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Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGFβ homologs during *Drosophila* embryogenesis

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SUMMARY

Differentiation of distinct cell types at specific locations within a developing organism depends largely on the ability of cells to communicate. A major class of signalling proteins implicated in cell to cell communication is represented by members of the TGFβ superfamily. A corresponding class of transmembrane serine/threonine kinases has recently been discovered that act as cell surface receptors for ligands of the TGFβ superfamily. The product of the *Drosophila* gene *decapentaplegic* (*dpp)* encodes a TGFβ homolog that plays multiple roles during embryogenesis and the development of imaginal discs. Here we describe the complex expression pattern of *thick veins* (*tkv*), which encodes a receptor for *dpp*. We make use of *tkv* loss-of-function mutations to examine the consequences of the failure of embryonic cells to respond to *dpp* and/or other TGFβ homologs. We find that while maternal *tkv* product allows largely normal dorsoventral pattering of the embryo, zygotic *tkv* activity is indispensable for dorsal closure of the embryo after germ band retraction. Furthermore, *tkv* activity is crucial for patterning the visceral mesoderm; in the absence of functional *tkv* gene product, visceral mesoderm parasegment 7 cells fail to express *Ultrabithorax*, but instead accumulate *Antennapedia* protein. The *tkv* receptor is therefore involved in delimiting the expression domains of homeotic genes in the visceral mesoderm. Interestingly, *tkv* mutants fail to establish a proper tracheal network. Tracheal braches formed by cells migrating in dorsal or ventral directions are absent in *tkv* mutants. The requirements for *tkv* in dorsal closure, visceral mesoderm and trachea development assign novel functions to *dpp* or a closely related member of the TGFβ superfamily.

Key words: transmembrane receptor, serine/threonine kinase, TGFβ signalling, cell migration, *decapentaplegic*

INTRODUCTION

A major goal of embryological studies is to understand the mechanisms by which the spatial organization of an animal emerges from a fertilized egg. A particularly important role in this process has been attributed to mechanisms whereby a signal generated by a cell (or a group of cells) controls the fate of neighboring cells, a phenomena referred to as induction (Spemann and Mangold, 1924; reviewed in Gurdon, 1992).

In vertebrates, the formation of most adult organs seems to depend on inductive interactions between mesenchymal and epithelial cells, i.e. between mesoderm and ectoderm/endoderm. Many of the early developmental decisions also involve induction. In amphibians, for example, the mesodermal germ layer is established via a system of intercellular signalling originating from the endoderm (Nieuwkoop, 1969). In invertebrates suitable for genetic analysis, the difficulty in manipulating embryos has been a major limitation to studies on induction. However, recent cell ablation and molecular genetic approaches have revealed that this mechanism also occurs in various developmental pathways in invertebrates. Examples include the formation of the vulva and the anterior pharynx in *Caenorhabditis elegans* (Priess and Thomson, 1987; Sulston and White, 1980), as well as cell fate induction in the developing compound eye (Dickson and Hafen, 1994; Greenwald and Rubin, 1992), embryonic segmentation (Ingham and Martinez-Arias, 1992) and gene regulatory processes across germ layers in *Drosophila melanogaster* (Bienz, 1994; Crabtree et al., 1992).

Biochemical and genetic studies have led to the identification of several secreted signalling molecules which mediate inductive interactions. In *Drosophila*, one such molecule is encoded by the gene *decapentaplegic* (*dpp*) (Ferguson and Anderson, 1992; Irish and Gelbart, 1987; Padgett et al., 1987). *dpp* encodes a member of the TGFβ superfamily of secreted signalling molecules, to which other known growth and differentiation factors belong (i.e. TGFβ, activins, Müllerian inhibiting substance (see Kingsley, 1994)). Numerous studies in invertebrate and vertebrate organisms have revealed a large number of diverse biological activities of TGFβ superfamily members, but it is largely unknown how they mediate communication between cells.

Recent studies in mammalian systems revealed that

members of the TGFβ superfamily interact with transmembrane proteins that have been classified according to their ligand-binding properties as type I or type II receptor serine threonine kinases (Attisano et al., 1993; Ebner et al., 1993; Franzen et al., 1993; Lin et al., 1992; Massagué, 1992; Mathews and Vale, 1991; ten Dijke et al., 1994). Whereas the type II receptors can bind their ligand independently, type I receptors appear to require the association of a type II receptor for ligand binding. Neither type I nor type II receptors appear to be able to signal alone. These proteins are therefore considered to form heteromeric signalling receptor complexes composed of a type I and a type II receptor (Wrana et al., 1992). How binding of these receptors to their ligands triggers the diverse biological activities of the signalling complexes is still unknown.

To initiate a genetic dissection of signalling processes involving members of the TGFβ superfamily, we have previously isolated two members of the type I receptor family in *Drosophila* and demonstrated that they are encoded by the genes *saxophone (sax)* and *thick veins (tkv)* (Nellen et al., 1994). Mutations in *sax* and *tkv* interact genetically with *dpp*. Both genes are required maternally for dorsoventral patterning of the early *Drosophila* embryo (Nellen et al., 1994), suggesting that *tkv* might act as a receptor for *dpp*. Indeed, *tkv* protein has been shown to bind *dpp* protein and BMP-2 with high affinity (Penton et al., 1994).

Although null mutations in *dpp* result in a strong ventralization of the embryo, *dpp* is also expressed, and presumably required, in various restricted sites later during embryonic development. However, due to the severe consequences of aberrant dorsoventral patterning of the early *dpp* mutant embryo, the function of *dpp* in these later processes could not be analyzed. The only two exceptions are *dpp*'s involvement in patterning the visceral mesoderm and the imaginal discs (Bienz, 1994, Crabtree et al., 1992, Spencer et al., 1982, Posakony et al., 1990). Fortuitously however, for both of these functions of *dpp* there are alleles available that affect the specific *dpp* expression pattern.

To investigate whether *dpp* plays additional roles during embryogenesis, we made use of mutations that inactivate the *dpp* receptor *tkv*. In contrast to *dpp*, which is expressed exclusively zygotically, *tkv* product required for the early dorsoventral patterning is provided maternally. Here we report the dynamic and spatially complex embryonic expression pattern of *tkv* and focus on the analysis of the zygotic functions of *tkv*. We found that *tkv* is required at multiple distinct steps during embryonic development. The earliest zygotic requirement of *tkv* activity appears to correspond to the process of dorsal closure of the epidermis. Later, *tkv* activity is crucial for visceral mesoderm and endoderm patterning, a function it seems to carry out primarily via the establishment of *dpp* expression in the visceral mesoderm. Interestingly, *tkv* mutant embryos fail to develop a functional tracheal system possibly due to a partial failure in cell migration. Our results assign novel functions to *dpp* or a closely related member of the TGFβ superfamily of signalling molecules in the process of dorsal closure, in the patterning of the visceral mesoderm and in trachea formation.

MATERIALS AND METHODS

Drosophila strains

The two embryonic lethal alleles tkv^{slater} ($tkv^{str\text{-}I}$ and $tkv^{str\text{-}II}$) were isolated by Nüsslein-Volhard et al. (1984). $tkv^{str\text{-}II}$ has a stop codon at amino acid position 144 resulting in a predicted protein that terminates immediately N-terminal of the conserved cysteine cluster in the extracellular domain of the encoded receptor serine/threonine kinase protein; this allele presumably represents a null mutation of *tkv* (Nellen et al., 1994). We therefore used this particular allele for our mutant analysis. However, all phenotypes reported also occur in the $tkv^{str\text{-}I}$ mutant, which changes a conserved glutamate residue 528 into a lysine residue at the C terminus of the kinase domain. The deficiency stock of *tkv* (Df(2L)$tkv^{sz\text{-}2}$) was obtained from J. Szidonya (Szidonya and Reuter, 1988). The tracheal system was visualized using the P-element-containing chromosome from the strain *1-eve-1* described in Perrimon et al. (1991).

Identification of mutant embryos

In general, mutant embryos were identified using CyO chromosomes carrying β-galactosidase-expressing P-element constructs (see Affolter et al., 1993). To identify homozygous *tkv*-deficient embryos at the blastoderm stage, we used a *tkv* cDNA probe and performed whole-mount hybridizations on embryo collections from the *tkv* deficiency carrying flies.

