ventral veinless, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in *Drosophila*

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SUMMARY

Cell migration is an important step in a variety of developmental processes in many multicellular organisms. A particularly appropriate model to address the study of cell migration is the tracheal system of *Drosophila*, whose formation occurs by migration and fusion from clusters of ectodermal cells specified in each side of ten embryonic segments. Morphogenesis of the tracheal tree requires the activity of many genes, among them breathless (btl) and ventral veinless (vvl) whose mutations abolish tracheal cell migration. Activation of the btl receptor by branchless (bnl), its putative ligand, exerts an instructive role in the process of guiding tracheal cell migration. vvl has been shown to be required for the maintenance of btl expression during tracheal tree formation. Here we show that, in addition, vvl is independently required for the specific expression in the tracheal cells of thick veins (tkv) and rhomboid (rho), two genes whose mutations disrupt only particular branches of the tracheal system. Indeed, we show that expression in the tracheal cells of an activated form of tkv, the putative decapentaplegic (dpp) receptor, is able to induce shifts in their migration, asserting the role of the dpp pathway in establishing the branching pattern of the tracheal tree. In addition, by ubiquitous expression of the btl and tkv genes in vvl mutant embryos we show that both genes contribute to vvl function. These results indicate that through activation of its target genes, vvl makes the tracheal cells competent to further signalling and suggest that the btl transduction pathway could collaborate with other transduction pathways also regulated by vvl to specify the tracheal branching pattern.

Key words: *Drosophila*, ventral veinless, decapentaplegic, rhomboid, cell migration, tracheal development

INTRODUCTION

Cell migration occurs in a variety of developmental processes in many multicellular organisms. For migration to occur, cells need to become specifically differentiated to acquire the functions implicated in cell motility (for a review, see Laufkenburger and Horwitz, 1996). Moreover, cells migrate in an ordered pattern and thus they also have to set their appropriate direction of migration, probably by the recognition of specific cues. The tracheal system of *Drosophila* is a particularly appropriate model with which to address these issues since formation of the tracheal tree occurs by cell migration and cell fusion from the tracheal placodes (reviewed by Manning and Krasnow, 1993). The tracheal placodes are clusters of ectodermal cells specified in each side of ten embryonic segments (from the second thoracic segment to the eighth abdominal segment). The function of the tracheless (trh) gene is crucial for this process and it has been proposed to act as an inducer of tracheal cell fates. Not only is trh necessary for tracheal fate determination, but also many specific tracheal markers are induced after ectopic expression of trh (Isaac and Andrew, 1996; Wilk et al., 1996).

*btl*, the *Drosophila* homologue of the FGF receptor, is one of the genes whose expression in the tracheal cells seems to be induced by trh (Wilk et al., 1996). Conversely to trh, btl itself is not necessary for tracheal cell determination since tracheal pits form in btl mutant embryos. Instead, btl is specifically required for migration of the tracheal cells; tracheal tree formation is abolished in btl mutant embryos because the tracheal pits form abnormal cavities that remain disconnected (Klämbt et al., 1992). Thus, differentiation of specific cell motility functions mediated by btl are linked to the genetic program of tracheal cell induction under the control of the trh gene.

Recent studies support the notion that spatial regulation of the btl receptor activity exerts an instructive role in the process of guiding tracheal cell migration (Lee et al., 1996). Further support for this hypothesis has been provided by the identification of branchless (bnl), the gene coding for the putative ligand of the btl receptor (Sutherland et al., 1996). bnl is expressed dynamically in clusters of cells surrounding the developing tracheal system at each position where a new branch will form and grow out. Moreover, localised misexpression of bnl can direct branch formation and outgrowth to new positions (Sutherland et al., 1996).

Besides btl and bnl, vvl, the gene encoding the Cf1α transcription factor, is also required for tracheal cell migration – vvl has been renamed drifter (dfr) by Johnson and collaborators (Anderson et al., 1995). The vvl mutant phenotype has already been described (Anderson et al., 1995; de Celis et al., 1995); in vvl mutant embryos, the tracheal pits do form but their cells do not migrate to form the tracheal tree, as is the case in btl mutant...
embryos. Although btl and vvl are not required for setting the initial expression of each other in the tracheal placodes (de Celis et al., 1995), recent results have demonstrated a role for vvl in the maintenance of btl expression at high levels during tracheal formation (Anderson et al., 1996).

