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Elbow and Noc define a family of zinc finger proteins controlling morphogenesis of specific tracheal branches

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SUMMARY

The *elbow* (*elB*) gene encodes a conserved nuclear protein with a single zinc finger. Expression of ElB is restricted to a specific subset of tracheal cells, namely the dorsal branch and the lateral trunks. Stalled or aberrant migration of these branches is observed in *elB* mutant embryos. Conversely, ElB misexpression in the trachea gave rise to absence of the visceral branch and an increase in the number of cells forming the dorsal branch. These results imply that the restricted expression of ElB contributes to the specification of distinct branch fates, as reflected in their stereotypic pattern of migration. As *elB* loss-of-function tracheal phenotypes are reminiscent of defects in Dpp signaling, the relationship between ElB and the Dpp pathway was examined. By using pMad antibodies that detect the activation pattern of the Dpp pathway, we show that Dpp signaling in the trachea is not impaired in *elB* mutants. In addition, expression of the Dpp target gene *kni* was unaltered. The opposite is true as well, because expression of *elB* is independent of Dpp signaling. ElB thus defines a parallel input, which determines the identity of the lateral trunk and dorsal branch cells. No ocelli (Noc) is the *Drosophila* protein most similar to ElB. Mutations in *noc* give rise to a similar tracheal phenotype. Noc is capable of associating with ElB, suggesting that they can function as a heterodimer. ElB also associates with the Groucho protein, indicating that the complex has the capacity to repress transcription of target genes. Indeed, in *elB* or *noc* mutants, expanded expression of tracheal branch-specific genes was observed.

Key words: Elbow, Noc, Zinc finger, Trachea, Branch migration, Groucho, *Drosophila*

INTRODUCTION

The *Drosophila* tracheal system is a stereotypical network of interconnected tubes that supplies air to all cells of the organism. Initially, ten tracheal placodes are defined on both sides of the embryo, each consisting of 20 cells. The placodes undergo two rounds of division, giving rise to the final number of tracheal cells. All subsequent events of tracheal morphogenesis and branch migration occur in the absence of any further cell division (reviewed by Manning and Krasnow, 1993).

The final structure of the tracheal tree is elaborate. Each tracheal pit gives rise to five different branches: dorsal branch (DB), dorsal trunk (DT), visceral branch (VB), lateral trunk anterior (LTa) and lateral posterior/ganglionic branch (LTp/GB). The number of cells allocated to each branch is fixed and the final structure of each branch is stereotyped, reflecting established migration routes. Within each branch, different cell types are formed from an originally equipotent population of tracheal cells (Samakovlis et al., 1996a). The cells at the termini of the branches differentiate as terminal cells that send long hollow extensions to hypoxic tissues (Guillemin et al., 1996). Another group of specialized cells, termed fusion cells, establishes connections between branches from adjacent segments (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996).

This elaborate tracheal structure is set up by the concerted activity of multiple signaling pathways, uncovered in the past decade (reviewed by Affolter and Shilo, 2000; Zelzer and Shilo, 2000b). The initial assignment of tracheal fates within the population of ectodermal cells is driven by the localized expression of the Trachealess and Drifter transcription factors (Anderson et al., 1995; Wilk et al., 1996; Llimargas and Casanova, 1997; Zelzer and Shilo, 2000a). Persistent expression of these genes in the trachea provides a 'cell context' for other signals that impinge on the trachea.

Prior to the onset of tracheal migration, the precise number of cells must be allocated to each future branch. Several signaling pathways contribute to this decision, and in many cases parallel inputs from different pathways are responsible for the assignment of a particular branch fate (Wappner et al., 1997; Vincent et al., 1998; Llimargas, 2000; Llimargas and Lawrence, 2001; Chihara and Hayashi, 2000; Glazer and Shilo, 2001).

The process of migration is guided by the FGF pathway. All tracheal cells express the FGF receptor, Breathless (Btl)

(Glazer and Shilo, 1991; Klämbt et al., 1992). The ligand, Branchless (Bnl), is expressed locally in adjacent ectodermal or mesodermal cells (Sutherland et al., 1996). This restricted ligand presentation is responsible for guided migration. In addition, as the branches elongate, the levels of Btl activation determine the fate of the cells as terminal or fusion cells. Additional accessory guidance systems are present, such as the presence of a mesodermal cell expressing Hunchback (Hb) that assists the migration of the dorsal trunk cells (Wolf and Schuh, 2000).

