Fig. 5D). In pharate adults, GAL-4 activity is maintained in the area around the wing margin, long after expression in the A/P border has disappeared (Fig. 5E). Animals that expressed the dominant negative *UAS-KM-Raf^{2.1}* along with *sca-GAL-4* lack pieces of L3 and L4 veins (Fig. 5F), which correlates well with the early GAL-4 expression in third

instar (Fig. 5C, arrow). Further, they show ectopic veins in positions that associate with the late pupal *sca-GAL-4* expression (Fig. 5F). Thus it appears that loss of RTK activity early in third instar results in loss of veins, while loss of RTK activity late in pupal development induces extra vein tissue.

The suppression of vein differentiation by Raf does not depend on a negative feedback loop mediated by *argos*

Aos is a secreted molecule that functions as an inhibitor of the signaling triggered by DER (Freeman et al., 1992). In the embryonic ventral ectoderm, *aos* is expressed in the ventralmost row of cells where it is induced by the DER pathway. In this way, the activity of the DER pathway is restricted through an inhibitory loop (Golembo et al., 1996).

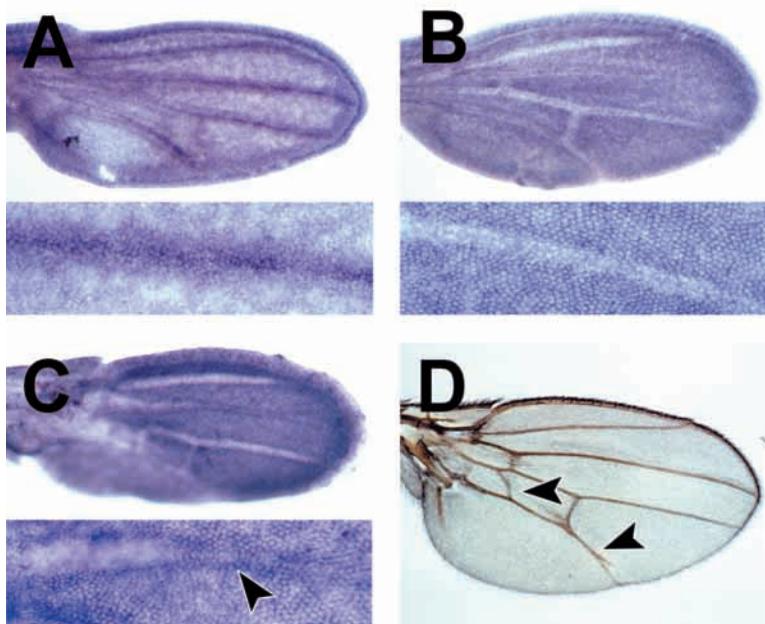


Fig. 6. D-Raf signaling activates the expression of *argos*. Ectopic pupal expression of *Argos* promotes veins. (A) Expression of *aos* in 24 hours APF pupal wings from wild-type animals. Low magnification ($\times 10$). The expression of *aos* is restricted to vein primordia. High magnification ($\times 60$). The expression of *aos* is visible in the cytoplasm of vein cells. (B) Expression of *aos* in 30 hours APF pupal wings from wild-type animals. Low magnification. The expression of *aos* is detected in interveins. High magnification. *aos* accumulates in the cytoplasm of intervein cells. (C) A pupal wing (30 hours APF) taken from a *UAS-ΔD-Raf^{20/+}; GAL-4^{604/+}* animal (low and high magnification). *aos* expression is detected in intervein cells and in all cells transformed to intervein fates. (D) Extra vein tissue (arrowheads) in the wing blade of *UAS-Aos/+; GAL-4^{604/+}* animals raised at 29°C. Ectopic vein cells developed in proximity to vein territories.