We have investigated the possibility that vvl might have additional roles in tracheal migration besides maintaining btl expression. Actually, these recent studies have also suggested the existence of additional factors that may modulate the effects of bnl or function with it to specify the branching pattern (Sutherland et al., 1996). We have found that indeed vvl is independently required for the specific expression of tkv and rho in tracheal cells. Since tkv codes for a putative dpp receptor (Brummel et al., 1994, Nellen et al., 1994; Benton et al., 1994) while rho codes for a transmembrane protein involved in the EGF transduction pathway (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993), our results suggest that through activation of its target genes, vvl makes the tracheal cells competent to further signalling. Indeed, tkv and rho mutant embryos display different defects in particular branches of the tracheal system (Bier et al., 1990; Affolter et al., 1994). Furthermore, here we show that expression of the activated tkv receptor in the tracheal cells is able to induce shifts in the branching of the tracheal tree, unveiling a new role for the dpp pathway in tracheal cell migration. Altogether our results point to a situation where more than a transduction pathway concur in the process of tracheal migration.

MATERIALS AND METHODS
Fly strains
We have used the following alleles: btlEG10 (Glazer and Shilo, 1991), vvl6A3 (de Celis et al., 1995), dpfEG2 (Anderson et al., 1995, 1996) tkv7 and tkvA (Nüsslein-Vöhlard et al., 1984; Nellen et al., 1994) and trh7 (Wilk et al., 1996). We used the H82 enhancer trap line that drives β-gal expression under the control of the btl promoter (Glazer and Shilo, 1991). Ectopic expression of either dpp or a constitutive form of the tkv receptor was achieved with the GAL4 system (Brand and Perrimon, 1993) using UAS-dpp lines (Capdevila and Guerrero, 1994) and UAS-tkv lines (Lecuit et al., 1996) activated either by a ptc-GAL4 line (Hinz et al., 1994), by the ubiquitous 69-GAL4 line (Brand and Perrimon, 1993) or by a hs-GAL4 line (from A. Brand). Activation in the tracheal placodes was achieved with btl-GAL4 lines (from S. Hayashi). The hs-btl line was provided by D. Montell and the hstk line by K. Basler. To identify homozygous mutant embryos we used blue balancers of the second and third chromosome: CyO-hb-lacZ, TM2-lacZ, TM3-ftz-lacZ and TM3-hb-lacZ.

Immunohistochemistry and in situ hybridisation
We used antibody #55 and #84 (provided by B. Shilo) and mAb2A12 (provided by N. Patel) which recognise the lumen of the tracheal tree. Embryos were stained according to standard protocols using the Vectastain ABC kit. However, embryos labelled with mAb2A12 were fixed for 40 minutes. Whole-mount in situ hybridisations were done following the method of Tautz and Pfeifle (1989) with minor modifications. Detection of β-gal with a specific antibody (Cappel) was performed to identify embryos carrying a blue balancer. An anti-rho antibody was kindly provided by Dr. E. Bier. A tkv probe was generated from a Neul/Clal fragment of the STK-A plasmid containing the tkv CDNA (Nellen et al., 1994).

Heat-shock treatments
Embryos were collected for 1 hour at 25°C. After 4 hours of development at 25°C, they were transferred to 37°C for 45 minutes. Subsequently, they were allowed to develop and were fixed and labelled with an antibody recognising the tracheal lumen.

Production of antiserum
To raise an antibody against vvl protein, the vvl DNA fragment that had been used previously as a probe (de Celis et al., 1995) was subcloned in PGEX-4T-1 and a protein containing the last 116 amino acids of the vvl peptide was produced. After induction and partial purification, this protein was used to raise rat polyclonal antibodies. The protein pattern detected with this antiserum is the same as the RNA expression pattern detected by in situ hybridisation with a vvl probe. Moreover, this antiserum fails to detect any protein expression in homozygous vvl6A3 embryos. Altogether, these results confirm the specificity of the antibody for the vvl protein.

