The *tracheae defective* gene encodes a bZIP protein that controls tracheal cell movement during *Drosophila* embryogenesis

Karsten G.Eulenberg and Reinhard Schuh[1]

Max-Planck-Institut für biophysikalische Chemie, Abt. Molekulare Entwicklungsbiologie, Am Fassberg 11, 37070 Göttingen, Germany

[1]Corresponding author

The *tracheae defective* (*tdf*) gene is required for the formation of the tracheal system during *Drosophila* embryogenesis. It encodes a putative bZIP transcription factor (TDF). Antibodies directed against TDF detect a nuclear protein in all tracheal cells before invagination and throughout tracheal system morphogenesis. Examination of *tdf* mutants revealed that *tdf* activity is not necessary for determining tracheal cell identity but for subsequent morphogenetic cell movements. *tdf* activity is under the control of *trachealess*, the key regulator gene for tracheal development. In contrast, *tdf* activity is not dependent on and does not interfere with the fibroblast growth factor- (FGF) and Decapentaplegic- (DPP) mediated signalling that direct guided tracheal cell migration. Our results suggest that lack of *tdf* activity affects tracheal cell migration in general rather than specific aspects of cell migration. *tdf* activity involves a maternal and zygotic component and its requirement is not limited to tracheal system formation. The complex spatiotemporal patterns of TDF expression in the embryo correspond to defects, suggesting that cell migration is impaired. We propose that the bZIP protein TDF functions as a co-regulator of target genes that provide cells with the ability to migrate.

Keywords: bZIP proteins/*Drosophila*/*tracheae defective*/tracheal system

Introduction

Cell migration processes play a central role in the embryogenesis of multicellular organisms. This involves coordinated cell movements during gastrulation and morphogenesis of the nervous and vascular system. The cellular mechanisms underlying migration of cells include path finding, target tissue recognition and cell shape changes (Gumbiner, 1992; Riesau and Flamme, 1995; Tanaka and Sabry, 1995; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996).

The formation of the *Drosophila* tracheal (respiratory) system provides an ideal model system for the study of processes that underlie cell migration. It develops from ten ectodermal lateral cell clusters on either side of the embryo, the tracheal placodes. The cells of the placodes invaginate into the underlying mesoderm forming small openings, the tracheal pits, along the lateral sides of the embryo. The segmentally clustered tracheal cells migrate out and form tubes which eventually fuse to give a complex three-dimensional network of tracheal tubes throughout the embryo. This embryonic tracheal system formation occurs in the absence of cell proliferation and involves cell migration and differentiation processes exclusively (for a detailed description, see Manning and Krasnow, 1993; Samakovlis *et al.*, 1996).

Outgrowth of tracheal branches, in a directed manner, involves two different signal transduction pathways. One signalling pathway is activated by the *branchless* (*bnl*) gene product, a homologue of mammalian fibroblast growth factors (FGFs), that is expressed in distinct clusters of cells surrounding the developing tracheal branches (Sutherland *et al.*, 1996). BNL attracts branch outgrowth in a concentration-dependent manner via the *breathless* (*btl*) gene product, a receptor tyrosine kinase (Glazer and Shilo, 1991; Klämbt *et al.*, 1992; Lee *et al.*, 1996). During later stages of tracheal development, BNL signalling is required for new gene expression that is necessary for the control of secondary and terminal branching events (Sutherland *et al.*, 1996). The second pathway is initiated by the morphogen Decapentaplegic (DPP), a member of the TGFβ superfamily, that is expressed on the dorsal and ventral side of the invaginating tracheal metameres (Affolter *et al.*, 1994; Vincent *et al.*, 1997). DPP signalling via receptor serine/threonine kinases (Ruberte *et al.*, 1995) directs tracheal cell migration along the dorsoventral body axis and it causes localized gene expression patterns in the developing tracheal placode (Vincent *et al.*, 1997). Such regionalized gene expression in response to DPP signalling is seen for the transcription factors *knirps* (*kni*) (Nauber *et al.*, 1988) and *spalt* (*sal*) (Kühnlein *et al.*, 1994). Recent studies have shown that *sal* is necessary for the formation of the dorsal trunk, the main anterior–posterior tracheal connection (Kühnlein and Schuh, 1996).

In addition to the signalling pathways, and the few genes that act at their receiving ends, required to mediate guided migration and/or differentiation of tracheal cells, a key regulatory gene, *trachealess* (*trh*), has been identified. *trh* controls tubulogenesis and tracheal cell fate determination. The *trh* gene encodes a transcription factor (TRH) of the basic helix-loop-helix-PAS family and shows high sequence homology to the human hypoxia-inducible factor-1 alpha (Isaac and Andrew, 1996; Wilk *et al.*, 1996).

Here we describe the molecular characterization of the *tracheae defective* (*tdf*) gene, which acts downstream of *trh*, that encodes a potential transcriptional regulator a nuclear localized bZIP protein (TDF). We show that TDF activity enables cells to migrate during tracheal branch outgrowth and tube formation. Furthermore, TDF activity is a prerequisite for tracheal cell migration but independent of directed cell migration in response to DPP and BNL signalling. TDF function is not restricted to the tracheal system but is also required in various other embryonic tissues where the gene is expressed.
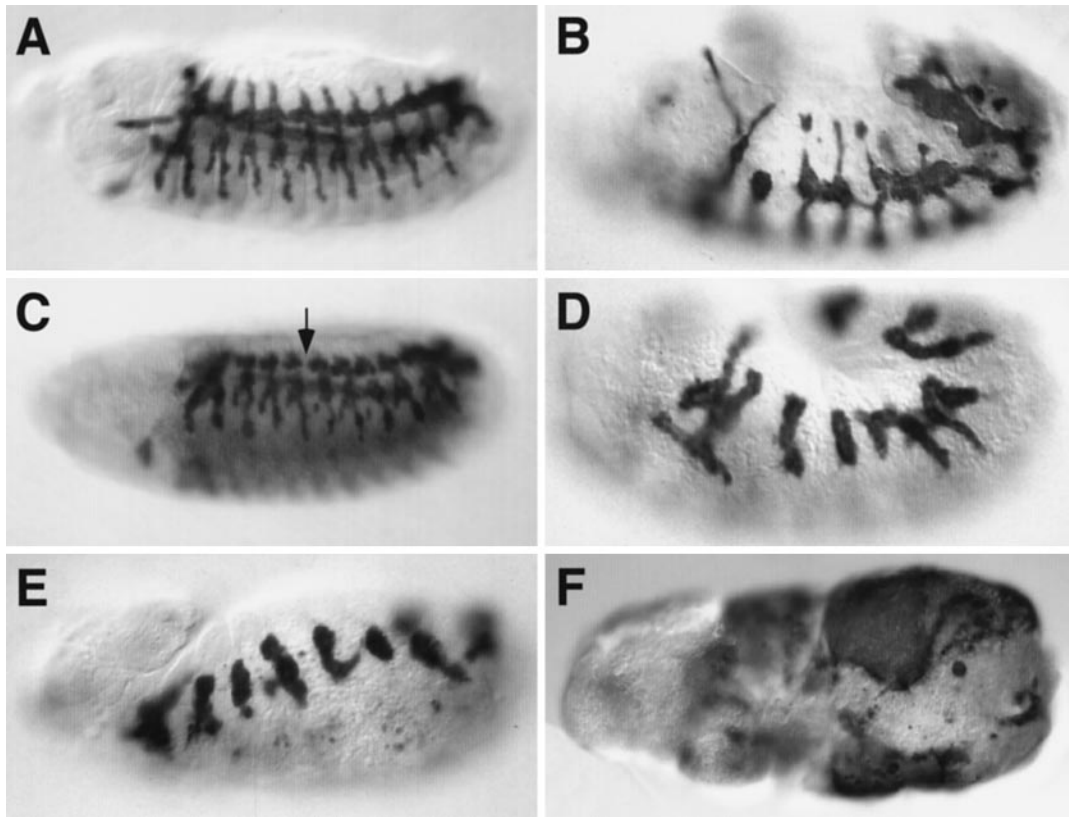
Fig. 1. Tracheal phenotype of *tdf* mutations. (A–F) Whole-mount anti-β-galactosidase staining of a stage 14 wild-type (**A**), a transheterozygous *tdf^{P1}/tdf^{P2}* (**B**), a weak (**C**) and a strong (**D**) phenotype of a *tdf^{P2}*, a *tdf$^{P\Delta3}$* (**E**) and a maternal and zygotic *tdf$^{P\Delta3}$* (**F**) embryo bearing the *1-eve-1* chromosome. The β-galactosidase expression of the *1-eve-1* enhancer trap line serves as a tracheal cell marker. Note the interconnected tracheal system in the wild-type embryo (A), a partial lack of primary branches and dorsal trunk fusion in the intermediate *tdf* phenotype (B) and stalled primary branch formation is found in the weak *tdf* mutant embryo (C). The arrow in (C) points to broken dorsal trunk structures. The strong *tdf* mutant embryo (D) developed a reduced number of primary branches and the zygotic lack-of-function *tdf* mutant embryo (E) shows tracheal cell invagination and sac formation but lacks branch outgrowth. The *tdf*-deficient embryo (F; lacking both maternal and zygotic *tdf* activity) lacks tracheal pit formation and the tracheal cells visualized by the *1-eve-1* marker (Perrimon *et al.*, 1991) remain within the epidermis, i.e. they fail to migrate internally. In addition, the *tdf*-deficient embryo develops fused tracheal placodes. We also noted that such embryos show other significant defects with respect to their ability to gastrulate properly. Thus, the fusion of tracheal placodes are likely to be caused by secondary effects that correspond to the blastodermal expression domains of *tdf* (see Figure 4B). (A–E) Anterior is left and dorsal is up. (F) Dorsal view. Note: embryos in B and D show stalled germ band retraction.