In situ hybridization

In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989) with minor modifications (Affolter et al., 1993). The entire plasmid carrying the *tkv* cDNA insert was used as a probe (Nellen et al., 1994). For *wg*, we have used a 2.9 kb cDNA insert (Rijsewijk et al., 1987). For *dpp*, a cDNA clone containing an approximately 4.5 kb insert covering the coding sequence was labelled (St. Johnston et al., 1990). For *lab*, the entire insert of the cDNA clone c241 was used (Mlodzik et al., 1988). For *pdm-1*, a bluescript vector containing a 1.7 kb *Eco*RI fragment encompassing the entire coding sequence was labelled (Affolter et al., 1993). For *pnr* and *zen*, a cDNA clone containing the entire coding sequence was labelled (Ramain et al., 1993; Rushlow et al., 1987).

Immunostainings and microscopy

Embryos were immunostained according to standard procedures (Ashburner, 1989), followed by the addition of a secondary antibody conjugated with biotin or with alkaline phosphatase. The distribution of the second antibody was revealed either by using the horseradish peroxidase ABC kit (Vectastain) or by staining for alkaline phosphatase activity. In double immunostainings, alkaline phosphatase staining was performed before the horseradish peroxidase reaction. The rabbit *lab* antiserum was kindly provided by Tom Kaufman, the *Ubx* monoclonal antibody and the rat *abdA* antiserum by Gines Morata, the rabbit *Scr* antiserum by Peter LeMotte and Walter Gehring, the monoclonal *Antp* antibody by Dan Brower and the rabbit *abdA* antiserum by François Karch. The monoclonal *crumbs* antibody was a generous gift of Elisabeth Knust (Tepass et al., 1990). To visualize β-galactosidase expression, a monoclonal antibody (Promega) was used. For light microscopy, immunostained embryos were viewed in a Zeiss Axiophot compound microscope using differential interference contrast. For documentation, images were photographed on EKTAR 100 (Kodak) film.

RESULTS

Expression of *tkv* during embryonic development

The distribution of *tkv* transcripts during embryonic development was analyzed using in situ hybridization to whole-

mount embryos (Tautz and Pfeifle, 1989). High, uniform levels of *tkv* transcripts are detected in unfertilized eggs (Fig. 1A). During cellularization after nuclear cycle 13 (stage 5: all stages according to Campos-Ortega and Hartenstein (1985)), the pattern of *tkv* transcript distribution is extremely dynamic. Before the maternal contribution of *tkv* RNA disappears completely, *tkv* transcripts accumulate at the dorsal side of the embryo (Fig. 1C,D). With the exception of two small gaps, transcripts build up dorsally along the entire anteroposterior axis. During the late phase of cellularization, *tkv* transcripts start to accumulate on the ventral side of the embryo in a pattern reminiscent of the *twist* gene product (Fig. 1F,G). During the process of mesoderm invagination, *tkv* transcripts are detectable at high levels in all mesodermal cells (Fig. 1H,I). During germ band extension, *tkv* is still expressed in the mesoderm (Fig. 1J) and transcript levels only decline in this germ layer during the last phase of extension (Fig. 1L and data not shown). As transcripts fade away in the mesoderm, *tkv* transcription is activated in cells located in the medial ectodermal region, the neurectoderm, with lower levels detected towards the midline (Fig. 1K,L). Until full extension of the germ band is reached, the expression in the neurectodermal region refines into a narrow stripe in the ventrolateral ectoderm on either side of the midline (Fig. 1M,N). Transcripts are not detectable in the anterior and the posterior midgut, neither at this stage nor at later stages (see Fig. 1J,L). In stage 11 embryos (full germ band extension), *tkv* is expressed in the tracheal placodes before and during the invagination of the epidermal tracheal cells (Fig. 1O-R). Shortly after, the continuous stripe of expression along the anteroposterior axis in the ventrolateral ectoderm is split in a segmentally repeated manner (Fig. 1Q,R). In stage 13 embryos, during the last phase of germ band retraction, *tkv* transcripts are readily detected in parts of the visceral mesoderm (Fig. 1U,V). The regions of the visceral mesoderm that express *tkv* in stage 15 embryos are located just anterior and posterior to the developing gastric caeca and in the posterior part of the midgut (Fig. 1W). No expression is observed in the endodermally derived midgut cells (Fig. 1J,W,X). Expression is still detectable in the ventrolateral and dorsolateral epidermis in one to two rows of cells flanking, both anteriorly and posteriorly, the segment grooves (Fig. 1S,T and data not shown). *tkv* expression is also detected in a complex pattern in the head region from early developmental stages on (Fig. 1). This has not been analyzed in detail.

Analysis of *tkv* mutant embryos

In a previous study, we have shown that the **maternal** expression of *tkv* (Fig. 1A) is required for patterning the entire domain of the presumptive ectoderm normally specified by *dpp* (Nellen et al., 1994). In order to learn more about the functional significance of the **zygotic** expression pattern of *tkv*, we have undertaken a detailed analysis of *tkv* mutant embryos using available molecular markers and have tried to correlate the observed phenotypes with the dynamic expression of *tkv*. We have identified three stages during embryonic development at which *tkv* activity (and therefore presumably also the activity of a TGFβ family member) is crucial for the proper specification of distinct cell types. Below, we will describe these *tkv* requirements separately and then discuss the possibility that *dpp* acts as the signalling molecule triggering these biological activities of *tkv*.

Zygotic *tkv* expression is required for dorsal closure but not for dorsoventral patterning of the embryo

The asymmetric accumulation of *tkv* transcripts on the dorsal side of the embryo at a time when cells are assigned to specific positions along the two body axis (i.e. during cellularization) prompted us to examine in detail the cuticular phenotypes of *tkv* mutant embryos. In particular, we wanted to determine whether mutant embryos displayed defects along the dorsoventral axis. Thus we scored cuticles of mutant first instar larvae for the presence or absence of cuticular structures that are derived from dorsal regions of the blastoderm map (Jürgens, 1987; Jürgens et al., 1986; Lohs-Schardin et al., 1979).

As reported by Nüsslein-Volhard et al. (1984), most of the cuticles of *tkv* mutant embryos display a prominent dorsal hole in the trunk region (Fig. 2A). In addition to this defect in dorsal closure, *tkv* cuticles lack parts of the dorsal hypoderm in the trunk region (Fig. 2A,B). Keilin's organs and both the ventral and lateral (T2 and T3) or dorsal (T1) black dots, respectively, are present in the thoracic region (data not shown). Outside the trunk region, all dorsally derived structures at the posterior end, such as the Filzkörper, the spiracular hairs, as well as the anal plates are retained in *tkv* mutants (Fig. 2B). In the head, mouth hooks and cirri are present, and both the antennal and the maxillary sense organs are formed. The cephalopharyngeal skeleton is severely disrupted or absent, and its remains are forced out of the body cavity.

The thoracic and abdominal regions of the dorsal epidermis do not contain scorable cuticular markers that define different dorsolateral positions. In the wild-type embryo, the dorsal-most blastoderm cells in the trunk region give rise to the extraembryonic amnioserosa, which does not contribute to the final cuticular structure of the larva, but appears to be necessary for the proper morphogenetic movements during gastrulation (Lohs-Schardin et al., 1979). We have used the expression patterns of *zerknüllt (zen)* and *Krüppel (Kr)* as markers for proper amnioserosa specification and differentiation, respectively. We found that *zen* and *Kr* are expressed in zygotic *tkv* mutant embryos in patterns indistinguishable from those observed in wild-type embryos (Fig. 3A,B). In addition, the *Krüppel (Kr)* expressing amnioserosa cells in *tkv* mutant embryos contain large and flattened nuclei which are characteristic of the amnioserosa (Fig. 3B). This indicates that the dorsal-most cells in the trunk region of the cellular blastoderm embryo differentiate properly in the absence of zygotic *tkv* activity.