RESULTS
dpp signalling recruits cells to form tracheal placodes and to express vvl
Development of tracheal placodes is induced by the expression of trh in ten pair of clusters in the ectoderm (Isaac and Andrew, 1996; Wilk et al., 1996). Another gene, vvl, which is also expressed at the same position independently of trh (Isaac and Andrew, 1996, Fig. 1C), is specifically required for tracheal cell migration; in vvl mutant embryos tracheal pits do form but their cells do not migrate (Anderson et al., 1995; de Celis et al., 1995). Thus, both formation of tracheal placodes (mediated by trh) and vvl expression are likely to occur independently in the same cells directed by the same positional cues. We have shown that dpp is one of such cues determining vvl expression along the embryonic dorsoventral axis; expansion of dpp expression results in an increased recruitment of cells to express vvl (de Celis et al., 1995; and Fig. 1E). These cells are allocated in the expanded tracheal placodes indicating that expansion of dpp expression along the dorsoventral axis causes a concomitant enlargement of the tracheal placodes and of vvl expression. We obtained similar results by ectopic expression of a constitutive form of tkv (Lecuit et al., 1996), a putative dpp receptor, indicating that early activation of the tkv receptor can induce vvl expression (Fig. 1F). However, vvl expression is normal in the tracheal placodes of vvl mutant embryos (Fig. 1B) arguing that zygotic expression of tkv is not involved in this process. Altogether, these results indicate that vvl activation and the setting of the tracheal placodes by dpp is mediated either by the early maternal component of tkv or by another unknown receptor.

vvl regulates the expression of tkv and rho in the tracheal cells
Migration of tracheal cells is dependent on the spatial regulated activity of the btl receptor, the Drosophila homologue of the FGF receptor, by its putative ligand, the product of the bnl gene (Klämbt et al., 1992; Lee et al., 1996; Sutherland et al., 1996). Although btl and vvl are not required for setting the initial expression of each other in the tracheal placodes (de Celis et al., 1995), vvl is required for the maintenance of btl transcripts at high levels during tracheal migration (Anderson et al., 1996), which could account for the vvl mutant phenotype.

We have analysed the possibility that vvl might have additional roles in tracheal migration besides maintaining btl expression since it has been suggested that additional factors may exist that would modulate the effects of bnl or function with it to specify the branching pattern (Sutherland et al., 1996). tkv is one of the genes expressed specifically in the tracheal pits and tkv mutant embryos display a particular failure
of the tracheal cells to migrate in the dorsal and ventral directions (Fig. 3B). Indeed, we have found that vvl is required for the specific expression of tkv in the tracheal pits (Fig. 2B) and thus tkv appears to be a target of vvl in the tracheal pits. tkv is expressed in the embryo in a regulated pattern in such a way that specific structures have a distinct level of expression (Affolter et al., 1994). However, it has also been shown that expression of tkv under the level of detection by in situ hybridisation techniques is sufficient to provide early embryonic wild-type gene function (Ruberte et al., 1995). Thus, while it is not possible to conclude that there is no tkv expression in the tracheal placodes of vvl embryos, we can establish that vvl is required for the specific tracheal expression of tkv. Accordingly, putative binding sites for the vvl transcription factor have been found in the upstream region of the tkv gene (Russell and de Celis, personal communication).

rho is another gene required for tracheal migration; rho mutations produce breaks and deletions mainly in the longitudinal dorsal trunks (Bier et al., 1990 and Fig. 3C,D). Similarly to tkv, rho is expressed in the tracheal pits (Bier et al., 1990 and Fig. 2E) but in the mutant not all tracheal migration is impaired, suggesting again that rho activity is only required in some tracheal cells. Therefore, we have analysed whether rho expression could be another target of vvl regulation and found that indeed vvl is required for appropriate rho expression in the tracheal placodes (Fig. 2F). However, rho is still expressed in other regions of vvl mutant embryos, pointing to a specific requirement of vvl activity for rho tracheal expression. Interestingly, vvl has also been shown to be required for rho expression in the wing vein primordia (Sturtevant and Bier, 1995), suggesting a conservation of the mechanism of regulation in the development of different structures.

tkv and rho tracheal expression is not affected in btl mutant embryos

As mentioned above, vvl is required for the maintenance of btl transcripts at high levels during tracheal migration (Anderson et al., 1996) and thus the defective expression of tkv and rho in vvl mutant embryos could be an indirect consequence of the failure to maintain btl expression in such embryos. Hence, we have analysed tkv and rho expression in btl mutant embryos. In contrast to the results obtained for vvl, tkv and rho appear to be normally expressed in the tracheal placodes of btl mutant embryos (Fig. 2C,G), indicating that the regulation of tkv and rho by vvl is independent of its effect on btl expression and suggesting that vvl might have additional roles in tracheal cell migration by means of regulation of other subordinate genes.