Morphogenesis of the tracheal system is determined by highly coordinated signaling events, which are restricted in both space and time (Affolter and Shilo, 2000; Zelzer and Shilo, 2000b). This prompted us to search for new genes regulating tracheal development using the EP misexpression screen (Rorth, 1996; Rorth et al., 1998). We used the midline- and tracheal-specific *btl-Gal4* driver (Shiga et al., 1996), and screened the collection of EP lines for those that will give rise to lethality because of aberrant development of the tracheal system, or other tissues expressing *btl-Gal4*. Known genes that regulate tracheal patterning, such as *dpp*, *bnl*, *rhomboid* and *escargot* were scored, validating the specificity of the approach. In addition, this screen identified new genes that were not previously known to be involved in patterning the trachea.

We present the analysis of the *elB* gene encoding a conserved nuclear protein with a single zinc finger. ElB misexpression in the trachea gave rise to loss of the visceral branch and expansion of the dorsal branch, while a mutation in the gene resulted in defective migration in the lateral and dorsal branches. These phenotypes are consistent with the restricted expression of *elB* in the lateral trunk and dorsal branch. While the *elB* mutant tracheal phenotypes are reminiscent of defects in Dpp signaling, the two inputs are independent. ElB thus defines a parallel pathway that determines the identity of the lateral trunks and dorsal tracheal branches. A similar phenotype was observed in mutations for the *noc* gene, which encodes a protein homologous to ElB. We show that the two proteins associate with each other, suggesting that they function as a complex. In addition. ElB associates with the Groucho protein, implying that the ElB/Noc complex can repress the expression of target genes. Indeed, in *elB* or *noc* mutants, expanded expression of tracheal branch-specific or cell-specific genes was observed.

MATERIALS AND METHODS

Fly strains

The EP lines used in this study include EP(2)2039 upstream of the *elB* gene, and EP(2)2000 and EP(2)2173 upstream of the *noc* gene. Lines containing full-length cDNA *elB* or *noc* in pUAST were also generated and tested. Expression was driven by *btl-Gal4* (Shiga et al., 1996) inserted on the third chromosome. Excisions in the *elB* and *noc* genes were generated by crossing the EP(2)2039 or EP(2)2173 lines to the Delta 2-3 transposase-bearing line on the second chromosome. Lethal excisions were characterized by Southern blots. The excision lines were also tested for lethality over *Df(2L)noc10* (34F1-35B1) (U#42449) or *Df(2L) fn2* (35A3-35B2) (BL#3583), removing both *elB* and *noc* loci. An excision line removing the 5′ region of each gene was characterized in detail (elB^{d47} and noc^{d64}). A line containing a *Gal4* insertion upstream of *elB* (provided by C. Desplan) was used to follow the expression pattern of the gene, following a cross to a line bearing *UAS-elB*. The lethal enhancer trap line *l(2)k07706*, inserted upstream to *elB* and partially recapitulating the expression pattern, was also used.

The following markers for gene expression in specific tracheal branches were used: *l(2)01351*, which expresses *lacZ* in the visceral branch and in pairs of cells in the dorsal trunk; and *kni-lacZ* (2.2 KI) inserted on the third chromosome (provided by R. Schuh), which is expressed in the dorsal branch, visceral branch and lateral trunks.

To test the relationship between the Dpp pathway and ElB, the following lines were used: tkv^{II}*/CyO* (provided by K. Basler), *UAS-tkv** (provided by S. Cohen) and *UAS-knrl* (provided by R. Schuh).

Sequences analysis

Protein sequences were scanned for homologies and conserved domains by protein BLAST (BLASTP) and by SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/interpro/scan.html). Multiple sequence alignments were made by PIMA protocol in the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). *elB* cDNA GenBank Accession Number, AY115567.

Antibodies and in situ hybridization

Rat anti-ElB was generated by injecting full length, His-tagged, recombinant protein expressed in a pRSET vector. To visualize tracheal nuclei and lumen we used rat anti-Trh and monoclonal 2A12. Rabbit anti-Sal antibody (provided by R. Schuh), and rabbit anti-β-gal (Cappel) or mouse anti-β-gal (Promega) were also used. Rabbit anti-pSmad1 antibodies (provided by P. ten Dijke) were used at a dilution of 1/200. Secondary antibodies were purchased from Jackson ImmunoResearch.