Our analysis of *tkv* mutant embryos and larvae demonstrates that most epidermal structures that derive from the dorsal region of the cellularizing embryo are present and that no expansion of ventral or lateral pattern elements occurs in zygotically mutant *tkv* cuticles. Furthermore, germ band extension and retraction occur normally in *tkv* embryos (see Figs 3, 4); mutants for genes that are known to affect the early patterning of the dorsal side show severe defects in these processes (Arora and Nüsslein-Volhard, 1992; Wharton et al., 1993). In conclusion, we find that zygotic *tkv* expression in dorsally located nuclei during cellularization is not required to specify epidermal cells with respect to their position along the dorsoventral axis. However, in the absense of maternal *tkv*

gene product, zygotic *tkv* activity can rescue some dorsolateral pattern elements and is thus able to play a role in early dorsoventral patterning (Nellen et al., 1994).

pannier expression decays prematurely in *tkv* mutants

The absence of dorsal closure is the only major defect of *tkv* mutants (aside from certain head defects), which might correlate with the lack of *tkv* expression on the dorsal side of mutant embryos during cellularization. A number of mutations have been isolated by Nüsslein-Volhard and colleagues, which fail to close up the hypoderm along the dorsal side *(anterior open, canoe, kayak, pannier, punt, schnurri, slater (tkv), yurt*; see Tearle and Nüsslein-Volhard, 1987). Beside *tkv* (this study), only *pannier (pnr)* has been analyzed at the molecular level. *pnr* encodes a protein containing two zinc fingers with high homology to those of the GATA-1 protein, a vertebrate transcription factor required for temporal regulation of the globin and other erythroid-specific genes (Evans and Felsenfeld, 1989; Ramain et al., 1993; Winick et al., 1993). *pnr* transcripts first appear in the dorsal portion of the embryos just prior to cellularization. As development proceeds, *pnr* RNA persists at high levels in the dorsal epidermis but is excluded from the region of the amnioserosa and is not detected elsewhere in the embryo (Winick et al., 1993).

The absence of *pnr* activity results in a phenotype that mimics aspects of the *tkv* mutant phenotype (i.e. incomplete dorsal closure). We therefore investigated whether *pnr* expression is affected in *tkv* mutants and whether this could explain the lack of dorsal closure in *tkv*. We found that *pnr* transcripts are distributed in *tkv* mutants at normal levels and following the spatial restriction observed in wild-type embryos up to full germ band extension (Fig. 3C-E). However, after the initiation of germ band retraction, there is a significant reduction not only in the level of *pnr* transcripts, but also in the number of dorsal epidermal cells along the dorsoventral axis which express *pnr* (Fig. 3F,H; compare Fig. 3H to G).

What aspect of the *tkv* expression pattern is required for the maintenance of *pnr* transcription in the dorsal epidermis? The late reduction of *pnr* transcription observed in *tkv* mutants suggests that the lack of the dorsal expression of *tkv* during cellularization is not responsible for the decay of *pnr* expression during germ band retraction. Indeed, *tkv* expression is also observed at low levels in the dorsal epidermal cells that accumulate *pnr* transcripts at the extended germ band stage in wild-type embryos (Fig. 3J). These observations suggest that it is this late aspect of the *tkv* expression pattern that, upon disruption in *tkv* mutants, leads to a reduction in *pnr* expression and to the failure to close up the hypodermis on the dorsal side (see Discussion).

Both the analysis of *tkv* cuticle preparations as well as molecular markers indicate that, due to the maternal rescue, dorsoventral patterning is largely normal in zygotic *tkv* mutants. We conclude that zygotic expression of *tkv* along the dorsal surface of the cellularizing embryo is not required to determine dorsoventral cell fates. However *tkv* is required zygotically for proper dorsal closure and appears to contribute to the maintenance of *pnr* activity in dorsal epidermal cells during germ band retraction.

tkv is required for *dpp* transcription in the visceral mesoderm

After the *tkv* RNA levels on the dorsal side of the embryo have declined during the late phase of cellularization, *tkv* transcripts appear in all ventral cells that will form the mesodermal cell layer (Fig. 1). Transcripts remain detectable in the mesoderm up to the stage of full germ band extension (Fig. 1J).

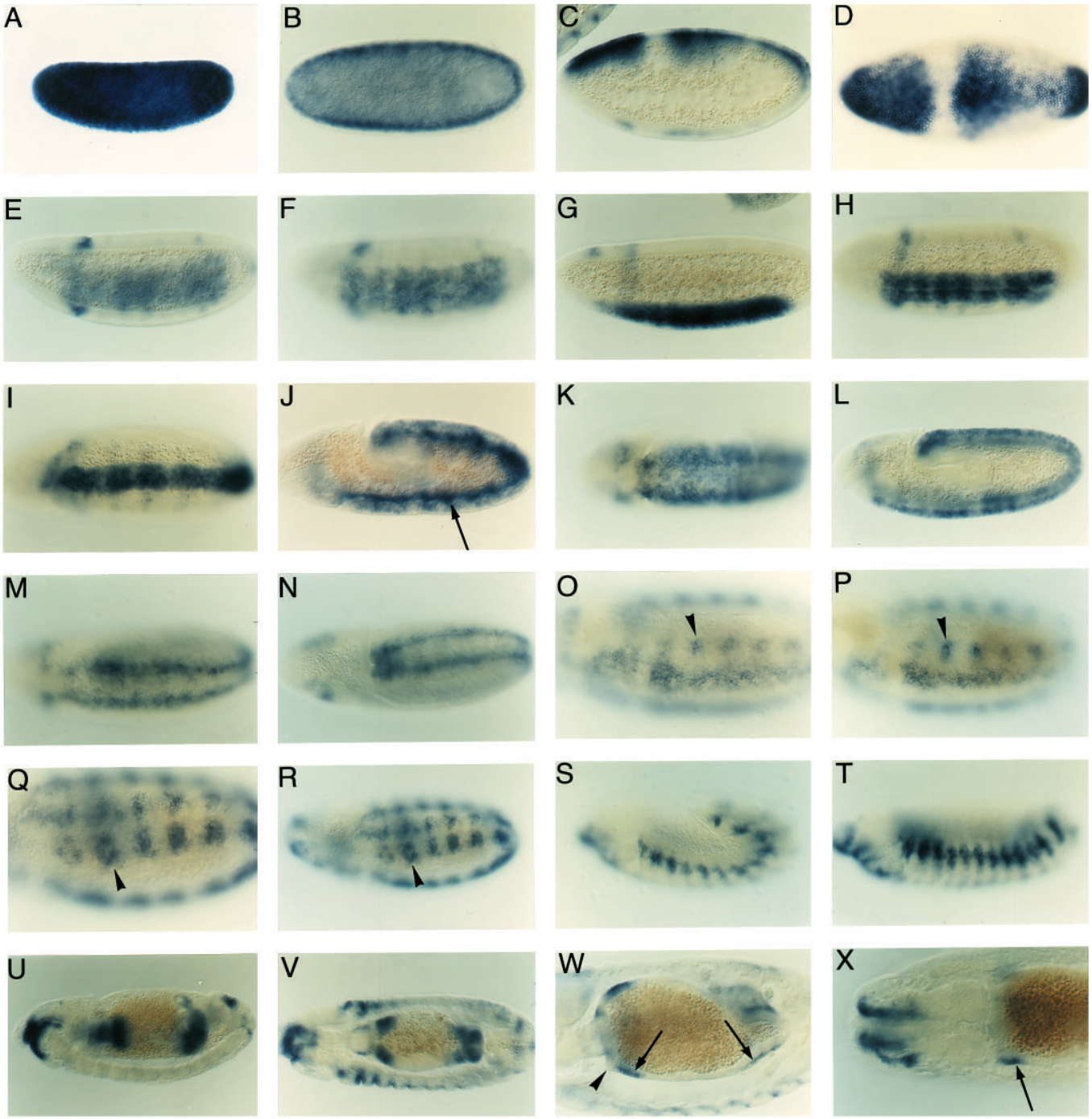
To elucidate the function of *tkv* during mesoderm differentiation, we have used various available antibodies and/or DNA probes to analyse different aspects of mesoderm differentiation in *tkv* mutants. We could not find any major defects in the early phases of cell specification in the somatic mesoderm. For example, both the early and the late expression patterns of *twist* (see Bate et al., 1991) are normal in *tkv* mutants. Furthermore, the segregation of mesodermally derived pericardial precursor cells is largely normal in the absence of *tkv* activity, as evidenced by the expression of the homeobox gene *even-skipped* (data not shown; see Azpiazu and Frasch, 1993). In