dpp signalling drives migration of tracheal cells along the dorsoventral axis

Our results point to tkv as a target of vvl and suggests that the failure to activate the tkv receptor in the tracheal placodes could
Fig. 2. tkv and rho tracheal expression in wild-type and mutant embryos. (A) tkv is expressed in the embryo in a regulated pattern in such a way that the tracheal pits (tp) have a distinct level of expression as detected by whole-mount in situ hybridisation (arrow). (B) Lack of tkv expression in the tracheal pits of mutant embryos for vvl^{vvl^3}, a null allele of vvl. Note that tkv expression is normal in cells of the mesoderm (as described by Affolter et al., 1994). (C) In contrast to the results obtained for vvl mutant embryos, tkv appears to be expressed normally in the tracheal pits of btl mutant embryos. (D) Homozygous embryos for dfr^{E82}, a hypomorphic vvl mutation, retain some tkv expression in the tracheal pits (arrow). (E) rho expression in a wild-type embryo as detected by an anti-rho antibody; at germ band extension rho is specifically expressed in the tracheal pits (arrow) and in cells that are considered to be the precursors of chordotonal organs (c; Bier et al., 1990; arrowhead). (F) vvl mutant embryos do not exhibit the distinct expression of rho in the tracheal pits, however expression is retained in the presumed chordotonal precursors cells. (G) In contrast to the results obtained for vvl mutant embryos, rho appears to be normally expressed in the tracheal pits of btl mutant embryos.

Fig. 3. tkv and rho mutant tracheal phenotype. (A) The tracheal system of a wild-type embryo as visualised by the antibody #55 or 84 which recognises the lumen of the tracheal tree. (B) tkv mutant embryos display tracheal tree defects consisting mainly of a failure to develop some particular branches in the dorsoventral axis. However, other tracheal branches in the anteroposterior axis still form in those embryos, although they may display an occasional gap. (C,D) rho mutant embryos display tracheal tree defects consisting mainly of breaks and deletions in the longitudinal dorsal trunk.
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... contribute to the vvl mutant phenotype. We have therefore addressed the question of what could be the role of the tkv receptor in tracheal migration. In spite of the general expression of tkv in the tracheal pits, its mutant phenotype is restricted to just some tracheal branches, arguing that activation of the tkv receptor is only required in a subset of the cells where it is expressed. This suggests that it is the limited distribution of the ligand that is responsible for the restricted activation of the receptor. In fact, dpp, the ligand for the tkv receptor, has been reported to be expressed in some cells just dorsal and ventral to the tracheal pits. This raises the possibility that it is the localised signalling of dpp from these nearby cells that directs some tracheal cells to migrate in the dorsoventral axis (Affolter et al., 1994). In order to assess this possibility we have examined the effect of ectopically expressing dpp on tracheal cell migration.

As described before, ectopic expression of dpp results in an enlargement of the tracheal placodes and of vvl expression. Interestingly, we also observe that ectopic expression of dpp has a later effect in tracheal development: only dorsoventral tracheal branches arise while longitudinal branches fail to form (Fig. 4B). This phenotype appears as the counterpart of that occurring in tkv mutant embryos (Fig. 3B), suggesting a specific effect of dpp on tracheal branching. Indeed, the shift on the branching of the tracheal tree is specifically due to changes in cell migration because nuclei of the tracheal cells can be detected in the dorsoventral axis but not between the original tracheal pits, as visualised with the H82 enhancer trap line (Klämbt et al., 1992) (Fig. 4F). Moreover, this phenotype is specifically due to dpp signalling as assessed by evaluating the role of the tkv receptor in the process: a reversion of dorsoventral migration and a restoration of migration in the anteroposterior axis can be observed in embryos with ectopic dpp expression (Fig. 4D).

**Specific activation of the tkv receptor in the tracheal cells induce shifts in their branching pattern**

Thus, under circumstances where all the tracheal cells should both receive the dpp signal and express the tkv receptor, cells migrate only dorsoventrally and no cells are detected along the anteroposterior axis. Similarly, expression of a constitutive form of the tkv receptor can also generate the same kind of tracheal pattern when ubiquitously expressed with the UAS/GAL4 system (Fig. 4C). Altogether, these results suggest that tkv activation in the