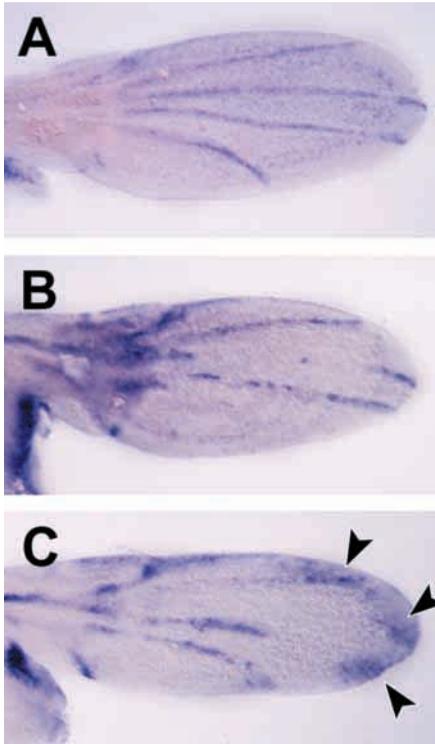


Fig. 7. Ectopic D-Raf signaling in vein territories inhibits the expression of *decapentaplegic*. (A) A pupal wing (24 hours APF) taken from a wild-type animal. Expression of *dpp* is restricted to vein primordia. (B) Strong reduction of *dpp* expression from veins in a pupal wing (24 hours APF) taken from an *UAS-ΔD-Raf^{F20/+}; GAL-4^{604/+}* animal. The loss of *dpp* correlates with the suppression of veins in adults of the same genotype (compare to Fig. 4A). (C) *dpp* pattern of expression in *scaGal-4/UAS-KM-Raf^{2.1}* flies raised at 29°C. Arrowheads point to the ectopic *dpp* expression in areas near the wing margin where *scaGal-4* is expressed at late stages (see Fig. 5F).

In the *aos^{sty1}* enhancer trap line, X-Gal staining appears to be restricted to the presumptive vein primordia throughout wing development (Sawamoto et al., 1994). Nonetheless, by *in situ* hybridization, we found that the expression of *aos* mRNA precisely follows the pattern of MAPK activation (Fig. 3). During pupal development, *aos* is first expressed in vein territories until 24 hours APF (Fig. 6A). From this time onwards, expression of *aos* in veins fades away while strong levels accumulate in the intervein tissue (Fig. 6B). To test if *aos* expression depends on MAPK activity, we overexpressed the *UAS-ΔD-Raf^{F20}* transgene with the *GAL-4⁶⁰⁴* insertion. In this condition, *aos* at 30 hours APF is expressed in all vein territories that are later transformed to interveins (Fig. 6C). This strongly suggests that the expression of *aos* is activated and maintained through the action of the Ras signaling.

We next overexpressed *Aos* with a *UAS-Aos* transgene under the control of the *GAL-4⁶⁰⁴* insertion. In this condition, wing veins developed normally and, in some cases, we found some extra vein tissue (Fig. 6D). This is reminiscent of those ectopic veins observed after the overexpression of dominant negative DER or Raf (see Fig. 5A,F). The overexpression of *Aos* during the larval period using the same transgene resulted in