Fig. 1. Expression of *tkv* during embryogenesis. The embryonic expression pattern of *tkv* has been analysed using whole-mount in situ hybridization with a DIG-labelled *tkv* cDNA probe. *tkv* RNA is readily detectable in the unfertilised egg (A). After fertilization, transcript levels decline during the early cleavage stages (B) until all-over expression is no longer observed during cellularization (C). The RNA distribution pattern of *tkv* is extremely dynamic during the process of cellularization (C-G). Before the maternally provided RNA store completely disappears, *tkv* transcripts accumulate in an anterior and a posterior domain on the dorsal side of the embryo (C is a lateral and D a dorsal view). As this expression fades, weak expression in seven stripes (of which the anterior most shows highest levels of transcripts) is seen (E-H; E, F and H represent ventrolateral and G a lateral view) and RNA starts to accumulate on the ventral side (E-G). Longer staining of the embryos demonstrates that, at a certain time during cellularization, all three aspects of the RNA distribution (expression on the dorsal and the ventral side as well as the seven stripes) can be detected in the same embryo (data not shown). During germ band extension, *tkv* transcripts are largely confined to the mesodermal cell layer (H,I; I shows a ventral view). At full germ band extension, *tkv* transcript is still detectable in mesodermal cells (arrow in J), but later the expression levels decline below detection limits. Dynamic expression is seen at the extended germ band stage in the region of the neuroectoderm (K-N). Although rather uniformly distributed in this region at first (data not shown), *tkv* transcript levels fade in the region around the midline (K) and finally refine to two narrow lateral stripes (M shows a ventral and N a dorsal view of embryos at the extended germ band stage). None of the segregated neuroblasts show detectable levels of *tkv* transcripts at this stage (data not shown). Slightly later, *tkv* transcripts appear in the tracheal placodes (see arrowheads in O to Q; O and P are ventrolateral and Q and R slightly older dorsolateral views). Longer staining of embryos shows that low levels of *tkv* transcripts can be detected in the two to three dorsal-most cells at that stage (see Fig. 3J). During germ band retraction (S and T), *tkv* expression is detectable in the lateral epidermis in one or two rows of cells just anterior and posterior to the segmental grooves. We do not believe that this expression is related to the earlier expression in the tracheal placodes, but rather represents de novo transcriptional activity of *tkv* in the lateral epidermis. Similar expression is seen in the ventral epidermis (data not shown). During and after germ band retraction, *tkv* transcripts are detectable just anterior and posterior to the developing gastric caeca (arrowhead in W) and in the posterior midgut (U-X). Expression appears to be confined to the visceral mesoderm (see arrows in W and X). We did not analyse in detail the expression pattern of *tkv* in the head region.

addition, the subdivision of the mesoderm into somatic and visceral mesoderm appears to occur quite normally in *tkv* mutants (see below). However, distinct alterations in gene expression patterns were found in the visceral mesoderm (and in the adjacent germ layer, the endoderm). As we demonstrate in the following paragraphs, most of the changes that we identified can be explained by the failure to establish expression of the gene *dpp* in the visceral mesoderm of *tkv* mutants.

After the process of gastrulation, the activity of *dpp* is required for midgut morphogenesis, presumably by controlling the spatially restricted expression of several genes in the visceral mesoderm (e.g. *Scr*, *Ubx*, *wg*). *dpp* is expressed in the developing visceral mesoderm in regions overlapping parasegment 3 and 7 and loss of this expression causes the lack of gastric caeca and second midgut constriction, respectively (Immerglück et al., 1990; Panganiban et al., 1990). In addition to its morphological function, *dpp* has been proposed directly to mediate the transfer of positional information from the visceral mesoderm to the endoderm, resulting in the restricted expression of the homeotic gene *labial (lab)* in endodermal cells of the central portion of the midgut (Immerglück et al., 1990; Panganiban et al., 1990).

tkv mutant embryos completely lack *dpp* transcripts in both the anterior domain (around parasegment 3) and the central



domain (parasegment 7) (Fig. 4B). *dpp* expression in the pharynx and the oesophagus is normal, but transcripts also fail to accumulate in the clypeolabrum (Fig. 4B and data not shown). Consistent with the midgut phenotypes observed in dpp^{shv} mutants (which lack *cis*-regulatory DNA elements responsible for *dpp* expression in the visceral mesoderm), *tkv* mutants fail to establish *wg* transcription in parasegment 8 (Fig. 4D) and lack induction of *lab* and repression of *pdm-1* (Affolter et al., 1993) in the endodermal cells adjacent to parasegment 7 (Fig. 4F,H). With respect to morphological criteria, *tkv* mutant embryos fail to form the second midgut constriction and, during later stages, to build the gastric caeca (data not shown). These phenotypes have previously been described for *dpp* mutants (Panganiban et al., 1990; Immerglück et al., 1990).

tkv mutants show a germ layer-specific homeotic transformation in the visceral mesoderm

It has been observed (Panganiban et al., 1990; Hursh et al., 1993) that the lack of *dpp* expression in parasegment 7 results in a reduction of the level of *Ubx* expression in the same parasegment of the visceral mesoderm. In contrast to *dpp* mutants, *tkv* mutants completely fail to activate *Ubx* (Fig. 5F). However, the homeotic genes *Scr*, *Antp* and *abd-A* are expressed in the visceral mesoderm indicating that patterning in this germ layer is not disrupted all together (Fig. 5B,D,H; see Tremml and Bienz (1989) for a detailed analysis of homeotic gene expression in the visceral mesoderm).

We wanted to find out whether the lack of *Ubx* expression in the visceral mesoderm of *tkv* mutants reflects the absence of parasegment 7 information in this cell layer or whether the cells normally forming parasegment 7 were respecified to a different identity. In *Ultrabithorax (Ubx)* mutants, which lack parasegment 7, the domain of the anteriorly expressed *Antennapedia (Antp)* gene is extended by one parasegment, bringing *Antp* expression adjacent to *abdominal-A (abd-A)* expression in the visceral mesoderm (Tremml and Bienz, 1989). Using double immunostainings, we observed the same phenomenon: the *Antp* and *abd-A* expression domains abut each other in *tkv* mutants (Fig. 5J). We therefore conclude that *tkv* is crucial for the establishment of visceral mesoderm parasegment 7 and rep-