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**Fig. 4.** dpp signalling induces directed migration of tracheal cells along the dorsoventral axis. (A) The tracheal tree of the *Drosophila* embryo at last stages of embryogenesis as visualised by an antibody recognising a lumen epitope (#55 or 84). (B) Ubiquitous expression of dpp causes a modification of the tracheal tree organisation such that only dorsoventral tracheal branches arise while longitudinal branches fail to be formed. (C) The same change in the tracheal tree is generated by ubiquitous expression of a constitutively active tkv receptor. (D) The tracheal phenotype caused by ubiquitous expression of dpp is specifically due to dpp signalling since a reversion of dorsoventral migration and a restoration of migration in the anteroposterior axis can be observed in embryos with ectopic dpp expression that are also mutant for tkv. (E) The H82 enhancer trap line allows the visualisation of the nuclei of the tracheal cells. (F) The shift of the branching of the tracheal tree caused by ubiquitous expression of dpp is specifically due to changes in cell migration, as nuclei of the tracheal cells can be detected in the dorsoventral axis but not between the original tracheal pits, as visualised with the H82 enhancer trap line. (G) Specific tracheal expression of a constitutively active tkv receptor induced by means of a btl-GAL4 line results in a failure of the tracheal cells to migrate anteroposteriorly to form the dorsal trunk.
tracheal placodes determines migration of tracheal cells to form specific dorsalventral branches. To establish that tkv activity is specifically required in the tracheal cells, the constitutively active tkv receptor was induced in those cells by means of a line in which GAL4 is under the control of btl. Indeed, expression of this form of the activated tkv receptor in all the cells of the tracheal placodes shifts the branching of the tracheal tree; in particular the tracheal cells fail to migrate anteroposteriorly to form the dorsal trunk (Fig. 4G). This phenotype is less severe than the one observed when the constitutive tkv receptor is expressed all over the embryo (Fig. 4C); in particular the visceral branches and the lateral trunk do usually form, although there are some embryos that exhibit a more extreme phenotype in which these anteroposterior branches are also abolished. Activation of the tkv receptor in cells other than the tracheal cells could account for the different phenotypes upon induction by an ubiquitous promoter or by a tracheal specific promoter. Besides, differences on the level or timing of dpp signalling could also explain the observation that distinct branches are differentially inhibited when different GAL4 lines are used. In sum, irrespective of a possible role of tkv for tracheal migration outside the tracheal cells, our experiments with the btl-GAL4 line show an autonomous effect of tkv activation in the tracheal cells for their correct migration.

Ubiquitous expression of btl and tkv in vvl mutant embryos

The results presented hitherto indicate that vvl regulates tkv expression in the tracheal pits and that activity of the tkv receptor in the tracheal pits has an effect on the branching pattern of the tracheal tree. In addition, as already mentioned, vvl has a role in the maintenance of btl transcripts at high levels (Anderson et al., 1996). However, the significance of these different roles of vvl for the process of tracheal migration remains unclear. While tkv appears to be a target of vvl regulation it is not clear whether its mere activity would be sufficient to confer some migration on tracheal cells in the absence of vvl function. Alternatively, other targets of vvl could also be required for cells to migrate. To discriminate between these possibilities we have expressed the tkv constitutive receptor in vvl mutant embryos to examine whether it could partially restore migration. No substantial migration was detected in those vvl mutant embryos upon expression of a constitutively active form of the tkv receptor in the same conditions that were sufficient to completely induce dorsoventral migration in a wild-type background (not shown). The same results were obtained even when the experiments were performed at 29°C (GAL4 seems to be more effective at this temperature) and more than one copy of the UAS-tkv expression was present in the genome. These results indicate that other functions of vvl, and probably its role in maintaining btl transcripts, are still required to substantiate the dorsoventral migration induced by tkv activity.

In contrast to the results obtained with tkv, ubiquitously expressed btl protein under the control of a heterologous promoter has been reported to be able to rescue the tracheal phenotype of a vvl mutation (Anderson et al., 1996), which contests the significance of tkv regulation by vvl for tracheal migration. Interestingly, the rescue of the vvl phenotype by ubiquitous expression of btl was obtained using a particular mutation of vvl (dfrE82), a mutation that has been shown to retain some vvl function (Anderson et al., 1996). Indeed, we have found that dfrE82 mutant embryos retain some tkv expression in the tracheal pits (Fig. 2D), suggesting that this residual expression could contribute to the ability of btl to restore tracheal migration in these embryos. Further support for this hypothesis comes from our experiments in which upon ubiquitous btl expression in embryos mutant for a null allele of vvl, we did not observe the same rescued phenotype as reported for the dfrE82 mutation.