Results

Genetic characterization of the tdf gene function

In a search for genes that function during tracheal branch outgrowth we examined collections of P-element enhancer trap lines to identify mutations which cause abnormal tracheal branching. We found two homozygous lethal enhancer-trap lines *l(2)07010* (Karpen and Spradling, 1992) and *l(2)k15608* (Torok *et al.*, 1993) with P-element insertions in the chromosome band 59F that failed to complement each other for lethality and tracheal branch formation. Based on the mutant tracheal phenotype (Figure 1A and B) we refer to the targeted gene locus as *tracheae defective* (*tdf*) and to the two mutant alleles as *tdf^{P1}* and *tdf^{P2}*, respectively. In homozygous *tdf^{P1}* and *tdf^{P2}* mutant embryos, as well as in transheterozygous *tdf^{P1}/tdf^{P2}* mutant embryos the formation of tracheal placodes, the invagination of tracheal cells and the generation of tracheal sacs occurs normally (data not shown). However, the subsequent stereotypic outgrowth of tracheal branches is severely disrupted in the *tdf* mutant embryos. Although the tracheal mutant phenotype is variable from embryo to embryo, severe defects in primary branching and a weak

stalled primary branches phenotype were observed consistently (Figure 1C and D). Both *tdf* alleles show in hemizygous conditions over the *tdf* deficiency *Df bw^{S46}* a weaker tracheal phenotype than embryos which are homozygous for the *tdf* deficiency (data not shown) suggesting that the *tdf^{P1}* and *tdf^{P2}* alleles caused by the P-element insertions (see below) represent hypomorphic *tdf* alleles.

In order to generate lack-of-function *tdf* alleles we mobilized the viable P-element insertion *C5-2-5* in chromosomal band 59F (Hartenstein and Jan, 1992) which contains a P-element insertion upstream of the *tdf* transcription unit (see below). After mobilization of the P-element (see Materials and methods) we obtained three lethal jump-out lines which failed to complement the *tdf^{P1}* and *tdf^{P2}* alleles as well as the *Df bw^{S46}*. Two of these lines, *tdf$^{P\Delta3}$* and *tdf$^{P\Delta4}$*, display in homozygous as well as hemizygous conditions over the *Df bw^{S46}* the same severity of tracheal phenotype as observed with homozygous *Df bw^{S46}* embryos (Figure 1E). This indicates that *tdf$^{P\Delta3}$* and *tdf$^{P\Delta4}$* represent lack-of-function alleles. However, as observed with the hypomorphic *tdf* alleles, the lack-of-function *tdf* mutant alleles and *Df bw^{S46}* embryos also show variable tracheal defects.
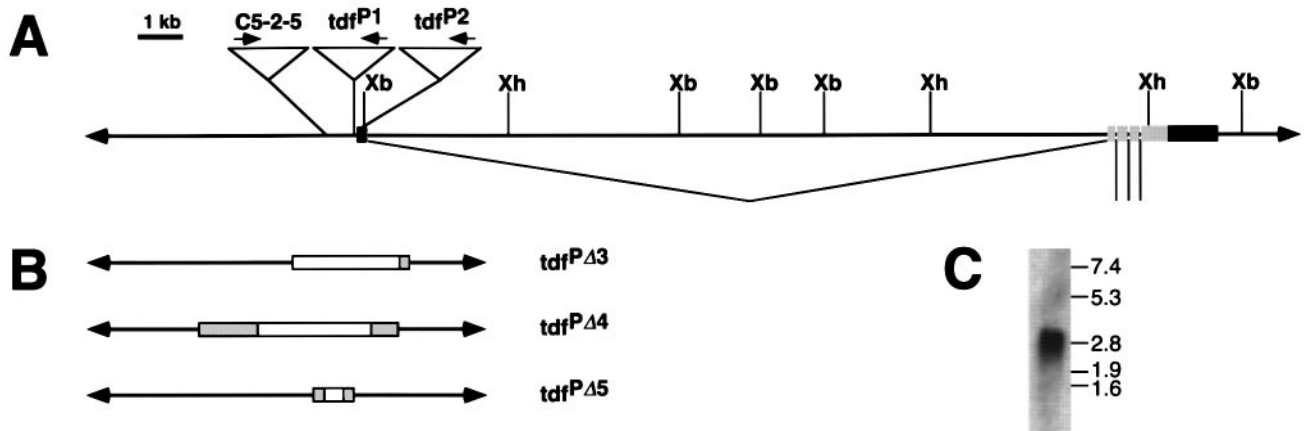
Fig. 2. Molecular organization of the *tdf* gene and DNA deletions associated with *tdf* mutants. (**A**) Physical map of the genomic region containing the *tdf* gene. The P-element insertion sites of the three enhancer trap lines *C5-2-5*, *tdf*[P1] and *tdf*[P2] are indicated. The direction of the *lacZ* expression is shown by arrows. The genomic organization of the *tdf* transcription unit was established by sequencing cDNAs and the corresponding genomic DNA including exon/intron boundaries. The DNA sequence information is available under accession No. AJ001615. Dotted bar: longest open reading frame (1452 bp) of the *tdf* transcript. Black bar: untranslated region of the *tdf* transcript. The introns of 16.5 kb, 62 bp, 63 bp and 62 bp are indicated. Xb, *Xba*I; Xh, *Xho*I. (**B**) Genomic DNA deletions associated with *tdf* mutations. Minimal (white bars) and maximal (dotted bars) genomic DNA deletions of the corresponding *tdf* jump-out mutations. The DNA deletion of *tdf*[PΔ3] includes the first exon and ~1 kb of the first intron. In *tdf*[PΔ4], DNA portions including the first exon are deleted. *tdf*[PΔ5] lacks DNA sequences upstream of the *tdf* transcription unit. *tdf*[PΔ3] and *tdf*[PΔ4] mutant embryos show no TDF expression while *tdf*[P1], *tdf*[P2] and *tdf*[PΔ5] embryos show strongly reduced TDF expression (data not shown). (**C**) Autoradiogram of a Northern blot with poly(A)[+] RNA from 0- to 18-hour-old embryos hybridized with ^{32}P-labelled *tdf* cDNA. The cDNA detects a single hybridization signal of 2.9 kb. The numbers refer to RNA molecular size markers in kb.

Maternal tdf expression contributes to tdf function

It is difficult to see how the absence of a gene product, as is the case with lack-of-function *tdf* alleles, could cause a variable mutant phenotype. One possible explanation is that *tdf* activity in the embryo is composed of maternal and zygotic activity and that a consistent phenotype can only be obtained when both activities are deleted. We tested this possibility by generating germline clones of the strong *tdf*[PΔ3] allele (see Materials and methods). Embryos that lack maternal and zygotic *tdf* function show a severe and consistent tracheal phenotype (Figure 1F). They lack tracheal branch outgrowth and tracheal pit formation does not occur. The tracheal cells remain clustered within the lateral epidermis, meaning that the cells fail to migrate inwards. However, they adopt tracheal cell fate as revealed by *1-eve-1* tracheal marker gene expression (Figure 1F). This suggests that the lack of maternal *tdf* enhances the zygotic *tdf* tracheal phenotype, indicating that maternally supplied *tdf* partially and variably rescues lack of zygotic *tdf* function. Furthermore, the lack of *tdf* activity does not affect the initiation of tracheal cell fate but affects morphological processes subsequent to the cellular differentiation.