DIG-labeled RNA antisense probes were prepared from *elB* or *noc* cDNAs, and processed and visualized according to standard protocols.

GST pull-down

The following GST fusion constructs were generated from *elB*: pGEX-4T3 containing ElB-69-553aa (generated by cloning an *Xho*I fragment). pGEX-4T3 containing ElB-287-553 amino acids (generated by cloning the 3′ *Bam*HI fragment). pGEX-4T3 containing ElB-287-480 amino acids (generated from the 3′ *Bam*HI-*Not*I fragment). The Noc GST fusion pGEX-4T3 contains 297-396 amino acids (generated from the *Eco*RV-*Not*I fragment). All GST fusion constructs were purified on Glutathione-agarose (Sigma) by standard procedures. ElB, Noc, CtBP and Groucho were labeled with ^{35}S-methionine in vitro by translation in TnT® T7RNA polymerase-coupled reticulocyte lysate system (Promega) according to manufacturer's instructions. *groucho* and *CtBP* cDNAs were provided by Z. Paroush.

GST pull-downs were performed in 10 mM Tris (pH 7.0), 150 mM NaCl, 0.1% Triton X-100 buffer. After 2 hours binding, beads were washed five times with the same buffer for 2 minutes each, followed by an additional 30 minute wash with 10 mM Tris (pH 7.0), 300 mM NaCl, 0.1% Triton X-100 buffer. Samples were separated on a 10% acrylamide gel by SDS-PAGE, fixed for 30 minutes in acetic acid:water:isopropanol (10:65:25) and washed for 15 minutes in Amplify solution (Amersham), prior to exposure.

RESULTS

Identification of *elB* in an EP tracheal misexpression screen

In a misexpression screen of the EP collection with the midline- and tracheal-specific *btl-Gal4* driver, the EP(2)2039 line gave rise to a lethal phenotype. Examination of the tracheal system of these embryos with antibodies recognizing the