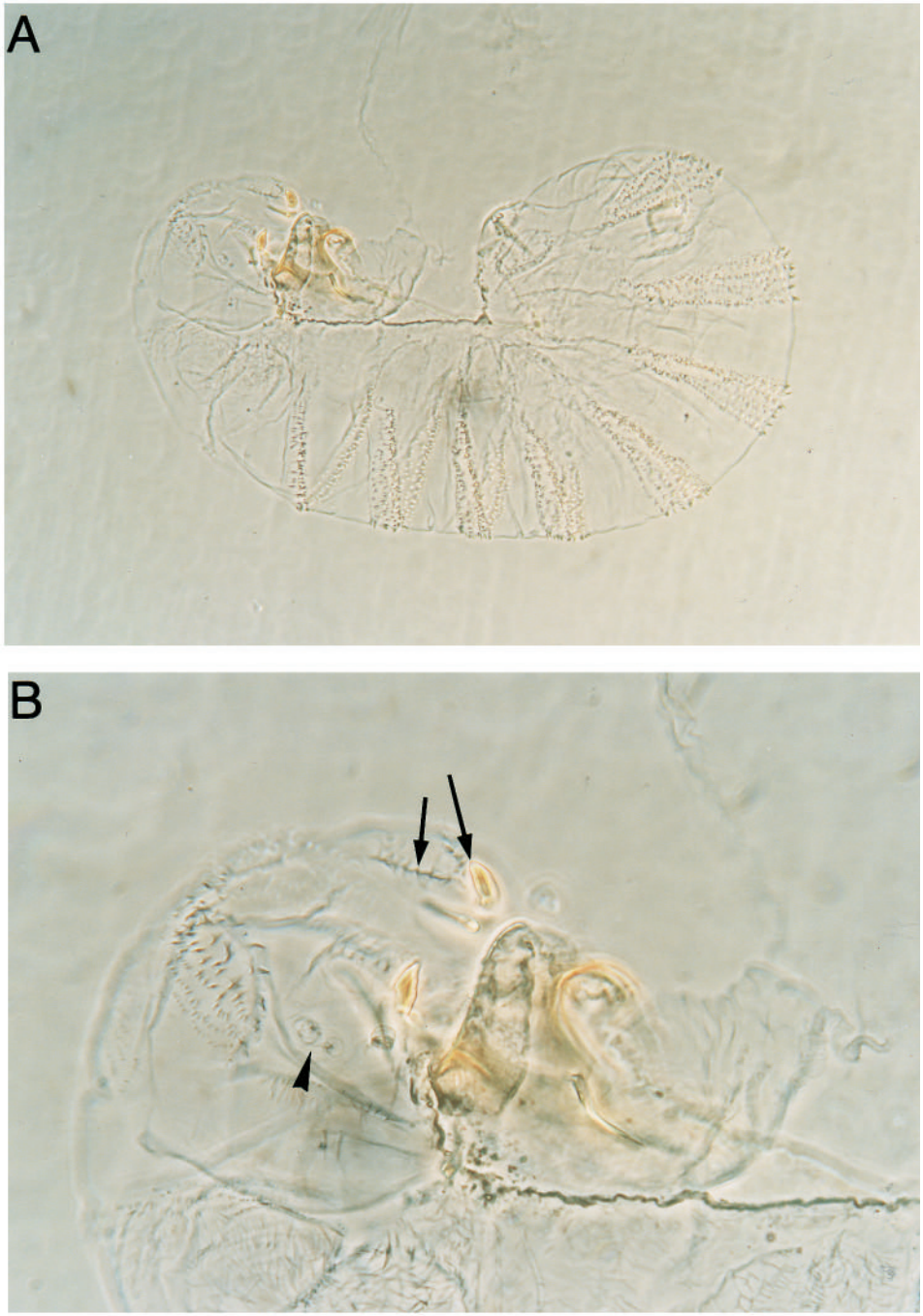


Fig. 2. Cuticle defects of *tkv* mutant larvae. Cuticle preparation of $tkv^{str\text{-}II}$ homozygous larvae at lower (A) and higher magnification (B) are shown. A prominent dorsal hole is visible and no dorsal hairs characteristic for the dorsal hypoderm are detectable (A). At the posterior end, Filzkörper and spiracular hairs can readily be distinguished (A). In all mutant cuticles, the anal plates are present (data not shown). In the head, mouth hooks and cirri (arrow), and both the antennal and the maxillary sense organs (arrowhead) are formed in *tkv* mutants (B). The remains of the cephalopharyngeal skeleton are forced out of the body cavity. The defects observed in the cephalopharyngeal skeleton might either be caused by the lack of *tkv* expression in *tkv* mutants in the anterodorsal region during cellularization (see Fig. 1C,D) or in the head region during later embryonic stages (see Fig. 1U to X).

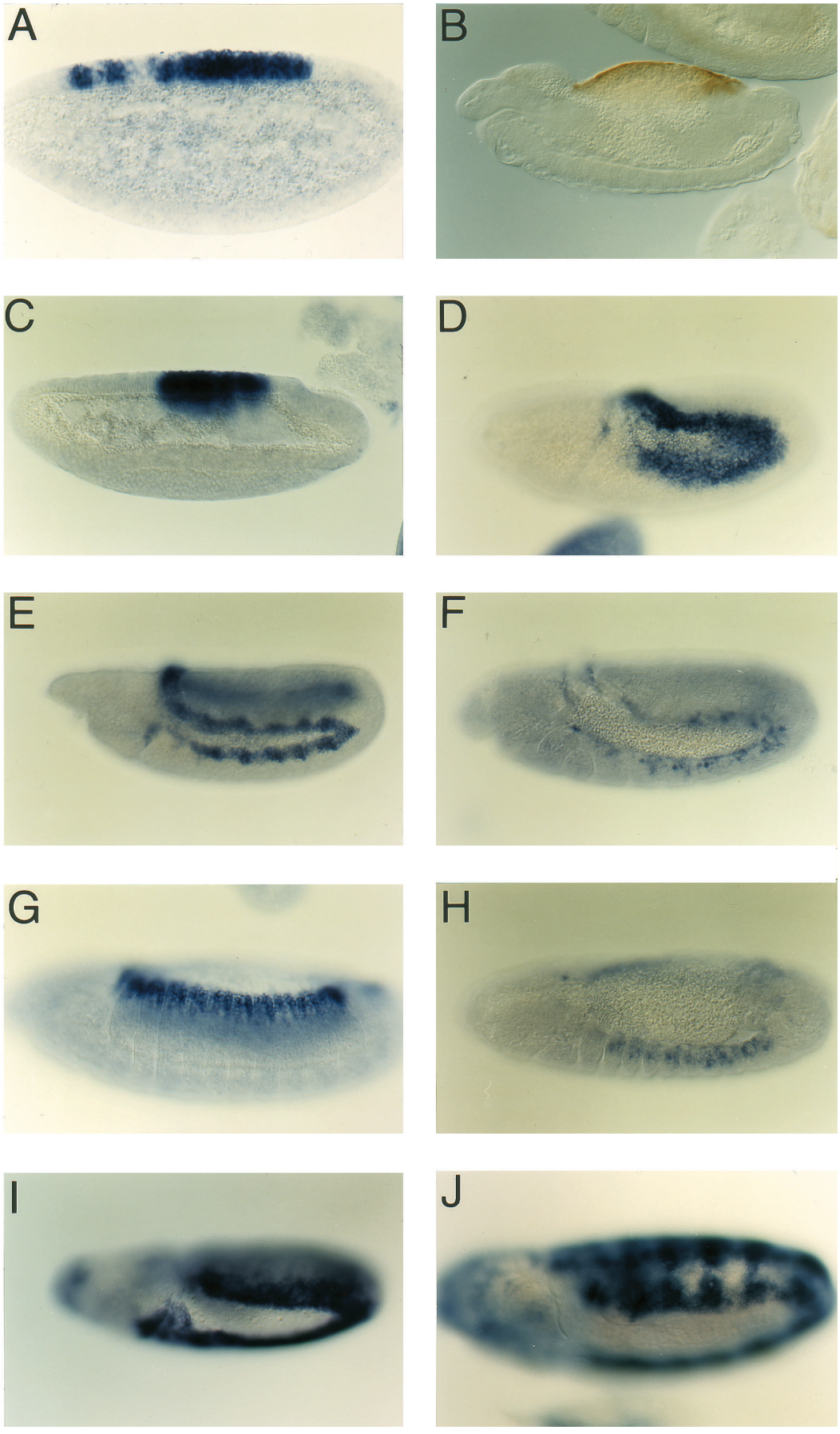


Fig. 3. *tkv* is required for the maintenance of *pnr* transcription. In situ hybridizations (A, C-J) or β-galactosidase antibody staining (B) to wild-type or mutant embryos are shown. Homozygous embryos were from Df(2L)$tkv^{sz\text{-}2}$ (A, C-F), $tkv^{str\text{-}II}$ (B,H) or wild type (G,I,J). To monitor the differentiation of amnioserosa cells, we used a *Kr-lacZ* marker gene which specifically labels these cells between stages 8 and 15 of embryogenesis (Ferguson and Anderson, 1992) and crossed a chromosome containing this marker into a $tkv^{str\text{-}II}$ mutant background (B). DNA probes used were *zen* (A), *pnr* (C-H), *dpp* (I) and *tkv* (J). During cellularization, the *zen* expression pattern refines normally in *tkv* deficient embryos (A). During further embryonic development, *tkv* mutants form a morphologically normal amnioserosa (B). *pnr* expression is normal in *tkv* deficient embryos up to full germ band extension (C-E and data not shown). During germ band retraction (F), only few of the dorsally located epidermal cells maintain weak *pnr* expression in *tkv* deficient embryos. At the retracted germ band stage, strong and uniform *pnr* expression is seen in the dorsal-most epidermal cells in wild type (G) but only weak and patchy expression is observed in $tkv^{str\text{-}II}$ homozygotes (H). At the extended germ band stage, *pnr* (E), *dpp* (I) and *tkv* (J) are all expressed in the dorsal epidermis.

resents the first receptor-encoding gene shown to be involved in mesoderm segmentation.

tkv is required for cell migration during tracheal development

Upon full extension of the germ band, *tkv* transcripts start to accumulate in segmentally repeated clusters of cells on the lateral side of the epidermal cell layer. Slightly later, the tracheal pits invaginate in the middle of these cell clusters (Fig. 1 and data not shown). *tkv* expression therefore marks the tracheal placodes slightly before the tracheal cells invaginate into the underlying mesoderm. After the invagination, the complex branching pattern of the tracheal system is established, without cell division, via cell migration and cell extension (for a detailed description of the development of the trachea, see Campos-Ortega and Hartenstein, 1985; Manning and Krasnow, 1993).