Embryos that lack maternal *tdf* function but retain zygotic *tdf* function develop into normal-looking adults. This suggests that maternal *tdf* expression is not essential for development if *tdf* activity is provided zygotically. However, only few eggs develop from females with *tdf*-deficient germlines, suggesting that maternal *tdf* activity impairs normal development. Furthermore, *tdf* requirement is not restricted to tracheal system development but is also necessary in several other tissues where the gene is expressed zygotically (see below). *tdf* mutant embryos show specific defects in the central nervous system, stalled germ band retraction and defects during head formation (unpublished data). Details of these phenotypes (K.G. Eulenberg and R.Schuh, in preparation) are beyond the

scope of our present study, but they are consistent with the proposal that the lack of *tdf* affects cell migration processes in the domains where the gene is normally expressed.

Molecular characterization of the tdf gene

Mobilization of the P-element in the *tdf*[P2] strain resulted in several independent excision lines that are homozygous viable and fertile. The restoration of the wild-type function by excision of the P-element indicates that the P-insertion had caused the *tdf* mutation. The cloning of the gene was initiated by 'plasmid rescue experiments' (see Materials and methods) resulting in genomic DNA fragments flanking the P-insertion sides. This DNA was used to establish a chromosomal walk in the region and nearby transcribed DNA sequences were characterized by cDNA and Northern blot analysis (see Materials and methods). The structure of the nearby transcription unit was determined by sequence analysis of three overlapping cDNAs and the corresponding portions of the genomic DNA (Figure 2A). The P-element-associated transcription unit contains four introns and is transcribed into a single 2.9 kb-long transcript matching the size of the embryonic poly(A)[+] RNA detected by Northern blot analysis (Figure 2A and C). To identify the sites of the three P-element insertions we sequenced from the ends of the P-elements into the flanking genomic DNA. The P-elements of the lethal *tdf*[P1] and *tdf*[P2] alleles were inserted 63 bp upstream and 167 bp downstream of the putative transcription start site, respectively. The viable *C5-2-5* P-element insertion lies ~1 kb upstream of the putative transcription start site (summarized in Figure 2A).

Several lines of evidence argue that this transcription unit carries the *tdf* function. First, the embryonic spatio-temporal expression pattern of the cDNA coincides with the β-galactosidase marker gene expression of the three P-element enhancer trap lines (data not shown). Second,
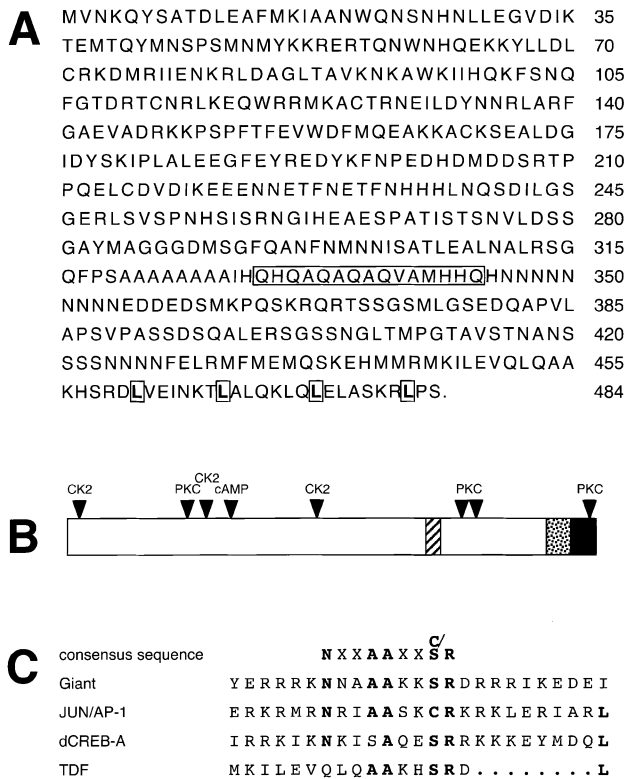
A
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MVNKQYSATDLEAFMKIAANWQNSNHNLLEGVDIK    35
TEMTQYMNSPSMNMYKKRERTQNWNHQEKKYLLDL    70
CRKDMRIIENKRLDAGLTAVKNKAWKIIHQKFSNQ   105
FGTDRTCNRLKEQWRRMKACTRNEILDYNNRLARF   140
GAEVADRKKPSPFTFEVWDFMQEAKKACKSEALDG   175
IDYSKIPLALEEGFEYREDYKFNPEDHDMDDSRTP   210
PQELCDVDIKEEENNETFNETFNHHHLNQSDILGS   245
GERLSVSPNHSISRNGIHEAESPATISTSNVLDSS   280
GAYMAGGGDMSGFQANFNMNNISATLEALNALRSG   315
QFPSAAAAAAAIH QHQAQAQAQVAMHHQ HNNNNN   350
NNNNEDDEDSMKPQSKRQRTSSGSMLGSEDQAPVL   385
APSVPASSDSQALERSGSSNGLTMPGTAVSTNANS   420
SSSNNNNFELRMFMEMQSKEHMMRMKILEVQLQAA   455
KHSRD L VEINKT L ALQKLQ L ELASKR L PS.   484
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B

C

consensus sequence	N X X A A X X S/R R
Giant	Y E R R R K N N A A A K K S R D R R R I K E D E I
JUN/AP-1	E R K R M R N R I A A S K C R K R K L E R I A R L
dCREB-A	I R R K I K N K I S A Q E S R R K K K E Y M D Q L
TDF	M K I L E V Q L Q A A K H S R D L

Fig. 3. The putative *tdf* protein. (**A**) Deduced amino acid sequence of the putative *tdf* protein. The leucine residues of the leucine zipper are indicated. The glutamine-rich region is boxed. (**B**) Diagram showing structural features of the predicted *tdf* protein. The location of the leucine zipper motif is indicated by a black box, the putative basic DNA binding domain is shown by a stippled box, and the glutamine-rich region is indicated by a hatched box. Potential phosphorylation sites of cAMP-dependent protein kinase (cAMP), Protein Kinase C (PKC) and Casein Kinase II (CK2) are indicated by arrowheads. (**C**) Sequence alignment of the consensus sequence of the basic DNA binding domain with corresponding regions of Giant (Capovilla *et al.*, 1992), JUN/AP-1 (Lamph *et al.*, 1988), dCREB-A (Smolik *et al.*, 1992) and TDF. Amino acids of the consensus sequence are shown in bold letters. Note: the glutamine in TDF represent a conservative amino acid exchange of the asparagine in the consensus sequence. The reduced distance of the leucine zipper and the basic region of TDF is indicated by dots.

tdf$^{P\Delta3}$ and *tdf*$^{P\Delta4}$ mutant embryos lack the corresponding protein expression (data not shown). Third, the jump-out P-lines with lethal *tdf* mutations exhibit genomic DNA deletions including portions of the transcription unit and may include protein coding sequences (for details see Figure 2B). Fourth, rescue experiments using the P-element-associated transcription unit unambiguously demonstrate that it carries *tdf* function since transgene-mediated cDNA expression can restore tracheal branching in *tdf* mutant embryos (see below).