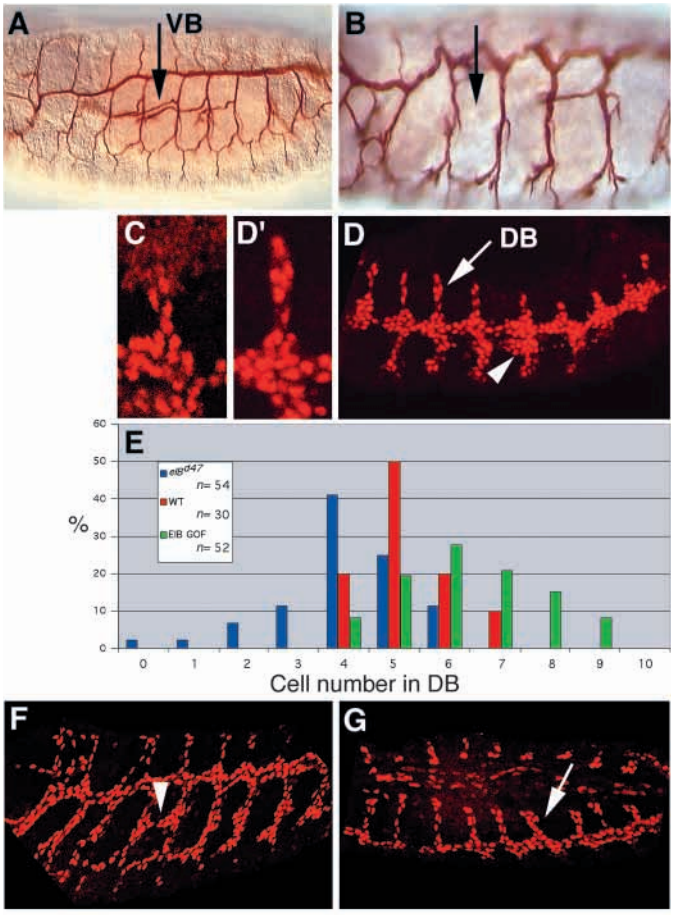

Fig. 1. Tracheal phenotype following *elB* overexpression. (A) Wild-type stage 14 embryo stained with anti-lumen antibody. Note the visceral branch (VB, arrow). (B) In *btl-Gal4*/EP-*elB* the VB is not formed in most metameres (arrow). (C) Wild-type stage 14 embryo stained with anti-Trh antibody, marking the tracheal nuclei. Note the presence of five or six nuclei in the dorsal branch formed by tracheal pit 3. (D,D′) After tracheal misexpression of ElB, embryos were stained with anti-ElB antibody. Note the nuclear staining and the presence of seven nuclei in the dorsal branch (DB) formed by tracheal pit 3 (arrow). The stalled visceral branch cells remain in the transverse connective (arrowhead). (E) The number of nuclei in the dorsal branch was quantitated in wild type (red bars, *n*=30), ElB overexpression (green, *n*=52) and *elB* mutant embryos (blue, *n*=54). (F,G) *btl-Gal4/UAS-elB* embryos were stained with anti-ElB antibodies. Note the reduced visceral branches (arrowhead) and the extended dorsal branches (arrow).

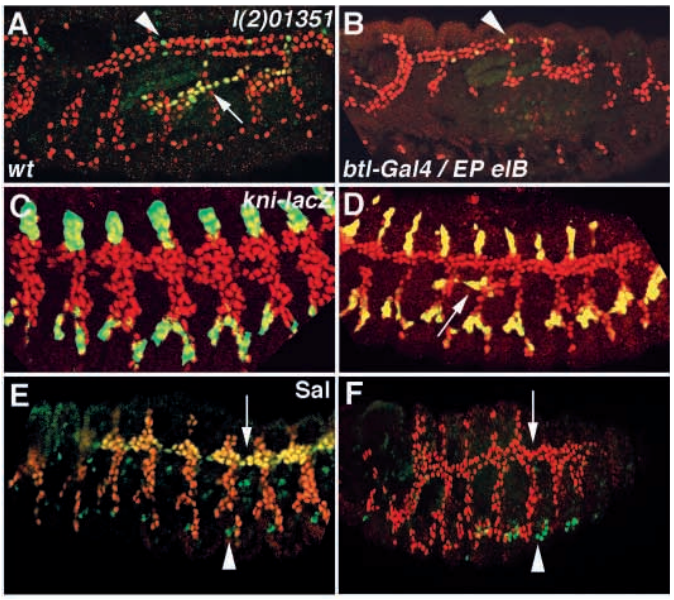

Fig. 2. Effects of *elB* overexpression on tracheal gene expression. (A) The enhancer trap line *l(2)01351* is expressed in a pair of cells in the dorsal trunk of each metamere (arrowhead) and in the visceral branch (arrow). The tracheal nuclei are marked by anti-Trh staining (red), and the enhancer trap by anti-β-gal (green). (B) After *elB* tracheal misexpression, the enhancer trap line (green) continues to be expressed in the dorsal trunk (arrowhead), but is not observed in the residual visceral branch cells. Tracheal nuclei (red) are marked by anti-ElB. (C) *kni-lacZ* (green) is a target of Dpp signaling in the trachea in the dorsal branch and lateral trunks. It is also expressed weakly in the visceral branch. Red: anti-Trh. (D) After *elB* tracheal misexpression, *kni* expression (green) is retained in the reduced dorsal branches and the residual visceral branch (arrow). Red: anti-ElB. *kni* expression in the visceral branch that is independent of Dpp signaling was previously reported in wild-type embryos (Vincent et al., 1997; Chen et al., 1998). (E) Sal is expressed in the dorsal trunk (arrow) and oenocytes (arrowhead). Red, anti-Trh; green, anti-Sal. (F) After *elB* tracheal misexpression, Sal protein is retained in the oenocytes (arrowhead), but is not detected in the trachea (arrow). Red, anti-Trh; green, anti-Sal.

tracheal lumen revealed a specific abolishment of the visceral branch (Fig. 1A,B). By immunohistochemical analysis using anti-Trh antibody that detects all tracheal nuclei, as well as anti-ElB antibody, which shows a similar pattern, we observed that cells that normally form the visceral branch remained in the central region of the transverse connective branch. In the dorsal branch, an increase in the number of cells was identified (Fig. 1C,D). Quantitation of the number of dorsal branch cells demonstrated that while in wild-type embryos each branch contains an average of five cells, in ElB misexpression embryos most dorsal branches contain five to eight cells (Fig. 1E).