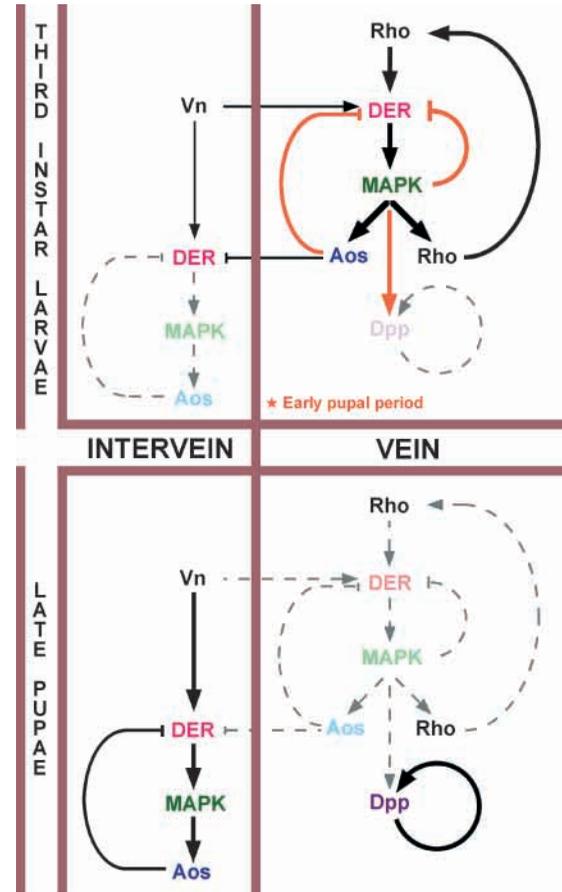


Fig. 8. A model for a developmental switch for DER signaling during wing development. During early larval development, we postulate that the DER pathway becomes activated in the vein presumptive territories by the actions of Vn and Rho (this one mediating the action of a still unknown ligand). This activation leads to a reinforcement of the expression of *rho*, triggering a positive feedback loop, that results in the allocation of vein cell fates. The DER signaling cascade induces *aos* expression, that starts to accumulate in vein territories. *Aos*, a secreted molecule, could diffuse from the provein territories limiting the action of the DER cascade to narrow domains centered around the veins. Early in pupal periods, DER expression in veins is transcriptionally downregulated as a consequence of the high levels of MAPK activity. This reduction in DER, together with the presence of *Aos* in vein territories, entirely suppresses the activity of the MAPK pathway in veins. As we have shown, this reduction is necessary for the differentiation of vein cells. In parallel, MAPK activity builds up in intervein territories, probably due to the presence of Vn, a weak DER activator. As a consequence, *aos* expression relocates to intervein territories. The final differentiation of vein cells depends on the action of the secreted molecule Dpp. The expression of *dpp*, triggered in veins by DER signaling at early pupal periods, is maintained through an autoregulatory loop. Our data suggest that this autoregulation is inhibited by DER in intervein cells, enforcing a refinement of vein territories.

suppression of veins (data not shown). These results suggest that a negative feedback loop mediated by *aos* is not responsible for the suppression of vein differentiation by Raf signaling.

D-Raf signaling represses *decapentaplegic* expression during pupal development

The mechanisms involved in the differentiation of wing cells are largely unknown. Detailed genetic analysis has demonstrated that *dpp* is essential for vein differentiation (Yu et al., 1996; De Celis, 1997). In the absence of *dpp* signaling during pupal stages, the vein cells do not differentiate, but rather form intervein structures. The activation of *dpp* expression, which initiates in wing vein territories from 18 hours APF appears to be mediated by the activity of DER (De Celis, 1997). Therefore, we have monitored the expression of *dpp* subsequent to altering Raf activity during pupal period. We have found that the overexpression of the *UAS-ΔD-Raf^{f20}* transgene with the *GAL-4⁶⁰⁴* insertion strongly reduces the expression of *dpp* at 20–24 hours APF (compare Fig. 7B to A). This reduction correlates with the suppression of vein territories shown above (Fig. 4A). Conversely, the downregulation of RTK signaling during pupal stages (using the *UAS-KM-Raf^{f2.1}/sca-GAL-4* combination) triggers ectopic *dpp* expression (Fig. 7C). These results suggest that the repression of vein differentiation by Raf activity during pupal stages is likely mediated by the downregulation of *dpp* signaling.

DISCUSSION

Vein cell fate specification

A very precise regulation of DER activity must be achieved to control its multiple roles triggering the development of different cell types. This regulation can be controlled at two levels: by transcription and by localized expression of its ligands. In *Drosophila*, one activating ligand for DER is Spitz, a transmembrane protein, that may be cleaved and act as a diffusible ligand (Schweitzer et al., 1995; Golembo et al., 1996). Based on the results of genetic epistasis, it has been proposed that the processing of Spitz requires the action of Rho. Vn, an additional ligand for DER, is a secreted growth factor. Genetic evidences suggest a functional link between both DER ligands; e.g. a reduction in *spitz* levels significantly enhances the lack of muscle precursors in *vn* mutant embryos (Yarnitzky et al., 1998). The levels of activated MAPK induced by the addition of secreted Spitz to DER-transfected S2 cells are significantly higher compared to those induced by Vn (Schnepp et al., 1998). This lower activation of DER by Vn may convey qualitative differences in terms of gene expression. Accordingly, it has been shown that unlike Spitz, Vn overexpression does not induce Aos expression in muscle founders (Yarnitzky et al., 1998). Furthermore, in some systems Vn appears to act after Spitz; e.g. *vn* mRNA in the somatic mesodermal cells follows Rho expression in the embryonic muscle progenitors (Buff et al., 1998). In addition, secreted Spitz emanating from the midline triggers the expression of *vn* in the ventral-most cells of the embryonic ectoderm (Golembo et al., 1999).