The *tdf* transcript contains a single open reading frame of 1452 bp. It codes for a putative *tdf* protein (TDF) of 484 amino acids with a leucine zipper domain (Landschulz *et al.*, 1988; Kerppola and Curran, 1991) at the C-terminus (Figure 3A and B). Leucine zippers are protein–protein interaction domains defined by amphipathic helixes containing a leucine residue at every seventh position. They define, combined with an adjacent region of basic amino acids that provide specific DNA binding, the subfamily of bZIP transcription factors (Nakabeppu and Nathans,

1989; Ellenberger *et al.*, 1992). The TDF basic domain fits the consensus sequence of the DNA binding domain (Hurst, 1995), including the known transcription factors dCREB-A (Smolik *et al.*, 1992), JUN/AP-1 (Lamph *et al.*, 1988) and Giant (Capovilla *et al.*, 1992; Figure 3C). It is interesting to note that the distance between the basic and the leucine zipper domain, which is often conserved in bZIP proteins, is reduced in TDF (Figure 3C). TDF also contains a glutamine-rich region that functions as a transactivation domain in many transcription factors (Gonzales *et al.*, 1991; Foulkes *et al.*, 1992). Furthermore, TDF contains potential phosphorylation sites for cAMP-dependent protein kinase, Protein Kinase C and Casein Kinase II (Figure 3C) which were found to be functional in the regulation of bZIP transcriptional activity (Hurst, 1995).

tdf transcript and protein expression patterns during embryogenesis

To localize the *tdf* transcripts during embryogenesis, *in situ* hybridizations on whole mount embryos were carried out using dioxigenin-labelled antisense *tdf* RNA probe (see Materials and methods). *tdf* transcripts are evenly distributed in unfertilized eggs (data not shown) and during syncytial blastoderm (Figure 4A), confirming that *tdf* is maternally expressed (see above). The maternal transcripts disappear during the syncytial blastoderm stage before zygotically expressed *tdf* transcripts accumulate during cellular blastoderm in three distinct domains (Figure 4B). At stage 11 (stages according to Campos-Ortega and Hartenstein, 1985), transcripts are observed in the developing tracheal placodes, in the head region and in dorsal vessel cells (Figure 4C). *tdf* transcripts are maintained in the tracheal cells after they migrate inwards and during tracheal branch outgrowth (Figure 4D). When the germ band is fully extended, *tdf* transcripts accumulate in the neuroectoderm, giving rise to a repetitive pattern in the developing CNS at stage 14 (Figure 4E). During stages 14–17 of embryogenesis, *tdf* is predominantly expressed in the CNS, in the developing head, in the tracheal system and in dorsal vessel cells that eventually form the *Drosophila* heart (Figure 4D and E).

In order to visualize the TDF expression we generated anti-TDF antibodies (see Materials and methods). TDF is evenly distributed in the cytoplasm of syncytial blastoderm stage embryos (Figure 4F). Afterwards, nuclear TDF antibody staining, consistent with the putative function of TDF as a bZIP transcription factor, is detected throughout embryogenesis (Figure 4G–J). TDF is observed in all nuclei of the blastoderm and maintained until gastrulation (Figure 4G). During stage 11, prominent TDF expression is found in the tracheal placodes and the head anlagen, while TDF in all other nuclei decreases and eventually fades away (Figure 4H). At later embryonic stages, TDF accumulates in the patterns of the *tdf* transcripts (Figure 4D, E, I and J). These results show that although the maternally supplied TDF has no apparent function in most cells of the embryo, it is nevertheless ubiquitously expressed. Zygotic TDF accumulates in the cell nuclei in patterns that follow *tdf* mRNA expression during embryogenesis, except in the blastoderm. The blastodermal TDF expression domains are most likely not seen because they are covered by maternally supplied TDF.
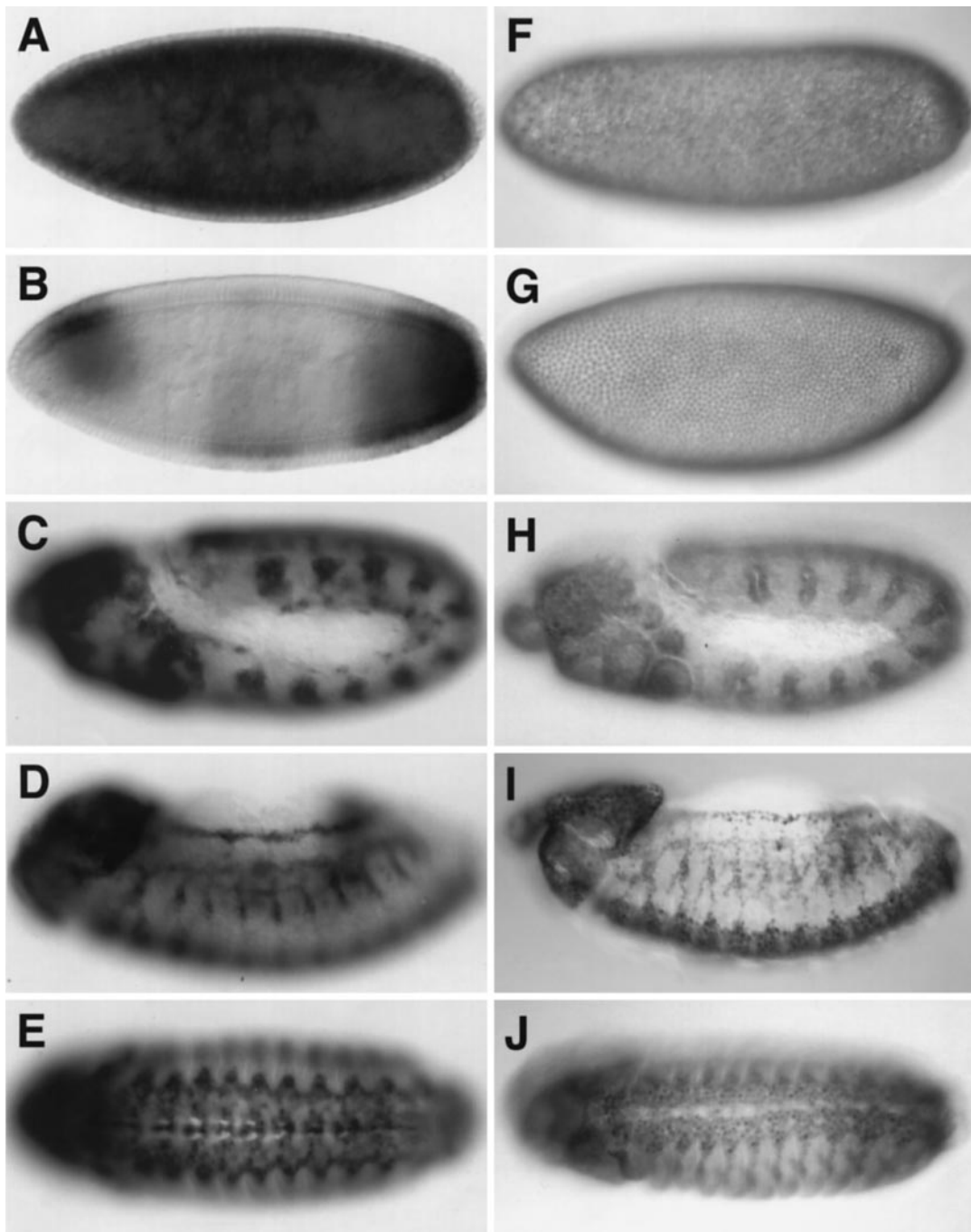
Fig. 4. *tdf* mRNA and protein expression during *Drosophila* embryonic development. (A–E) Whole-mount *in situ* hybridizations of wild-type embryos were performed with a digoxigenin-labelled *tdf* antisense RNA. (**A**) Stage 4, syncytial blastoderm. Homogeneous distribution of maternally supplied *tdf* RNA. Pole cells lack *tdf* transcripts. (**B**) Stage 5, cellular blastoderm. *tdf* expression in an anterior–dorsal, a ventral and a posterior domain. Maternally supplied *tdf* RNA fades out. (**C**) Stage 11, fully extended germ band. *tdf* expression in all tracheal placodes, the anlagen of the head, the dorsal vessel cells. (**D**) Stage 14. *tdf* expression persists in the tracheal system, the head region and the dorsal vessel cells. (**E**) Stage 14, ventral view. Segmentally repeated *tdf* expression in restricted parts of the ventral nerve cord. (F–J) Whole-mount antibody staining of wild-type embryos with anti-TDF antibodies. (**F**) Stage 4. Homogeneous distribution of TDF. (**G**) Stage 5. Focus on the lateral epidermis to visualize TDF accumulation in nuclei. No TDF domains as found for *tdf* transcription (B) are detectable. (**H**) Stage 11. Maternally supplied TDF starts to fade out. TDF accumulates in the tracheal placodes and in the head region. (**I**) Stage 14. TDF is predominantly expressed in the tracheal system, the developing head and the dorsal vessel cells. (**J**) Stage 14. Ventral view to visualize the distinct nuclei accumulation of TDF in restricted regions of the CNS. Stages are described according to Campos-Ortega and Hartenstein (1985).