To find out whether *tkv* is essential for the establishment of the trachea, we have visualized the developing tracheal system in wild-type or mutant embryos using the anti-*crumbs* monoclonal antibody (Tepass et al., 1990). The branching pattern of a wild-type stage 14 embryo is shown in Fig. 6A (see Figure legend and Manning and Krasnow (1993) for nomenclature used). *tkv* mutants reveal striking defects in the tracheal system (Fig. 6B). Whereas the lumina of the dorsal trunk and the visceral branches were virtually intact, there is a complete absence of the dorsally directed dorsal branches and the remainder of the tracheal lumen is only established in a rudimentary fashion (Fig. 6B).

To investigate in more detail whether and how tracheal cell migration is affected in *tkv* mutants, we have introduced into this mutant background a chromosome (*1-eve-1)* that provides early and persistent accumulation of β-galactosidase immunoreactivity in the cytoplasm of all tracheal cells (see Materials and Methods). This allows the migration pattern of tracheal cells during development in mutant embryos to be followed and to compare it to the wild-type situation.

Consistent with the observations made with the lumen-specific *crumbs* antibody, the distribution of β-galactosidase protein demonstrates that *tkv* mutant embryos do not develop dorsal branches and lack both the ganglionic branches and the

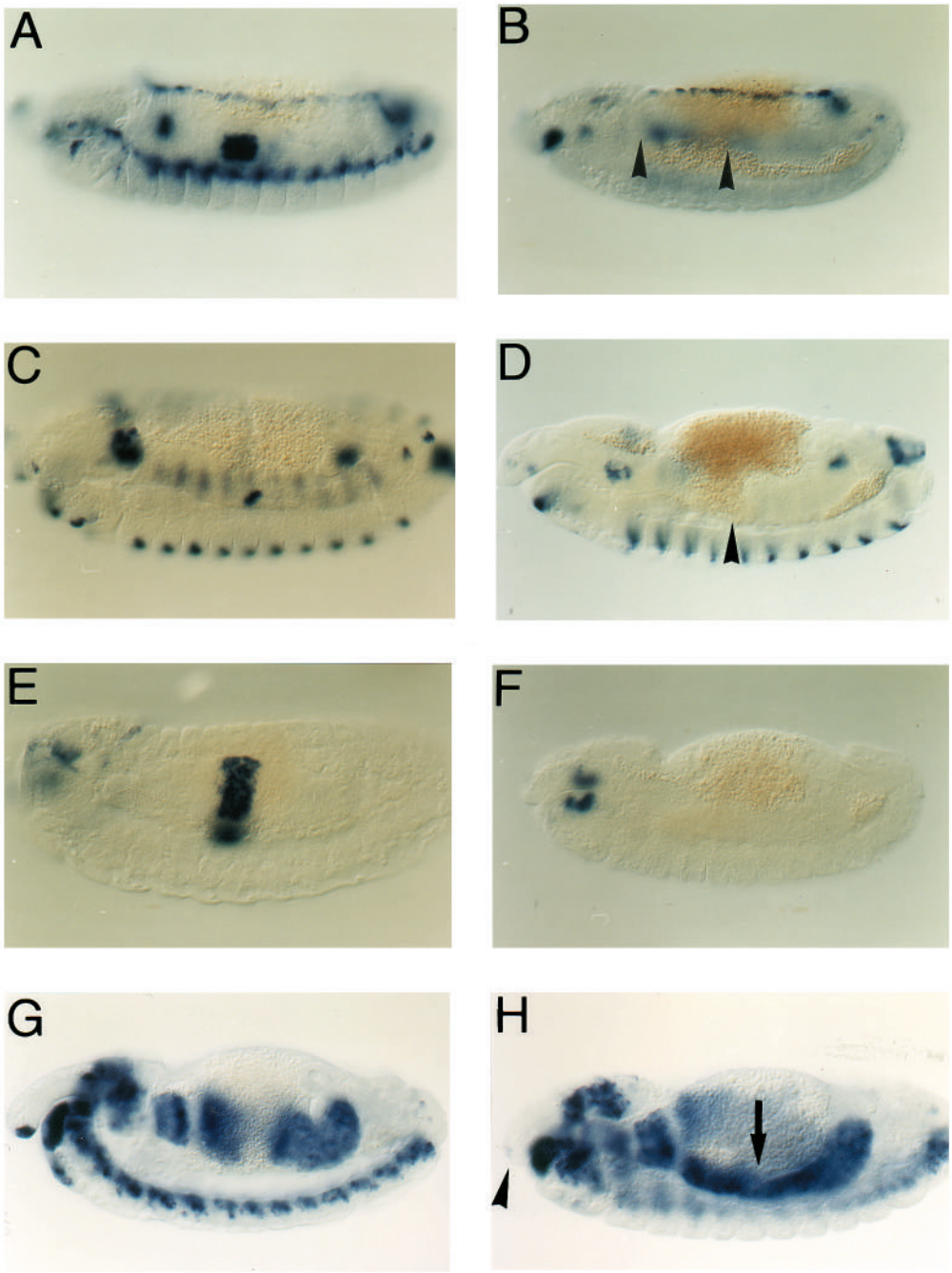


Fig. 4. *tkv* is required for *dpp* expression in the visceral mesoderm. Using whole-mount in situ hybridization, expression of *dpp* (A,B), *wg* (C,D), *lab* (E,F) and *pdm-1* (G,H) was analysed in $tkv^{str\text{-}II}$ mutants (B,D,F,H) and compared to wild-type stage 14 embryos (A,C,E,G). In $tkv^{str\text{-}II,}$ both *dpp* (arrowheads in B) and *wg* (arrowhead in D) are not detectable in the visceral mesoderm (apparent weak staining in the anterior expression domain of *dpp* in the visceral mesoderm in panel B represents *dpp* expression in the dorsal-most cells on the other side of the embryo). In addition, *lab* expression is not induced in the adjacent endoderm (F) and *pdm-1* is not repressed in the central midgut (arrow in H). Note that the expression of *dpp* (B and data not shown) and *pdm-1* (arrowhead in H) in the clypeolabrum is absent in *tkv* mutants.