It has been previously demonstrated that signaling from DER is required for the establishment of ‘vein competency’ and the specification of wing vein cells (Clifford and Schüpbach, 1989; Díaz-Benjumea and García-Bellido, 1990; Sturtevant et al., 1993; Díaz-Benjumea and Hafen, 1994). However, DER is widely expressed throughout the wing and

the regulation of the activity of the signaling cascade is thought to be accomplished by localized release of its ligands. While *spitz* is ubiquitously expressed all over the wing pouch, *rho* is expressed in narrow vein stripes in all vein primordia and *vn* is expressed first in a broad central region and then in most intervein cells. A direct role for Spitz in wing development has not yet been revealed and, indeed, clones of null alleles of *spitz* do not have wing defects (Nagaraj et al., 1999). *rho*, however, is necessary in conjunction with DER for the specification of vein cells (Sturtevant et al., 1993) and directs the local activation of the DER signaling pathway (Fig. 1). Nonetheless, the complete suppression of *rho* expression in wing discs (in a *rho^{ve}* mutant) does not result in a full repression of MAPK activity from presumptive vein territories. This suggests that the activation of MAPK depends on an additional input. Indeed, MAPK activation in wing veins also requires the presence of Vn (Fig. 1). In all, these results lead us to suggest a model in which Vn is required to supplement Rho activities in the specification of vein cells. Vn could act synergistically or sequentially to Rho to provide a low, but continuous level of DER activation. Beyond reinforcing the DER pathway activity in neighbouring vein cells, Vn expression could provide an autocrine function in interveins where it will direct low level activation of DER and be necessary for intervein cell specification.

An early positive feedback loop

Signals passing between adjacent sectors in the wing primordia activate the expression of ‘vein-organizing genes’ (as *rho*) in sharp vein stripes. These genes would be involved in the activation of secondary signals and the development of pro-vein territories (Biehs et al., 1998). The crossregulation between ‘vein-organizing genes’ and late effectors is thought to establish and refine the vein pattern (e.g. Roch et al., 1998).

rho expression and the specification of vein cells can be compromised by different mutations affecting distinct vein-promoting activities (Sturtevant and Bier, 1995). Among the genes involved in the activation of *rho* expression is *vn*. *vn* allelic combinations reduce the expression of *rho* in the wing. Moreover, insufficient levels of *D-Raf* expression strongly correlate with a general failure to express *rho* (Fig. 2). These results indicate that *rho* expression is downstream of RTK signaling, and is therefore likely to be downstream of DER signaling activity. These findings are consistent with the regulation of *rho* expression in the ovary, where the activation of DER by Gürken, an ovary-specific DER ligand, leads to *rho* expression activation and the determination of the dorsal follicle cells (Sapir et al., 1998).

rho expression is ectopically induced following general DER signaling activation. Interestingly, *rho* is upregulated only in areas in proximity to the veins (Fig. 2C,D). This suggests that there may be a convergent signaling pathway providing spatial and temporal information. An elevation of vein ‘competence’ via increased DER signaling would permit the development of extra vein cells upon input of a localized second signaling pathway. In accordance with this model, one would predict that the ectopic vein tissue produced by artificially elevated RTK signaling during the second instar would be found in close proximity to normal veins, as it is observed, rather than randomly distributed in the wing blade. We suggest that the function of a positive regulatory loop

between DER and *rho* will be the reinforcement of the activation of the pathway that will eventually result in the establishment of vein competent territories.

A biphasic response to DER/D-Raf signaling

It has been recently demonstrated the reiterated use of DER as a common effector of differentiation. In the *Drosophila* eye, DER is required for the determination of all cell types. In this system, cell fate depends on the developmental stage when the receptor is activated (Freeman, 1996).

By interfering with DER signaling activity, it can be found that the specification of veins respond to the activation of RTK signaling during larval stages, but that continued activation of RTK signaling results in a failure of vein cells to differentiate (see Fig. 4). One explanation for these opposite effects could be that early activation of RTK signaling would specify vein cells, while late RTK signaling would implement intervein cell fates. Several observations provide support for this model.

In pupae, MAPK is repressed in veins and activated in intervein cells. This activation of MAPK (and the expression of downstream genes, such as *argos*) responds to Ras signaling activity (Figs 4B, 6C), and appears to be involved in the suppression of vein cell fates. Indeed, after ectopical activation of D-Raf during the pupal period, promoting intervein cell fates, the MAPK activity remains stimulated all over the wing blade (Fig. 4B).

It seems that DER is the only receptor tyrosine kinase at work in the wing, able to activate Ras and Raf. While DER is ubiquitously expressed during larval imaginal disc development, DER mRNA levels are downregulated in the pupal period in presumptive vein cells (Sturtevant et al., 1994). This downregulation of DER could be involved in the suppression of MAPK activity in vein territories (Fig. 3). Furthermore, when a DN-DER molecule is overexpressed, titrating the endogenous DER, in pupae, extra vein tissue is induced (Fig. 5A). MAPK dephosphorylation in veins could also be induced by other mechanisms; for instance, the early expression of the inhibitor ligand Argos in veins up to 24 hours APF (Fig. 6A) could cooperate in the inactivation of MAPK in these territories.