To further prove the functional significance of tkv regulation by vvl we have analysed whether tkv and btl activity could provide some migration capability to the tracheal cells of vvl mutant embryos. In a first set of experiments, we expressed btl and tkv under the control of a heat shock promoter in vvl mutant embryos. As mentioned above, btl expression on its own produces a very weak rescue of the tracheal defects of vvl null mutants (Fig. 5B). However, this rescue is significantly enhanced when both tkv and btl are expressed in such embryos (Fig. 5C); in particular, there is an increase in the formation of dorsal and ganglior branches, which are branches that are missing in tkv mutant embryos. Similarly, expression of a constitutive form of the tkv receptor does not produce a substantial migration of tracheal cells in vvl mutants unless it is coexpressed with the btl receptor, in which case we can detect significant migration of tracheal cells along the dorsoventral axis (Fig. 5D). Thus, expression of neither tkv or btl on their own can substitute for the lack of vvl function but coexpression of both of them partially restores tracheal migration. All these results indicate that regulation of its different target genes by vvl is functionally significant for the process of tracheal migration. Besides, they also indicate that vvl might exert other functions in addition to those of tkv and btl as the rescue we observe is not complete.

DISCUSSION

Tracheal tree formation occurs by cell migration and cell fusion from the tracheal placodes (Manning and Krasnow, 1993). btl and vvl are both required for formation of the tracheal tree but they are not involved in tracheal placode determination. Some tracheal markers are expressed in vvl and btl mutant embryos indicating that some differentiation takes place in those tracheal cells but one of the characteristic of tracheal cells, its ability to migrate, is impaired. Thus, both btl and vvl appear to be specifically required in the tracheal cells for their migration. Recent work has made it possible to propose a model where btl activation by its putative ligand bnl is able to direct the migration of the tracheal cells; the localised activation of the btl receptor by the restricted domains of bnl expression from the nearby cells would dictate where tracheal branches would bud, and their direction of migration.

vvl regulates the expression of different target genes active in tracheal development

Since vvl codes for a transcription factor, it is likely that it is involved in regulating the tracheal expression of subordinate genes required for cell migration. Although vvl is not required for setting btl expression in the tracheal cells (de Celis et al., 1995) it has recently been shown that vvl has a role in the maintenance of btl expression in the tracheal cells (Anderson et al., 1996). Thus, the extinction of btl expression in vvl mutant embryos could account for their tracheal phenotype. However, our experiments have identified two additional targets of vvl: one, tkv, is a gene that codes for a putative dpp...
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receptor (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994) and the other \textit{rho}, a gene that codes for a transmembrane protein involved in the EGF transduction pathway (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993). On the contrary, \textit{tkv} and \textit{rho} expression are not altered in \textit{btl} mutant embryos. Hence, the effect of \textit{vvl} on \textit{tkv} and \textit{rho} identify new aspects of \textit{vvl} activity in tracheal cells besides its previously reported role in the maintenance of \textit{btl} expression.

It has to be noted that \textit{vvl} has a more general expression in the embryo that does not always coincide with expression of either \textit{tkv} or \textit{rho}. Besides, it is the tracheal expression of both \textit{tkv} and \textit{rho} that is dependent on \textit{vvl}, emphasising that the interaction between \textit{vvl} and \textit{tkv} and \textit{rho} in the tracheal pits could be tissue specific, possibly requiring other factors. However, at least in the case of \textit{rho}, \textit{vvl} activity is also required for its wing vein expression (Sturtevant and Bier, 1995) pointing to a conserved regulatory mechanism of \textit{rho} expression by \textit{vvl}, linking positional information and cell differentiation (de Celis et al., 1995).

The observation that \textit{vvl} is required for regulation of \textit{tkv} in the tracheal pits together with the phenotypes associated with \textit{tkv} loss-of-function and constitutively activated \textit{tkv} mutations suggest that regulation of \textit{tkv} expression by \textit{vvl} is relevant to the process of tracheal migration. Two additional observations stress the functional significance of \textit{tkv} regulation by \textit{vvl} in the tracheal pits. First, the rescue of the \textit{vvl} tracheal defects by expression of \textit{btl} is achieved in a mutant background where there is still \textit{tkv} expression. And second, the very weak rescue of the tracheal defects associated with \textit{vvl} null mutations by \textit{btl} is significantly enhanced when both \textit{btl} and \textit{tkv} are expressed in such embryos.