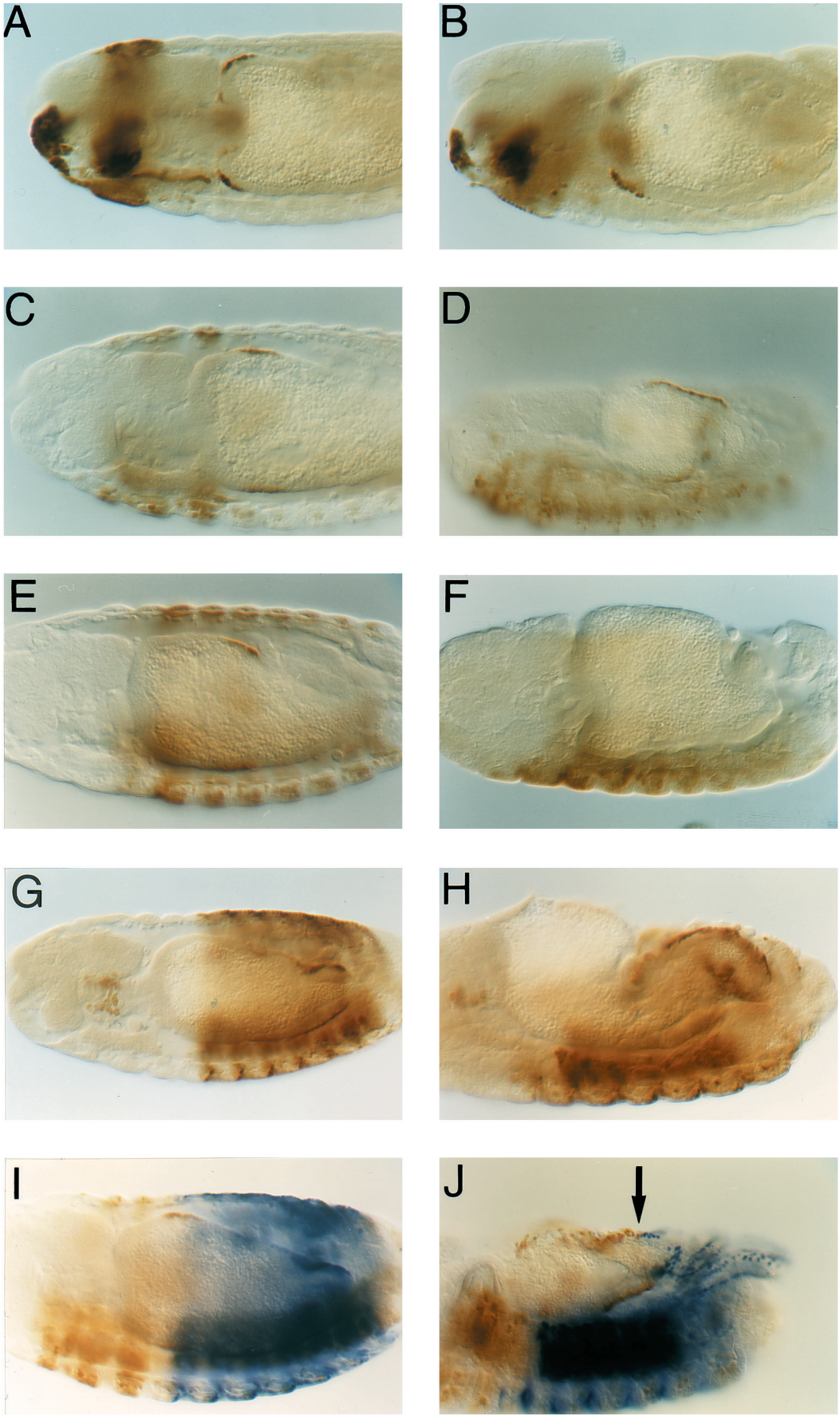


Fig. 5. Expression of homeotic genes in the visceral mesoderm of *tkv* mutants. Visceral mesoderm expression of the homeotic genes *Scr* (A,B), *Antp* (C,D), *Ubx* (E,F) and *abdA* (G,H) was analysed in $tkv^{str\text{-}II}$ homozygous embryos (B,D,F,H,J) and compared to wild type (A,C,E,G,I). *Scr* is expressed in *tkv* mutants and is expanded anteriorly (compare B to A; data not shown). *Antp* is also expressed in the visceral mesoderm, and is clearly expanded towards posterior (compare D to C). *Ubx* is not detectable in *tkv* mutants (F), whereas *abdA* appears to be expressed normally (H). Double immunostainings with *Antp* (brown) and *abdA* (blue) antibodies (I,J) clearly demonstrate that *Antp* and *abdA* expression abut each other in *tkv* mutants (arrow in J), presumably due to the lack of *Ubx* expression and the concomitant posterior expansion of *Antp*.

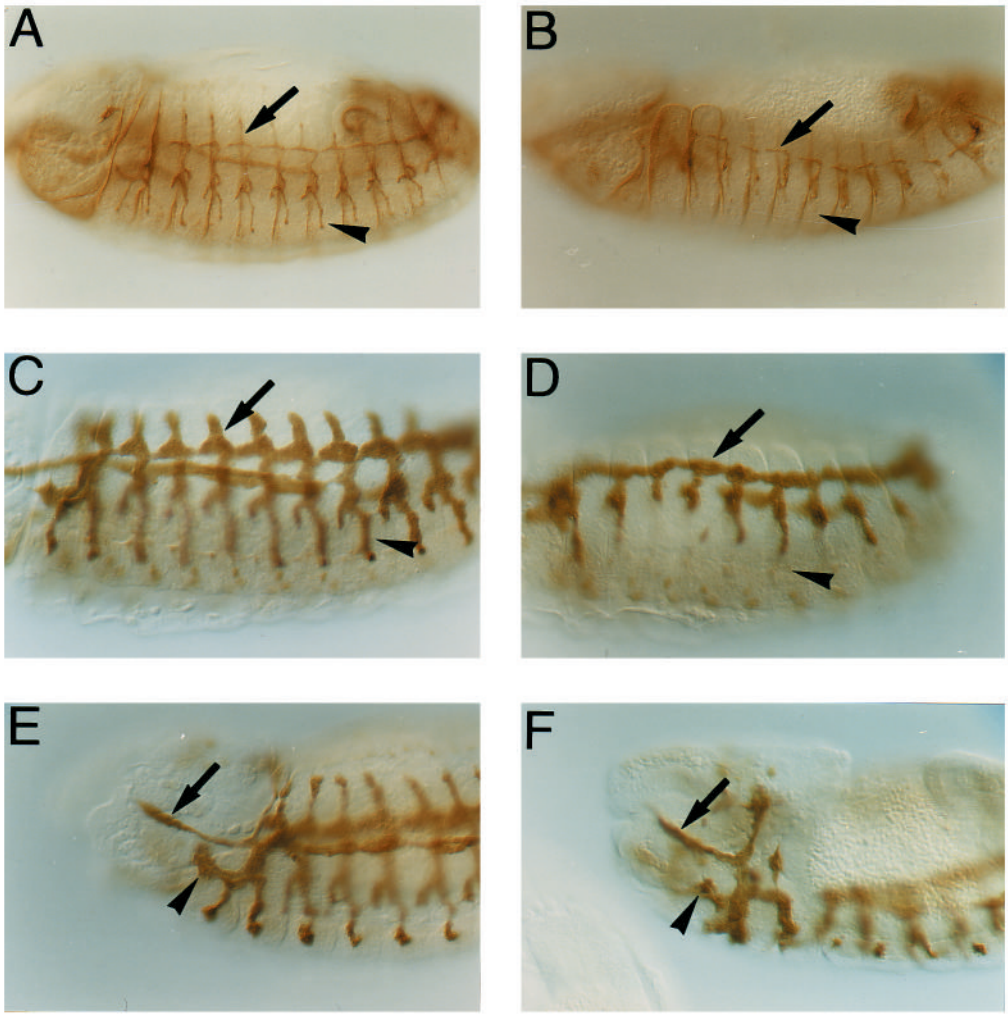


Fig. 6. *tkv* is required for tracheal development. In panels A and B, the tracheal system of stage 13 embryos is visualised with the *crumbs* monoclonal antibody; in panels C to F, tracheal development is followed using the *1-eve-1* chromosome (see Materials and Methods). A,C and E are wild type, B, D, and F are $tkv^{str\text{-}II}$ mutant embryos. The staining with *crumbs* and the staining with β-galactosidase antibodies reveal a lack of dorsal branches (arrows in A to D) and ganglionic branches (arrowhead in A to D) in *tkv* mutants. The dorsal trunk fuses in *tkv* homozygotes (see D). In the head, the tracheal system is not perturbed to a great extent; e.g. the pharyngeal branch (arrow in E,F) and the ventral cephalic branch (arrowhead in E,F) are formed, as are all the other branches (data not shown).

lateral trunk (Fig. 6D). The dorsal trunk and the visceral branches that develop along the anteroposterior axis (cells migrate from posterior towards anterior) are properly formed and the dorsal trunk derivatives of the ten trachomeres subsequently fuse, as they do in wild-type embryos (compare Fig. 6D to C). In the head region, most of the tracheal branches appear largely intact (Fig. 6F). For example, the pharyngeal branch that connects to the pharyngeal muscle and originates in the first thoracic segment reaches the pharynx during stage 15. In addition and as found in wild-type embryos, the tracheal cells that are in direct contact with the pharyngeal muscle express the DSRF gene product (data not shown; see Affolter et al., 1994). Therefore neither pathfinding nor migration of tracheal branches in the head region are affected in *tkv* mutant embryos. Defects are thus largely confined to those branches that in the early phase of cell migration grow in either dorsal or ventral direction, i.e. dorsal branch, lateral trunk and ganglionic branch.

