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

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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 tracheless, the key regulator gene for tracheal development. In contrast, tdf activity is not dependent on and does not interfere with the fibroblast growth factor-1 alpha (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/Drosophilatracheae 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 metaneres (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, tracheless (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.
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Fig. 1. Tracheal phenotype of tdf mutations. (A–F) Whole-mount anti-β-galactosidase staining of a stage 14 wild-type (A), a transheterozygous tdfP1/tdfP2 (B), a weak (C) and a strong (D) phenotype of a tdfP2, a tdfPΔ3 (E) and a maternal and zygotic tdfPΔ3 (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 tdfP1 and tdfP2, respectively. In homozygous tdfP1 and tdfP2 mutant embryos, as well as in transheterozygous tdfP1/tdfP2 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 bw346 a weaker tracheal phenotype than embryos which are homozygous for the tdf deficiency (data not shown) suggesting that the tdfP1 and tdfP2 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 tdfP1 and tdfP2 alleles as well as the Df bw346. Two of these lines, tdfPΔ3 and tdfPΔ4, display in hemizygous as well as hemizygous conditions over the Df bw346 the same severity of tracheal phenotype as observed with homozygous Df bw346 embryos (Figure 1E). This indicates that tdfPΔ3 and tdfPΔ4 represent lack-of-function alleles. However, as observed with the hypomorphic tdf alleles, the lack-of-function tdf mutant alleles and Df bw346 embryos also show variable tracheal defects.
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\textsuperscript{D1} 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\textsuperscript{D2} 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)\textsuperscript{+} 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\textsuperscript{P1} and tdf\textsuperscript{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,
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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 for cAMP-dependent protein kinase, Protein Kinase C and Casein Kinase II (Figure 3B) 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 dioxygenin-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.
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 tdfΔ35 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
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Fig. 5. TDF is necessary for tracheal branch formation. Whole-mount anti-β-galactosidase staining of a stage 14 l-eve-1 embryo (A), a tdfΔ3 l-eve-1 embryo (B) and a tdfΔ3 l-eve-1 embryo bearing btl–Gal4 driver and UAS–tdf effector construct (C; see Materials and methods). The l-eve-1 tracheal marker P-line was used to mark tracheal cells. The tdfΔ3 embryo in (B) develops rudimentary tracheal branches, while the rescued tdfΔ3 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
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...
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 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 tdpF1, tdpF2 and CS-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 (Deverex et al., 1984). To generate a UAS-tdf construct the tdf coding region of cDNA S3 was excised from Bluescript vector as an EcoRI-KpnI restriction fragment and inserted into the EcoRI-KpnI 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., 1996). Immunostained embryos were viewed with a Zeiss Axioshot 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), btfl (Klämbt et al., 1992), kni (Nauber et al., 1988), lacZ, tdf and trh (Wilk et al., 1996).

Generation of antisera and antibody purification
A Sall-BarnH restriction fragment of tdf cDNA S1 coding for the amino acids 32–260 of the predicted tdf protein was subcloned into pRSSET-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 Eurogencent (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 homologous tdfΔS3 and DfΔm–46 embryos showed exclusively maternal TDF staining and no staining during later embryogenesis (unpublished data).

Fly stocks and generation of germline clones
The tdpF1 and tdpF2 alleles correspond to the P-element insertion lines l(2)70701 (Karpen and Spradling, 1992) and l(2)kJ15608 (Torok et al., 1993) respectively. Mobilization of the singe, 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 tdpΔS3, tdpΔS4 and tdpΔS5 alleles were identified by Southern blot analysis. The lacZ enhancer trap line eve-l 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 p[Antip-l(3)17835] was used to mark secondary branch formation (Sutherland et al., 1996). We used the tdpΔIGIV (Klämbt et al., 1992), the tkvΔ7•Δ8 (Nüsslein-Volhard et al., 1984) and the trhΔ3 (Wilk et al., 1996) alleles to analyse tdf expression.

Female germline clones of tdpΔS3 were generated by the DSF-FLP method using tdpΔS3 recombinated 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 tdpΔS3/Cyo males, while those mutants that lack only maternal tdf were generated by fertilization with wild-type males.

Acknowledgements
We thank G.Dowe, R.P.Kühnlein and M.Pankratz for critical reading of the manuscript. We are grateful to H.Jäckle for critical comments and discussions on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 271).

References
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Received on August 12, 1997; revised on August 28, 1997

**Note added in proof**

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