tdf is necessary for tracheal cell migration

The stalled tracheal branch outgrowth in *tdf* mutant embryos is consistent with zygotic TDF expression in early and late tracheal cells. In order to see whether TDF activity provides those cells with the ability to migrate to their normal positions we performed a tissue-specific rescue experiment with homozygous *tdf^{PΔ3}* embryos. Such embryos fail to perform germ band retraction and they develop only rudimentary branches that lack interconnections (compare Figure 5A and B). To provide these embryos with *tdf* activity we expressed a UAS–tdf effector gene construct under the control of a tracheal-specific
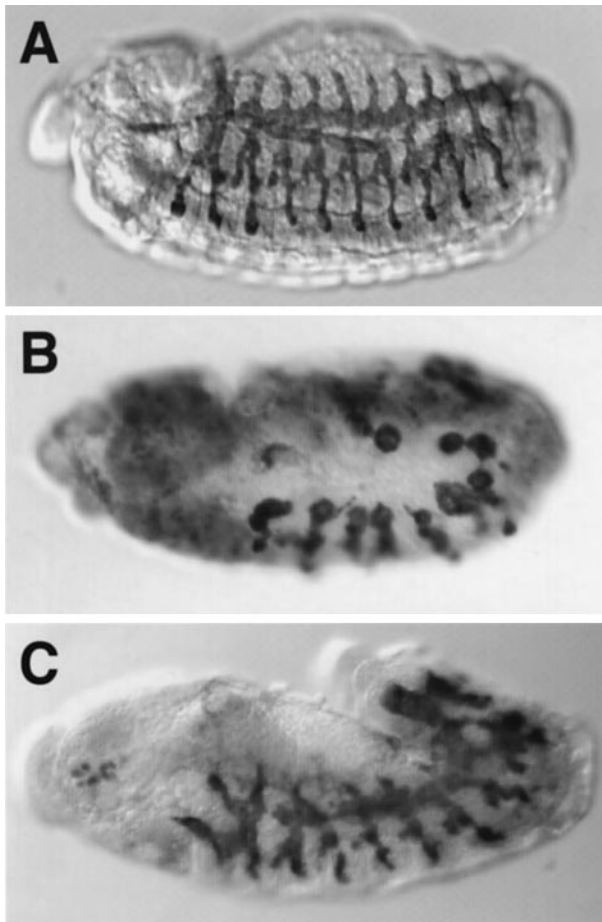
Fig. 5. TDF is necessary for tracheal branch formation. Whole-mount anti-β-galactosidase staining of a stage 14 *1-eve-1* embryo (**A**), a *tdf^{PΔ3}* *1-eve-1* embryo (**B**) and a *tdf^{PΔ3}* *1-eve-1* embryo bearing btl–Gal4 driver and UAS–tdf effector construct (**C**; see Materials and methods). The *1-eve-1* tracheal marker P-line was used to mark tracheal cells. The *tdf^{PΔ3}* embryo in (B) develops rudimentary tracheal branches, while the rescued *tdf^{PΔ3}* embryo in (C) develops an interconnected three-dimensional tracheal network. Note: the embryos in (B) and (C) both show a lack of germ band retraction.

Gal4 driver line (btl–Gal4; see Materials and methods). In such embryos the tracheal branches grow out and develop the tracheal network including a dorsal trunk similar to the wild-type embryos (Figure 5C). However, as expected, the lack of germ band retraction is not rescued in such embryos (Figure 5C). This result indicates that *tdf* expression in tracheal cells enables them to migrate and to form the stereotyped tubular array of the tracheal system.

tdf expression is dependent on *trh* but independent of processes leading to directed tracheal branch outgrowth

Tracheal system development is initiated by the induction of tracheal cell fates from ectodermal precursor cells followed by the invagination process. Both processes depend strictly on *trh* activity as the key regulatory gene for tracheal development (Isaac and Andrew, 1996; Wilk *et al.*, 1996). In embryos lacking *trh* activity, zygotic *tdf* expression is absent in places of the tracheal placodes (Figure 6A). Conversely, *trh* expression defining the tracheal placodes is normal in *tdf* mutant embryos (Figure 6B). This indicates that *tdf* is a direct or indirect target of

trh activity. Since *tdf* is necessary for the migration of tracheal cells and since their migration is controlled by both BNL and DPP signalling (Sutherland *et al.*, 1996; Vincent *et al.*, 1997), we next asked whether *tdf* expression depends on BNL signal transduction. Therefore, we examined *tdf* expression in *btl* mutant embryos which interrupt BNL signalling due to a defective receptor (Klämbt *et al.*, 1992). In such embryos *tdf* expression is normal, indicating that *tdf* expression is independent of BNL signalling (Figure 6C). The DPP-dependent aspects of tracheal system formation are later than TDF expression in tracheal cells (see below). In addition, *tdf* is expressed normally in tracheal cells which are mutant for *tkv* (Figure 6H), a gene encoding an essential DPP receptor (Nellen *et al.*, 1994). Thus, these observations rule out the possibility that the expression of tracheal TDF is controlled by DPP signalling.

tdf tracheal function acts in parallel to the BNL and DPP signalling pathways

The finding that *tdf* expression is not controlled by BNL and/or DPP signalling does not rule out the possibility that TDF might be an integral component of one or both signalling pathways. Furthermore, TDF may control the expression of such components in response to *trh* and thereby provide the link between *trh* activity and directed cell migration.

In order to test the interference of TDF with BNL signalling we examined the expression of *btl* and *pointed* (*pnt*), which is expressed in response to BNL signalling (Sutherland *et al.*, 1996), in *tdf* mutant embryos. Figure 6D and F show that both *btl* and *pnt* are expressed normally in *tdf* mutant embryos. In addition, stalled branch outgrowth in *tdf* mutant embryos directs to cells expressing *bnl* activity as observed in wild-type (Figure 6E). This indicates that the *tdf* function does not interfere with BNL signalling that leads to guided branch outgrowth.

Tracheal cell migration along the dorsoventral body axis of the embryo is under the control of the DPP signalling pathway (Affolter *et al.*, 1994; Vincent *et al.*, 1997). In order to see whether the lack of *tdf* activity interferes with the proper activity of the DPP pathway during tracheal system development, we examined DPP target gene expression in *tdf* mutant embryos. We analysed *kni* expression in tracheal cells which is activated in response to DPP and *sal* gene expression which is repressed by DPP (Vincent *et al.*, 1997). Both genes are expressed in tracheal cells of *tdf* mutant embryos (Figure 6G and data not shown). Thus, the lack of *tdf* activity does not interfere with the DPP pathway in the tracheal system, indicating that *tdf* acts in a DPP-independent manner in the tracheal system.

The results suggest that *tdf* enables cells to migrate by controlling the activity of the appropriate target genes and that the two signalling pathways act on top of this property of cells by instructing them which route to take.