DISCUSSION

The *tkv* mutant phenotypes may reveal novel functions of *dpp*

All aspects of signalling by TGFβ1 and activin in tissue culture cells can be accounted for by the presence and activity of their type I and type II transmembrane serine/threonine kinase receptors (Wrana et al., 1992; Attisano et al., 1993). There is no evidence so far that cells mutant for one of these receptors would be affected in any other aspect than by the lack of responsiveness to factors of the TGFβ superfamily. The zygotic defects that we observed in *tkv* mutant embryos are thus most likely due to the lack of responsiveness of certain cells to a TGFβ homolog.

Since *dpp* has been shown, both by genetic and biochemical means (Nellen et al., 1994; Penton et al., 1994), to constitute a ligand for the *tkv* receptor, we will discuss the zygotic defects in *tkv* mutants in the light of the possibility that they are caused by a failure to respond to *dpp* activity. These additional functions of *dpp* might have been masked due to the severity of the early dorsoventral defects in *dpp* null mutants. The *tkv* receptor required for *dpp*-mediated dorsoventral patterning is provided maternally (Nellen et al., 1994), which accounts for the largely normal patterning along the dorsoventral axis in *tkv* mutant embryos derived from heterozygous mothers. However, we cannot exclude the possibility that *tkv* functions additionally as a receptor for another as yet unidentified TGFβ homolog.

tkv activity regulates the expression of *pnr*

The most obvious defect observed in *tkv* mutants is the lack of

dorsal closure. To our knowledge, *pnr* is the only gene (besides *tkv*) that has been analysed molecularly and that is specifically required for the process of closing up the dorsal hypoderm of the embryo. We found that the expression of *pnr*, which encodes a putative transcription factor, is not maintained in *tkv* mutants at high levels and declines during germ band retraction. Interestingly, both *tkv* and *dpp* are expressed in the dorsal epidermal cells during the last phase of germ band extension (see Fig. 3I,J; Jackson and Hoffmann, 1994; St. Johnston and Gelbart, 1987). It is therefore possible that maintenance of *pnr* expression requires *dpp* activity and the receptor serine/threonine kinase encoded by *tkv*. To date, no mutations have been described in the *dpp* locus that remove selectively the expression in the dorsal epidermal cells. It is interesting to note, however, that the early expression of *pnr* during cellularization requires *dpp* activity (Winick et al., 1993), which in turn is mediated by *tkv* (Nellen et al., 1994).

tkv mutants lack parasegment 7 of the visceral mesoderm

It has been proposed that secreted *dpp* protein regulates gut morphogenesis, in part, by regulating homeotic gene expression in the visceral mesoderm and endoderm of the developing midgut (Immerglück et al., 1990; Panganiban et al., 1990; Bienz, 1994). Interestingly, most of the midgut defects that we observe in *tkv* mutants also occur in *dpp* mutants lacking *dpp* expression exclusively in the visceral mesoderm: (1) *tkv* mutants lack *wg* and have reduced *Ubx* expression in the visceral mesoderm, (2) *lab* induction as well as *pdm-1* repression no longer occur in the adjacent endodermal cells, and (3) *tkv* mutants lack the second midgut constriction and do not develop the gastric caeca. Not only are the *dpp*-mediated effects on midgut gene regulation and morphology disrupted in *tkv* mutants, but *dpp* expression in the visceral mesoderm is missing all together. This would place the putative receptor (*tkv*) upstream of its possible ligand (*dpp*) and would suggest that *tkv* activity is required for the establishment of *dpp* expression rather than for mediating its signalling capacities during midgut development. Consistent with this idea, it has been shown that *dpp* expression in the visceral mesoderm in both parasegments 3 and 7 is autoregulated; DNA fragments from the *dpp* locus which generate parasegment 3 and 7 expression patterns, strictly require endogenous *dpp* activity in order to be expressed in these two parasegments (Hursh et al., 1993). It is thus possible that *tkv* expression during the early development of the mesodermal cell layer is involved in *dpp*-mediated autoregulation in visceral mesoderm parasegments 3 and 7. Due to the failure of *dpp* to autoregulate its expression, *tkv* mutations result in a lack of *dpp* transcription.

In contrast to *dpp* mutants, *tkv* mutants completely lack *Ubx* expression in the visceral mesoderm and *Antp* is expressed in the cells that are devoid of *Ubx*. This represents a homeotic transformation of visceral mesoderm parasegment 7 into parasegment 6 caused by the absence of the *tkv* receptor kinase. Therefore, *tkv* appears to be tightly linked to the segmentation process of the visceral mesoderm and might be involved in the initial setting up of the parasegment 7 restricted expression of the *Ubx* gene. It has been shown that the *dpp* ligand mediates *Ubx* autoregulation through a defined *dpp* response element within upstream sequences of Ubx (Thüringer and Bienz, 1993; Thüringer et al., 1993). Further studies of *tkv* and its ligand(s) should provide insight into the establishment of parasegment 7 identity in the visceral mesoderm.

Although *tkv* may be involved in mediating *dpp* signalling in the visceral mesoderm, *tkv* is not expressed in the embryonic endoderm. It thus appears that *dpp*-mediated induction of the *lab* gene across germ layers must involve an additional type I receptor(s) expressed in the endodermal portion of the midgut. *lab* induction is significantly reduced in mutants of *saxophone (sax)* which encodes another type I receptor serine threonine kinase (Nellen et al., 1994). *sax* is expressed in the visceral mesoderm and in the endoderm and could thus be involved in either of the two cell layers in establishing *lab* expression in the endoderm. Alternatively, it is possible that *lab* induction results as an indirect consequence of *dpp* expression in the apposing visceral mesoderm and is mediated by a different signalling molecule.

tkv is required during tracheal development

Analysis of *tkv* mutant embryos revealed striking alterations in the development of the tracheal system. Although some aspects of tracheal development appear to be unaffected by the lack of *tkv* activity (i.e. the dorsal trunk and the visceral branches are properly established), other aspects such as the formation of the ventral trunk and the ganglionic and dorsal branches are strongly perturbed. *tkv* is expressed in the tracheal placodes slightly before the tracheal cells invaginate into the underlying mesoderm and start to migrate. *dpp* is not expressed within the tracheal placodes, but its transcript appears immediately prior to germ band shortening in a segmentally repeated pattern in the lateral ectoderm aligned with the ventral margin of the tracheal pits (St. Johnston and Gelbart, 1987; Jackson and Hoffman, 1994). Double labelling experiments revealed that these lateral ectodermal domains of *dpp* expression do not overlap with, but are adjacent to, the tracheal cells in the placodes (Nicole Grieder and M. A., unpublished observation). This is also the case for the *dpp* expression pattern in the dorsal-most cells which are located just dorsal of the tracheal placodes. The spatial apposition of cells that express *tkv* or *dpp* around the tracheal placodes suggests that the *dpp* signalling molecule might trigger the activity of the *tkv* kinase in adjacent cells and contribute thereby to the migration behaviour of tracheal cells. In this respect, it is interesting to note that most of the tracheal structures established along the dorsoventral axis (i.e. dorsal branch, ganglionic branch) are absent in *tkv* mutant embryos, whereas the structures extending along the anteroposterior axis (i.e. dorsal trunk, visceral branch) are not affected at all. In contrast to *tkv*, the *Drosophila* FGF receptor encoded by the *breathless* gene is required for migration of all tracheal cells (Klämbt et al., 1992, Reichman-Fried et al., 1994). To confirm a functional requirement of *dpp* in tracheal development, mutants that specifically remove *cis*-regulatory sequences responsible for expression in the lateral and dorsal epidermal region of germ band extended embryos will have to be constructed; such mutants are not available at present (see Jackson and Hoffmann, 1994).

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