The phenotypes of ElB tracheal misexpression were verified by crossing the *btl-Gal4* line to lines bearing a pUAST construct driving an *elB* full-length cDNA. Similar phenotypes of reduced visceral branches and enlarged dorsal branches were observed (Fig. 1F,G).

To obtain a finer resolution, we followed molecular markers for the different branches. By using the *l(2)01351* enhancer trap line that marks the visceral branch cells, we show that this marker is not expressed in the cells remaining in the transverse connective following ElB misexpression. Overexpression indeed abolished expression of a gene normally marking the visceral branch cell fate (Fig. 2A,B).

knirps (*kni*) is normally expressed in the lateral trunks (Lta and LTp), dorsal branch and visceral branch (Vincent et al., 1997; Chen et al., 1998). Uniform expression of ElB in the trachea did not alter the pattern of *kni* expression in cells that usually form the LT. *kni* expression is also observed in visceral branch cells that are retained in the transverse connective upon ElB misexpression. Thus, not all visceral branch markers are abolished. Finally, in the dorsal branch more cells express *kni* due to ectopic migration of cells at the expense of the dorsal trunk. (Fig. 2C,D).

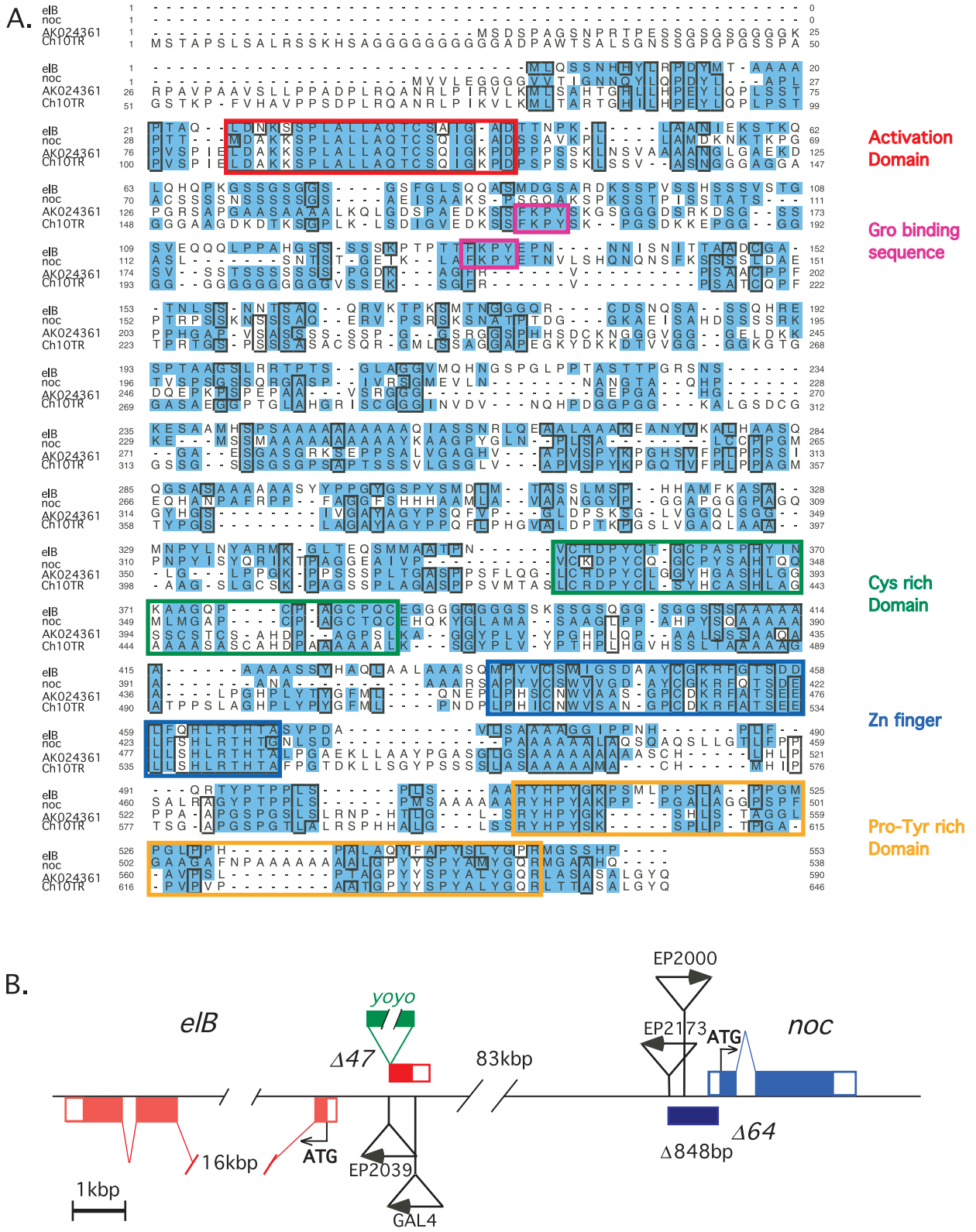

Fig. 3. Sequence of ElB and Noc and gene structure. (A) Alignment of the protein sequence of ElB, Noc and the two human homologs. The following domains are marked: activation domain (red), Groucho binding sequence (purple), cysteine-rich domain (green), Zn finger (blue) and proline-tyrosine rich domain (orange). (B) Gene structures of *elB* and *noc*, and the position of the EP and Gal4 elements. *elB* and *noc* are positioned in opposite transcriptional orientations, and the ATG codon of each positioned within the first exon. The imprecise excisions generated in *elB* and *noc* are shown.

In ElB misexpression embryos, several cells normally assigned to the dorsal trunk appear to be migrating into the dorsal branch, thus increasing the cell number in that branch. This suggested that there may also be a defect in specifying the dorsal trunk fate. Therefore, we followed a dorsal trunk marker, Spalt (Sal), in embryos misexpressing ElB. Sal is a transcription