What is the function of this change of expression? The first effect of this developmental switch is a modification in the expression of downstream targets. As a consequence of the reduction in MAPK activity from vein cells, *aos* is eliminated from veins between 24 and 30 hours APF. Conversely, it is upregulated in intervein territories (see Fig. 6). This scenario is reminiscent of the induction of DER ligands in the ventral ectoderm. Here, the primary signal, Spitz induces a relay mechanism by triggering the expression of Vn (and Aos) in adjacent cells. Aos reduces the overall level of DER signaling, whereas Vn provides a lower level of activation, capable of inducing only the lateral cell fates (Golembo et al., 1999). In the larval wing, high levels of DER signaling are achieved in veins through a positive feedback loop (see above). Here, DER activity promotes the expression of Aos. We suggest that Aos diffusion from veins could prevent adjacent cells from responding to the vein inductive signals and producing high levels of DER activity ('remote inhibition' – Freeman, 1996). Consistently, *aos* mutant flies display small deltas and extra veins clustered around vein territories. On the contrary, Aos overexpression in larval stages induces the suppression of veins

(Sawamoto et al., 1994). We also propose that, in pupae, while DER activity (and Aos) in veins are lost, Vn and Aos expression in intervein cells will reach a competitive balance leading to the activation of DER and MAPK, and intervein cell specification (see above and Fig. 8).

In an alternative model, different threshold requirements for DER signaling might be necessary at different stages during the development of the wing. DER activity will promote vein specification and induce a negative feedback loop at all times (i.e. by inducing the overexpression of Aos). In this scenario, ectopic veins resulting from the downregulation of the pathway (see Fig. 5) would be a consequence a reduction of DER activity by just the right amount to eliminate negative feedback loops without disrupting positive effector functions. These positive functions would eventually be implemented by a signaling event dependent on DER, but independent of Raf/MAPK activity. Is worth noting that this model is not supported by the consequences of Aos overexpression in pupal stages. At these stages, Aos ectopic expression does not result in the suppression of vein fates (Fig. 6D).

Cross talk between *dpp* and DER signaling during pupal development

Several types of cell-cell communication have been proposed to be required during the latter stages of pupal wing development. The *dpp* gene encodes a member of the TGF β superfamily and is expressed during early pupal development in vein primordia (Yu et al., 1996). A class of loss-of-function *dpp* alleles and certain combinations of Dpp receptor mutants lead to vein-loss phenotypes (Burke and Basler, 1996). Mosaic analysis of *dpp*^s allele show that mitotic clones affect the differentiation of veins. Meanwhile, the effects of overexpression of *dpp* or an active form of its receptor *thick veins* (*tkv*) indicate that Dpp directs vein differentiation through activation of Tkv in pupal stages (De Celis, 1997).

The initiation of *dpp* expression in pupal stages depends on the activity of early acting genes, and in particular DER activity (Yu et al., 1996; De Celis, 1997). However, although DER signaling is downregulated in vein territories during pupariation, *dpp* expression is maintained through an autoregulatory loop and remains high in vein cells until their final differentiation. Interestingly, in intervein cells, *dpp* expression is not activated in response to the DER activity described above. On the contrary, these cells express *short gastrulation* (*sog*), a gene that exerts an opposing effect to *dpp* (François et al., 1994). *sog* plays a role restricting vein formation to the center of the provein regions. *dpp* and *sog* interact antagonistically during vein differentiation (Yu et al., 1996). Ectopic activation of DER signaling in pupal stages abolishes *dpp* expression from veins (Fig. 7). This suppression of *dpp* correlates with the loss of veins observed in this condition and it is reminiscent of the effect of Sog overexpression in pupal wings (Yu et al., 1996). Moreover, vein plexates induced by compromising DER activity in pupal wings, associate with a broadening of *dpp*-expressing areas. We suggest that DER signaling downregulation from vein territories allows *dpp* to autoregulate its expression (Fig. 8). It remains to be determined whether *sog* expression depends on DER in intervein territories, or is a consequence of the activity of intervein-specific genes such as *blistered* (Roch et al., 1998).

The model presented here on how a single receptor (DER),

triggering a conserved signal transduction pathway, is used reiteratively to implement two different cell fates in the development of the fly wing serves to reconcile many observations that have been made regarding cell fate specification in the wing. This may well provide a paradigm for the regulation of DER signal transduction in other developmental events.

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