Altogether, these experiments suggest that \textit{vvl} is regulating transduction pathways in tracheal development and that its role in tracheal migration could be achieved at least in part by making tracheal cells competent to signalling. However, other functions of \textit{vvl} may also be required for tracheal branching since even coexpression of \textit{tkv} and \textit{btl} do not completely rescue the tracheal phenotype in \textit{vvl} null mutant embryos.

The role of the dpp pathway in tracheal tree formation

Our results suggest that dpp is a substantial cue for determining both the appropriate setting of tracheal primordia and \textit{vvl} expression along the dorsoventral axis. Besides this early role, we have found that dpp is also involved in branching of the tracheal tree. It was already known that migration to form branches along the dorsoventral axis is impaired in \textit{tkv} mutant embryos. Indeed, while \textit{tkv} is expressed in all the tracheal pit its activity seems to be required only in a subset of cells: those migrating to form specific branches in the dorsoventral axis. This observation prompted the suggestion that it is the local distribution of dpp just dorsal and ventral to the tracheal pits that might trigger the activity of the tkv kinase in adjacent cells and contribute thereby to the migration behaviour of tracheal cells (Affolter et al., 1994). However, since dpp signalling is required for dorsal-ventral patterning in the embryo (Irish and Gelbart, 1987), the tracheal phenotype of \textit{tkv} mutant embryos could be due to modification in embryonic polarity. Our experiments support the suggestion of Affolter et al. (1994) and show that activation of the tkv receptor in the tracheal cells has an effect on the morphogenesis of the tracheal tree. Thus, they argue against an interpretation of the tracheal phenotypes of \textit{tkv} mutant embryos as a mere outcome of a general effect of dpp in embryonic patterning. In sum, these experiments support the hypothesis that there is a specific requirement for dpp signalling in the tracheal cells for specification of the branching of the tracheal tree. However, these results do not exclude the possi-

![Fig. 5. Effect of ubiquitous \textit{btl} and \textit{tkv} expression in \textit{vvl} null mutant embryos. Lateral view of embryos labelled with mAb2A12 specific for the tracheal lumen that visualises the tracheal tree. (A) \textit{vvl}\textsuperscript{6A3} mutant embryo; in \textit{vvl} null mutants, there is no tracheal cell migration and the tracheal pits form abnormal cavities that remain disconnected. Note that in \textit{vvl} mutant embryos the mAb2A12 antibody preferentially recognises the posterior tracheal pits. (B) \textit{vvl}\textsuperscript{6A3} mutant embryo carrying a hs-\textit{btl} transgene and subjected to a heat-shock treatment for 45 minutes. Under these conditions, a very weak rescue of the tracheal phenotype can be observed, basically some extension of the lateral trunk. (C) \textit{vvl}\textsuperscript{6A3} mutant embryo carrying a hs-\textit{btl} and a hs-\textit{tkv} transgene and subjected to the same heat-shock treatment as the embryo in B. There is an increase in the rescue of the tracheal phenotype, particularly in the dorsal and ganglionar branches (arrowheads), which are the branches that are dependent on \textit{tkv} function (Affolter et al., 1994). (D) \textit{vvl}\textsuperscript{6A3} mutant embryo carrying a hs-\textit{btl}, a hs-Gal4 and a UAS\textit{tkv*} transgene subjected to the same heat-shock treatment as the embryos in B and C. The concomitant expression of \textit{btl} and a constitutive from of \textit{tkv} restores dorsoventral migration in tracheal cells of \textit{vvl} mutant embryos generating a very similar phenotype to that observed for the expression of a constitutive form of the tkv receptor in an otherwise wild-type embryo (as in Fig. 4C).]
bility that accurate formation of the tracheal tree requires in addition dpp signalling outside the tracheal placodes.