factor that is specifically expressed in the dorsal trunk cells and determines their identity (Kuhnlein and Schuh, 1996). Ectopic ElB expression abolished all Sal expression in the trachea (Fig. 2E,F). Surprisingly, this did not lead to an absence of the dorsal trunk, which is typical of *sal* mutant embryos, presumably because the *btl-Gal4* driver induced the accumulation of ElB and abolishment of Sal expression after execution of the normal Sal function in the dorsal trunk.

These experiments suggest that ElB expression must be excluded from the dorsal trunk and visceral branch, in order to specify their proper identities.

Elbow is a single zinc-finger protein

Plasmid rescue of the EP(2)2039 element showed that it is inserted at chromosomal position 35B, previously known as the *elbow-noc* (*elB-noc*) region (Davis et al., 1990; Davis et al., 1997; Ashburner et al., 1999). The EP line is inserted 965 bp upstream to the 5′ UTR and 1365 bp upstream to the translation start site of *elB*, and is homozygous viable. As no publicly available ESTs were found, we screened an embryonic cDNA library with the EP(2)2039 genomic rescue fragment. Three positive clones were sequenced and analyzed, all of them were 2550 bp and encoded a protein of 553 amino acids. The cDNA translation shows that there is a different exon-intron structure compared with both the published GeneScan predicted DS06238.3 (Ashburner et al., 1999), and to CG4220 in the current GadFly genomic annotation (http://hedgehog.lbl.gov:8001/cgi-bin/annot/query/).

BLAST analysis of the cDNA sequence was performed and shows that ElB belongs to the Sp1 transcription factor family. However, it contains a single zinc finger (C2H2 type), while Sp1 transcription factors generally posses several zinc fingers. Antibody staining of ElB in embryos overexpressing *elB* indeed showed a nuclear localization pattern (Fig. 1D). The closest homolog of ElB is the *Drosophila* Noc protein, which shares 50% identity (Cheah et al., 1994). It has previously been reported that Noc contains two zinc fingers, but the algorithms we used detect only one zinc finger. The *noc* gene is located in the same chromosomal region at a distance of 82 kb from *elB*, suggesting that it arose by a gene duplication event.