Discussion

Our results provide evidence that the bZIP protein encoded by the gene *tdf* is required for the migration of tracheal cells to form a tubular network in the *Drosophila* embryo, the tracheal system. In the absence of both maternal and
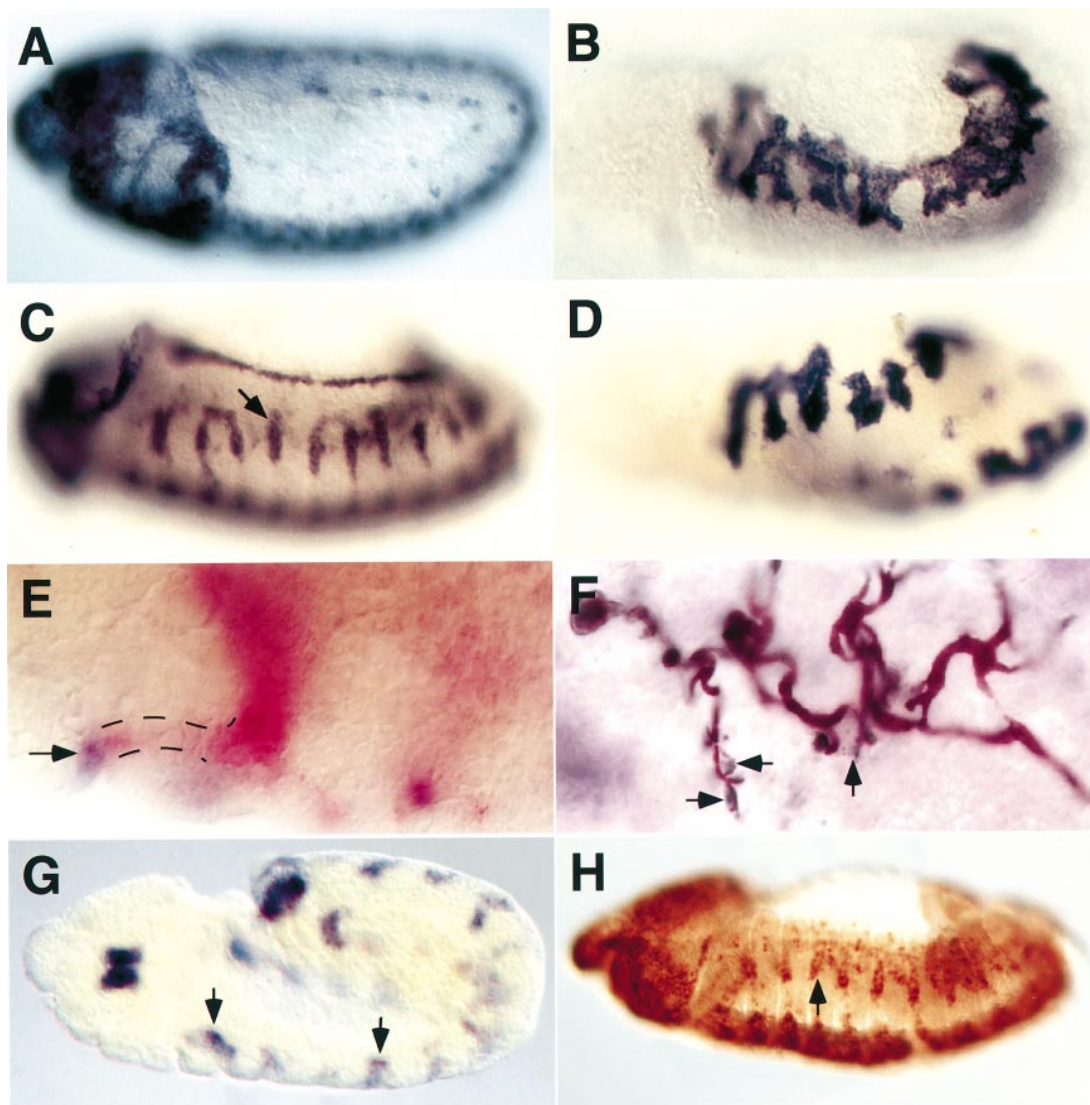
Fig. 6. *tdf* functions in general cell migration. (A–D, G) Whole-mount *in situ* hybridizations of homozygous *trh³* (A), *tdf^{PΔ3}* (B, D and G) and *btl^{LG19}* (C) embryos were performed with digoxigenin-labelled *tdf* (A and C), *trh* (B), *btl* (D) and *kni* (G) antisense RNA. No tracheal *tdf* expression is found in the *trh* mutant embryo in (**A**). Tracheal *trh* expression is present in the lack-of-function *tdf* mutant embryo in (**B**). *tdf* expression is present in the rudimentary tracheal metameres (arrow) of a *btl* mutant embryo in (**C**). Tracheal *btl* expression is present in the lack-of-function *tdf* mutant embryo in (**D**). (**E**) Whole-mount double *in situ* hybridization of a homozygous *tdf^{PΔ3}* embryo bearing the *1-eve-1* chromosome using *lacZ* (red) and *bnl* (blue) antisense RNA. Rudimentary branch outgrowth (broken lines) is guided to *bnl* expressing cells (arrow). (**F**) Whole-mount antibody double staining of a homozygous *tdf^{PΔ3}* embryo bearing the *pnt–lacZ* chromosome using anti-β-galactosidase (blue) and antibody 2A12 (brown). The rudimentary tracheal system is outlined by the 2A12 antibody. Marker gene expression for secondary tracheal branches is indicated by arrows. Tracheal *kni* expression (arrows) is found in the lack-of-function *tdf* mutant embryo in (**G**). (**H**) Whole-mount antibody staining of a homozygous *tkv^{str–1}* embryo using anti-TDF antibodies. TDF-expressing nuclei (arrow) are found within the rudimentary tracheal structures.

zygotic *tdf* activity tracheal cells form normally but remain in the epidermis. In the absence of only zygotic *tdf* activity the maternal component of *tdf* activity is sufficient to initiate migration, meaning that the invagination process appears normal. However, branching in stereotypical patterns is strongly reduced, resulting in stalled tracheal branches. Transgene-mediated *tdf* activity rescues the ability of tracheal cells to continue to migrate and to form branches. In addition to showing that *tdf* activity is necessary for tracheal cell migration, the tissue-specific rescue experiment unambiguously demonstrates that the bZIP protein is indeed the factor that is encoded by the *tdf* gene.

The role of tdf in tracheal system formation

Tracheal system development is initiated by the differentiation of tracheal cells from segmentally arranged ectodermal precursor cells and is followed by the invagination of tracheal cell groups into the underlying mesoderm. The prominent mechanism of tracheal morphogenesis is cell migration (Manning and Krasnow, 1993; Montell, 1994). The gene *trh*, a transcription factor of the basic helix-loop-helix-PAS family, acts as the key regulator of both tracheal cell fate determination and tubulogenesis (Isaac and Andrew, 1996; Wilk *et al.*, 1996). Tracheal *tdf* expression, as shown here, coincides with the spatial limits of *trh* expression and is dependent on *trh* activity. This
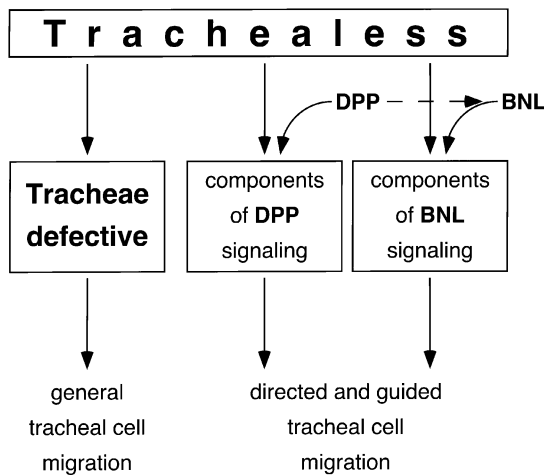
Fig. 7. *tdf* function is required for general tracheal cell migration and acts in parallel to DPP and BNL signalling that leads to guided and directed tracheal cell migration. The *trh* function directs the expression of TDF and components of the DPP and BNL signalling pathways that act autonomously in tracheal cells. The DPP and BNL signalling molecules emanate from specific non-tracheal cells and direct the guided tracheal branch outgrowth. Certain aspects of BNL expression are dependent on DPP signalling and are indicated by a broken arrow (Vincent *et al.*, 1997). In contrast, TDF directs general tracheal cell migration independently of either signalling pathways.

indicates that *tdf* represents a potential direct downstream target of *trh*. Embryos mutant for *trh* activity lack tracheal pit formation and tubular structures are not discernible (Isaac and Andrew, 1996; Wilk *et al.*, 1996). The cells that would normally migrate inwards remain clustered at their site of invagination. This phenotype is reminiscent of the *tdf* lack-of-function tracheal phenotype, consistent with the finding that *tdf* acts downstream of *trh*. Although the analysis of the tracheal phenotype in *tdf* deficient embryos is hampered by other gross morphological defects, it is nevertheless apparent that these mutants lack pit formation and that the tracheal cells remain at the lateral surface of the epidermis. However, despite these common features of the *tdf* and *trh* mutant embryos, *tdf* does not seem to mediate all aspects of *trh* function. For example, DPP and BNL signalling pathways strictly require *trh* in order to steer guided and directed tracheal branch migration (Sutherland *et al.*, 1996; Vincent *et al.*, 1997) but do not involve *tdf* function. This indicates that the *trh* activity is not mediated by *tdf* function in this case, and that at least three features of tracheal cells depend on *trh* activity and only one of them is mediated by *tdf*. The determination of tracheal cell fate and the ability of cells to respond to external signals to direct guided migration are processes independent of *tdf* activity. However, the third feature, the ability of cells to migrate, depends on *trh* and is mediated by *tdf*. This makes it likely that *tdf* functions as a *trh*-dependent transcription factor which regulates genes that are needed for basic features of cell movements (Figure 7). To enable cell migration, TDF may control or interfere with factors that affect the architecture or function of the cytoskeleton (Gumbiner, 1992; Kemler, 1993) and to cause or mediate defined cell shape changes (Manning and Krasnow, 1993). Furthermore, *tdf* function may participate in the control of cell adhesion molecules, such as *Drosophila* E-cadherin, which are required for the dynamic rearrangement mechanism of tracheal cells (Tanaka-

Matakatsu *et al.*, 1996; Uemura *et al.*, 1996). Further studies are necessary to disclose how *tdf* activity is involved in such processes that ultimately lead to tracheal cell migration.