The graded activity of dpp has been proposed as a key factor in generating pattern in some developmental processes (Nellen et al., 1996). Thus, it could be argued that the graded distribution of dpp is also important to establish positional values guiding migration. Alternatively, dpp signalling could single out a particular subset of the tracheal cells specifically modifying them. Accordingly, dpp from nearby cells would reach only some tracheal cells enabling them to recognise a localised cue that would be involved in determining branching activity by the localised expression of bnl, its putative ligand, exerting an instructive role in the process of guiding tracheal cell migration (Sutherland et al., 1996). Independently, here we show that dpp has a role in setting the dorsoventral branching of the tracheal tree. A first step, the activity of dpp (probably mediated by the maternal component of the tkv) and some polarity genes (i.e., wg and hh) are involved in the appropriate setting of the tracheal placode (circle). The same genes are also responsible for the setting of trh and vvl expression (de Celis et al., 1995; Wilk et al., 1996), that respond independently to the same positional cues (Fig. 6A). While initial expression of btl in the tracheal placodes is independent of vvl (de Celis et al., 1995) and seems to depend on trh (Wilk et al., 1996), maintenance of btl expression in the tracheal cells requires vvl (Anderson et al., 1996). Subsequent activation of the btl receptor by bnl exerts an instructive role in the process of guiding tracheal cell migration (Sutherland et al., 1996). Independently, vvl is also required for the regulation of other genes in the tracheal cells, as we have shown is the case for tkv and rho, tkv, whose gene product enables cells to become competent to dpp signalling has a role in dorsoventral branching of the tracheal tree. A similar mechanism could be occurring in other specific branches, since rho codes for a product operating on the EGF-R pathway and whose mutant phenotype affects migration.

**Different transduction pathways operate in the branching of the tracheal tree**

It has been demonstrated that regulation of the btl receptor activity by the localised expression of bnl, its putative ligand, exerts an instructive role in the process of guiding tracheal cell migration (Sutherland et al., 1996). In addition, here we show that vvl is required in the tracheal cells for other functions independent of maintenance of btl expression, namely for setting the expression of tkv, the putative dpp receptor. Furthermore, we also show that dpp has a role in setting the dorsoventral branching of the tracheal tree. How can the additional roles of vvl and the dpp pathway be interpreted in the light of the proposed model of btl activation in tracheal migration?

Interestingly, Sutherland et al. (1996) have corroborated the evidence for additional branch patterning factors that may modulate the effects of bnl or cooperate with it in the specification of tracheal branching. In particular, they observed that not all the cells responding in the same way to ectopic patches of bnl expression and also that localised activation of the receptor by bnl may not always be essential for all the tracheal branches. Our results support this conclusion and suggest that different transduction pathways could be collaborating to define the precise branching of the tracheal cells. For instance, as discussed above, dpp signalling could single out a particular subset of the tracheal cells modifying their response to other signals. In fact, mutants for tkv and rho, the two new targets we have identified for vvl, display defects associated with particular branches of the tracheal tree; tkv mutants mainly have defects in dorsoventral branches whereas rho have defects mainly in the dorsal trunk. Thus, the btl transduction pathway could collaborate respectively with the dpp and the EGF transduction pathways for the proper establishment of the different branches of the tracheal tree. Since both tkv and rho seem to be expressed in all the tracheal pit, it could well be that the local source of ligand would be important for the activation of one or other pathway required for cells to acquire particular identities or branching behaviours. However, additional experiments are clearly required to ascertain the role of the EGF transduction pathway in tracheal migration.

In summary, we would like to propose the following model for the role of vvl in branching of the tracheal tree (Fig. 6). In a first step, the activity of dpp (probably mediated by the maternal component of the tkv) and some polarity genes (i.e., wg and hh) are involved in the appropriate setting of the tracheal placode (circle). The same genes are also responsible for the setting of trh and vvl expression (de Celis et al., 1995; Wilk et al., 1996), that respond independently to the same positional cues (Fig. 6A). While initial expression of btl in the tracheal placodes is independent of vvl (de Celis et al., 1995) and seems to depend on trh (Wilk et al., 1996), maintenance of btl expression in the tracheal cells requires vvl (Anderson et al., 1996). Subsequent activation of the btl receptor by bnl exerts an instructive role in the process of guiding tracheal cell migration (Sutherland et al., 1996). Independently, vvl is also required for the regulation of other genes in the tracheal cells, as we have shown is the case for tkv and rho, tkv, whose gene product enables cells to become competent to dpp signalling has a role in dorsoventral branching of the tracheal tree. A similar mechanism could be occurring in other specific branches, since rho codes for a product operating on the EGF-R pathway and whose mutant phenotype affects migration.
mainly in the longitudinal dorsal trunk (Fig. 6B). Thus, the combined activation by specific localised ligands of the btl pathway and other transduction pathways also regulated by vvl would be responsible for the branching pattern of the tracheal system. Cooperation between TGF-β and other growth factors in promoting cell migration has also been established, for instance, in mouse primordial germ cells (Godin and Wylie, 1991). Of particular interest, interactions between TGF-β and FGF have been established in cell migration in angiogenesis (Gadjusek et al., 1993), arguing for a more general role of the combined activity of these two transduction pathways in inducing cell migration.

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