A human protein termed AK024361, which is localized to 8p11.2, and a putative transcript from chromosome 10 show a high degree of similarity to ElB and Noc proteins. Alignment of these four proteins allowed identification of additional conserved domains (Fig. 3). In the N terminus there is a putative activation domain, while a proline- and tyrosine-rich domain is located in the C terminus. In addition, there is a cysteine-rich domain in the middle of ElB protein. Between the activation domain and Cysteine-rich domain are several stretches of Alanine repeats. Previous studies suggested that Noc functions as a repressor of transcription due to the high amount of the A-repeats (Cheah et al., 1994). A putative FKPY Groucho-binding motif (Zhang et al., 2001; Hasson et al., 2001) is also found in all four proteins (see below).

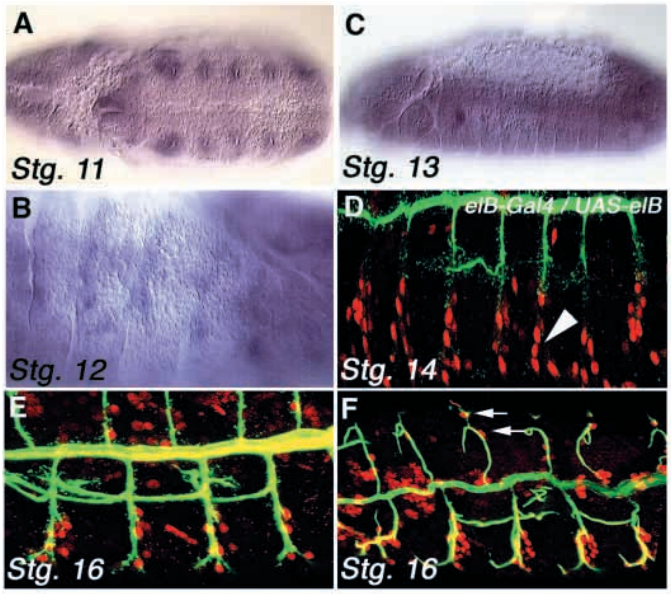

Fig. 4. Expression of ElB. (A) At stage 11, *elB* RNA is detected in all tracheal pits. (B) By stage 12, expression is reduced in the central part of the pit, and retained in the dorsal branch and lateral trunks. (C) At stage 13, expression is detected only in the lateral trunk, most prominently in the first metamere. (D) Insertion of a Gal4 element upstream to *elB* induced (at stage 14) expression of *UAS-elB* in the lateral branches (arrowhead), but not in the visceral branch or dorsal trunk. Red, anti-ElB; green, 2A12 (which marks the tracheal lumen). (E) At stage 16 the expression of *UAS-elB* in the spiracular branch, transverse connective, lateral trunk and ganglionic branch was observed. (F) At the same stage, the dorsal branch fusion cells also expressed ElB (arrows).

elbow expression pattern

In situ hybridization with an *elB* RNA probe reveals expression of *elB* in all tracheal pit cells, starting at stage 11. There is a higher level in the first and the tenth pits (Fig. 4A). At stage 12, as the primary branches form, *elB* expression is reduced within the central part of the pit, and becomes restricted to the lateral anterior and posterior branches and to the dorsal branch cells (Fig. 4B,C). The *elB* probe is specific and does not crossreact with *noc* RNA, which exhibits a different distribution (see below).

Anti-ElB antibodies were capable of detecting ectopically expressed protein (Fig. 1D), but marginally detected the endogenous protein levels. However, a Gal4 element inserted 1260 bp upstream of the 5′ UTR provided a sensitive expression pattern marker that correlated with the RNA pattern. A Gal4-responsive *UAS-elB* element showed that at stage 14 *elB* expression is detected in the lateral branches and excluded from the dorsal trunk and visceral branch. By stage 16 expression in the transverse connective, spiracular branch, lateral and ganglionic branches was observed. Within the dorsal branch, ElB was specifically expressed at this stage in the fusion cell (Fig. 4D-F). Restriction of ElB expression is indeed necessary, as expression of ElB in all branches was sufficient to eliminate the visceral branch, and lead to defects in the dorsal trunk (Figs 1, 2).