Maternal and zygotic *tdf* function is necessary in defined tissues

Although *tdf* function is important for tracheal development, its function is not restricted to tracheal development. In the embryo, zygotic TDF is expressed in various tissues while maternal TDF is homogeneously distributed in the egg and accumulates in all nuclei until germ band extension stage. This suggests that TDF may be necessary in many, possibly all, cells during early development. However, the lack of maternal *tdf* function does not affect development in a discernible manner, suggesting that maternal TDF is not essential for developmental processes, if TDF is zygotically provided. Since zygotic TDF expression is restricted to specific tissues, such as tracheal and the central nervous system, it appears that *tdf* has no essential function outside the zygotic expression domains. This conclusion is consistent with the result that the development of embryos into adult flies is not affected when TDF is ectopically expressed in cells of the developing embryo (unpublished results). Thus, lack-of-function and gain-of-function experiments argue that TDF does not interfere with cellular processes outside its zygotic domains of expression. This conclusion apparently conflicts with the observation that the lack of both zygotic and maternal TDF results in a more severe tracheal phenotype than a lack of zygotic TDF alone. Also, the defects in the central nervous system as well as in head development of zygotic *tdf* mutant embryos are enhanced by the lack of maternal TDF (K.G.Eulenberg and R.Schuh, in preparation). This indicates that although maternal *tdf* activity is not necessary for embryonic development it can partially compensate for the lack of zygotic *tdf* activity, while zygotic *tdf* activity is both necessary and sufficient to provide the *tdf* function in the embryo.

How does TDF control developmental processes?

The nuclear location of TDF and its diagnostic bZIP protein motif suggest that TDF mediates its function by transcriptional regulation. The leucine zipper at the C-terminal end of TDF may serve as a dimerization motif allowing homo- and/or heterodimers to form, as it has been shown for GCN4 (Ellenberger *et al.*, 1992). The major function of this dimerization is to bring together the basic regions of each monomer in the correct register to allow DNA binding. The specificity of DNA binding is a consequence of the homomeric or heteromeric complex formation. Homodimers recognize either a pseudopalindromic DNA sequence where the two half-sites overlap at a central base pair, or a palindromic DNA where the two half-sites abut one another (Hurst, 1995). In contrast, heterodimer recognition sites can differ from that of either homodimer and resemble instead a non-dyad symmetric site incorporating the two distinct monomeric DNA binding sites. Thus, heterodimer formation greatly expands the number of target sequences that may be recognized by bZIP proteins (Lamb and McKnight, 1991). The finding that maternal TDF, which is detectable in all nuclei up to germ band extension, is not sufficient to provide full *tdf*

function and that ectopic TDF expression does not interfere with normal development (see above), argues that TDF does not alone enable cells to migrate. This suggests that TDF requires one or several other factors to mediate its function in target cells or that TDF is modified in a tissue-specific manner, for example by phosphorylation (Gonzalez *et al.*, 1991). The ability of bZIP proteins to dimerize does not favour one of the above possibilities, but provides an attractive and testable model of the biochemical mode of TDF action. TDF may interact with one or several tissue-specific bZIP regulatory proteins via leucine zipper modules (Landschulz *et al.*, 1988). Heterodimerization may then result in a functional regulator that controls tissue-specific target genes. Cells that fail to provide partners for TDF would not respond to *tdf* activity, which may explain why TDF is not functional in those cells.

Irrespective of how TDF provides its function, our results strongly suggest that the formation of the tracheal system involves two linked cellular features to generate a stereotyped architecture. One feature is the ability of cells to migrate and this is dependent on *tdf* function, likely to act in a cell-autonomous manner. The second feature which builds upon this ability is that migration becomes guided and directed and this is dependent on external signals provided by DPP and BNL as shown recently (Sutherland *et al.*, 1996; Vincent *et al.*, 1997).

Materials and methods

DNA analysis
Preparation of DNA, isolation of genomic and ten different cDNAs, Southern and Northern blot analysis were performed according standard protocols (Sambrook *et al.*, 1989). Genomic DNA flanking the P-insertion lines *tdf^P1^*, *tdf^P2^* and *C5-2-5* were obtained by plasmid rescue experiments. Three cDNA sequences and the corresponding genomic DNA sequences, including intronic DNA sequences, were determined by the dideoxynucleotide method (Sanger *et al.*, 1977) employing automated sequencing. Sequence comparison with databases was performed using the HUSAR software package (Devereux *et al.*, 1984). To generate a UAS-tdf construct the *tdf* coding region of cDNA S3 was excised from Bluescript vector as a *Eco*RI–*Kpn*I restriction fragment and inserted into the *Eco*RI–*Kpn*I site of the P-element vector pUAST (Brand and Perrimon, 1993). The resulting pUAST–tdf plasmid was used for P-element mediated transformation of flies (Rubin and Spradling, 1983). Several independent fly strains bearing pUAST–tdf on different chromosomes were generated (UAS–tdf effector fly lines).

Immunostaining
Whole-mount immunostaining was performed as described (Ashburner, 1989). Biotinylated secondary antibodies were revealed using the VECTASTAIN Elite ABC-peroxidase system (Vector Laboratories). In double-staining experiments, alkaline phosphatase staining was performed after horseradish peroxidase staining. To stain tracheal lumen the monoclonal antibody 2A12 (Developmental Studies Hybridoma Bank, Iowa) was used. A polyclonal antibody (Cappel) was applied to detect β-galactosidase expression. To detect SAL the affinity-purified rabbit anti-SAL antibody was used (Kühnlein *et al.*, 1994). Immuno-stained embryos were viewed with a Zeiss Axiophot microscope.

In situ hybridization
In situ hybridization of whole-mount embryos were done with digoxigenin- and fluorescein-labelled RNA probes as described (Tautz and Pfeifle, 1989). The RNA probes used in our experiments derived from *bnl* (Sutherland *et al.*, 1996), *btl* (Klämbt *et al.*, 1992), *kni* (Nauber *et al.*, 1988), *lacZ*, *tdf* and *trh* (Wilk *et al.*, 1996).

Generation of antisera and antibody purification
A *Sal*I–*Bam*HI restriction fragment of *tdf* cDNA S1 coding for the amino acids 32–260 of the predicted *tdf* protein was subcloned into pRSET-A (Invitrogen). The histidine-tagged TDF fusion protein was produced in *Escherichia coli* BL21-lysS as described. The resulting recombinant protein was purified on ProBondTM Resin (Invitrogen) according to the manufacturer's protocol, separated from possible contaminants by SDS–PAGE and used for immunization of rabbits at Eurogentec (Brussels). The serum was affinity-purified on a CNBr-activated Sepharose (Pharmacia) coupled with histidine-tagged TDF fusion protein. The purified antibodies detect TDF specifically, since homozygous *tdf^PΔ3^* and *Df bw^S46^* embryos showed exclusively maternal TDF staining and no staining during later embryogenesis (unpublished data).

Fly stocks and generation of germline clones
The *tdf^P1^* and *tdf^P2^* alleles correspond to the P-element insertion lines *l(2)07010* (Karpen and Spradling, 1992) and *l(2)k15608* (Torok *et al.*, 1993) respectively. Mobilization of the single, viable P-element insertion of the *C5-2-5* line (Hartenstein and Jan, 1992) that is inserted in the *tdf* gene locus resulted in three embryonic lethal *tdf* alleles. The deletions associated with the *tdf^PΔ3^*, *tdf^PΔ4^* and *tdf^PΔ5^* alleles were identified by Southern blot analysis. The *lacZ* enhancer trap line *l-eve-1* which is integrated in the *trh* gene was used to mark tracheal cells with cytoplasmic β-galactosidase expression (Perrimon *et al.*, 1991). The *pnt–lacZ* enhancer trap line [Pantip-1/*l(3)7835*] was used to mark secondary branch formation (Sutherland *et al.*, 1996). We used the *btl^LG19^* (Klämbt *et al.*, 1992), the *tkv^str–1^* (Nüsslein-Volhard *et al.*, 1984) and the *trh^3^* (Wilk *et al.*, 1996) alleles to analyse *tdf* expression.

Female germline clones of *tdf^PΔ3^* were generated by the DSF-FLP method using *tdf^PΔ3^* recombined onto the FRT G13 chromosome and the P(ovoD1) insertion on the FRT G13 chromosome. Heat shock was delivered at 37°C for 2 h during first and second instar larval stage. Embryos mutant for both maternal and zygotic *tdf* were generated by fertilizing mutant germline clones with *tdf^PΔ3^/CyO* males, while those mutants that lack only maternal *tdf* were generated by fertilization with wild-type males.

References

Affolter,M., Nellen,D., Nussbaumer,U. and Basler,K. (1994) Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGFβ homologs during *Drosophila* embryogenesis. *Development*, **120**, 3105–3117.

Ashburner,M. (1989) *Drosophila, a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brand,A.H. and Perrimon,N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401–415.

Campos-Ortega,J.A. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster.* Berlin: Springer-Verlag.

Capovilla,M., Eldon,E.D. and Pirotta,V. (1992) The *giant* gene of *Drosophila* encodes a b-zip DNA-binding protein that regulates the expression of other segmentation gap genes. *Development*, **114**, 99–112.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Ellenberger,T.E., Brandl,C.J., Struhl,K. and Harrison,S.C. (1992) The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted a helices: crystal structure of the protein–DNA complex. *Cell*, **71**, 1223–1237.

Foulkes,N.S., Mellstrom,B., Benusiglio,E. and Sassonecorsi,P. (1992) Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature*, **355**, 80–84.

Glazer,L. and Shilo,B.Z. (1991) The *Drosophila* FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.*, **5**, 697–705.

Gonzales,G.A., Menzel,P., Leonard,J., Fischer,W.H. and Montminy,M.R. (1991) Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.*, **11**, 1306–1312.

Gumbiner,B.M. (1992) Epithelial morphogenesis. *Cell*, **69**, 385–387.

Hartenstein,V. and Jan,Y.N. (1992) Studying *Drosophila* embryogenesis with P-lacZ enhancer trap lines. *Roux's Arch. Dev. Biol.*, **201**, 194–220.

Hurst,H.C. (1995) Transcription factors 1: bZIP proteins. *Protein Profile*, **2**, 101–168.

Isaac,D.D. and Andrew,D.J. (1996) Tubulogenesis in *Drosophila*: a requirement for the *tracheless* gene product. *Genes Dev.*, **10**, 103–117.

Karpen,G.H. and Spradling,A.C. (1992) Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P-element insertional mutagenesis. *Genetics*, **132**, 737–753.

Kemler,R. (1993) From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.*, **9**, 317–321.

Kerppola,T.K. and Curran,T. (1991) Transcription factor interactions: basics on zippers. *Curr. Opin. Struct. Biol.*, **1**, 71–79.

Klämbt,C., Glazer,L. and Shilo,B.Z. (1992) *breathless*, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes Dev.*, **6**, 1668–1678.

Kühnlein,R.P. and Schuh,R. (1996) Dual function of the region specific homeotic gene *spalt* during *Drosophila* tracheal system development. *Development*, **122**, 2215–2223.

Kühnlein,R.P., Frommer,G., Friedrich,M., Gonzalez-Gaitan,M., Weber,A., Wagner-Bernholz,J.F., Gehring,W.J., Jäckle,H. and Schuh,R. (1994) *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J.*, **13**, 168–179.

Lamb,P. and McKnight,S.L. (1991) Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerisation. *Trends Genet.*, **16**, 417–422.

Lamph,W.W., Wamsley,P., Sassone-Corsi,P. and Verma,I.M. (1988) Induction of proto-oncogene JUN/AP-1 by serum and TPA. *Nature*, **334**, 629–631.

Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science*, **240**, 1759–1764.

Lauffenburger,D.A. and Horwitz,A.F. (1996) Cell migration: a physically integrated molecular process. *Cell*, **84**, 359–369.

Lee,T., Hacohen,N., Krasnow,M.A. and Montell,D.J. (1996) Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the *Drosophila* tracheal system. *Genes Dev.*, **10**, 2912–2921.

Manning,G. and Krasnow,M.A. (1993) Development of the *Drosophila* tracheal system. In Bate,M. and Martinez Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 609–685.

Mitchison,T.J. and Cramer,L.P. (1996) Actin-based cell motility and cell locomotion. *Cell*, **84**, 371–379.

Montell,D.J. (1994) Moving right along: regulation of cell migration during *Drosophila* development. *Trends Genet.*, **10**, 59–62.

Nakabeppu,Y. and Nathans,D. (1989) The basic region of Fos mediates specific DNA binding. *EMBO J.*, **8**, 3833–3841.

Nauber,U., Pankratz,M.J., Kienlin,A., Seifert,E., Klemm,U. and Jäckle,H. (1988) Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. *Nature*, **336**, 489–492.

Nellen,D., Affolter,M. and Basler,K. (1994) Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by decapentaplegic. *Cell*, **78**, 225–237.

Nüsslein-Volhard,C., Wieschaus,E. and Kluding,H. (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Biol.*, **193**, 267–282.

Perrimon,N., Noll,E., McCall,K. and Brand,A. (1991) Generating lineage-specific markers to study *Drosophila* development. *Dev. Genet.*, **12**, 238–252.

Riesau,W. and Flamme,I. (1995) Vasculogenesis. *Annu. Rev. Cell Dev. Biol.*, **11**, 73–91.

Ruberte,E., Marty,T., Nellen,D., Affolter,M. and Basler,K. (1995) An absolute requirement for both the type II and type I receptors, *punt* and *thick veins*, for *dpp* signaling *in vivo*. *Cell*, **80**, 889–897.

Rubin,G.M. and Spradling,A.C. (1983) Vectors for P element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res.*, **11**, 6341–6351.

Samakovlis,C., Hacohen,N., Manning,G., Sutherland,D.C., Guillemin,K. and Krasnow,M.A. (1996) Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development*, **122**, 1395–1407.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with the chain terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Smolik,S.M., Rose,R.E. and Goodman,R.H. (1992) A cyclic AMP-responsive element-binding transcriptional activator in *Drosophila melanogaster*, dCREB-A, is a member of the leucine zipper family. *Mol. Cell. Biol.*, **12**, 4123–4131.

Sutherland,D., Samakovlis,C. and Krasnow,M.A. (1996) *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell*, **87**, 1091–1101.

Tanaka,E. and Sabry,J. (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell*, **83**, 171–176.

Tanaka-Matakatsu,M., Uemura,T., Oda,H., Takeichi,M. and Hayashi,S. (1996) Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. *Development*, **122**, 3697–3705.

Tautz,D. and Pfeifle,C. (1989) A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma*, **98**, 81–85.

Torok,I., Tick,G., Alvarado,M. and Kiss,I. (1993) P-lacW insertional mutagenesis on the second chromosome of *Drosophila melanogaster*: isolation of lethals with different overgrowth phenotypes. *Genetics*, **135**, 71–80.

Uemura,T., Oda,H., Kraut,R., Hayashi,S., Kataoka,Y. and Takeichi,M. (1996) Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Dev.*, **10**, 659–671.

Vincent,S., Ruberte,E., Grieder,N.C., Chen,C.-K., Haerry,T., Schuh,R. and Affolter,M. (1997) DPP controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. *Development*, **124**, 2741–2750.

Wilk,R., Weizman,I. and Shilo,B.-Z. (1996) *tracheless* encodes a bHLH-PAS protein which is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.*, **10**, 93–102.

Note added in proof

Protein and DNA sequence comparison suggest that the *tracheae defective* gene is allelic to the *apontic* gene recently described by Gellon *et al.* (*Dev.*, **124**, 3321–3331; 1997).