elbow mutant phenotype

To determine the role of ElB in the branches where it is

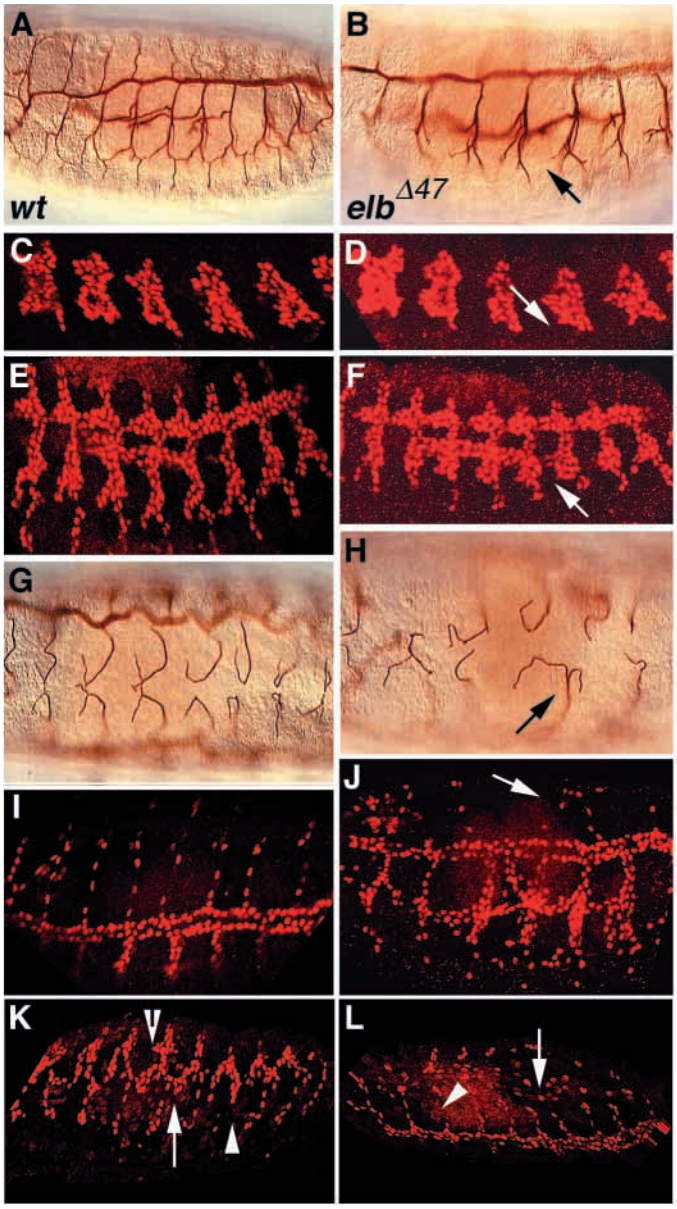

Fig. 5. *elB* mutant tracheal phenotypes. (A) Lumen staining of wild-type embryo at stage 14. (B) In $elB^{\Delta 47}$ mutant embryos, the lateral trunk anterior branch is not detected (arrow), and fusion between lateral trunks in adjacent metameres is not observed. (C) Anti-Trh staining of a wild-type embryo at stage 12 shows the initial stages of branching. (D) In *elB* mutants at the same stage, the lateral trunk anterior branch is stalled. (E) Wild-type embryo at stage 14. (F) In *elB* mutants, the lack of lateral trunk anterior migration is evident by stage 14. (G,I) Lumen and anti-Trh staining of the dorsal branches of a wild-type embryo. (H,J) In *elB* mutants, fusion between adjacent dorsal branches is observed (arrows). (K,L) Rescue of the tracheal phenotype of $elB^{\Delta 47}$ mutant embryos by *btl-Gal4/UAS-elB* was examined. Embryos misexpressing ElB were identified by anti-ElB staining, and stalled visceral branch migration (upper arrowhead in K). Over 120 such embryos were examined. $elB^{\Delta 47}$ embryos were scored by subtle defects in dorsal and ganglionic branch migration (arrowheads). Complete rescue of LTa migration (arrow in K) and partial rescue of dorsal branch migration (arrow in L) were observed.

normally expressed, analysis of mutations in the *elB* locus was carried out. Several mutant fly lines in the *elB* region are available; however, they are not precisely mapped. In order to create a line with a specific *elB* mutation, we excised the EP element, using a Delta 2-3 transposase. Only one lethal excision line (termed elB^{d47}) showed rearrangements in Southern blot analysis in the region of EP2039. Inverse PCR of the rearranged genomic fragment and sequencing provided the following molecular details. The EP2039 element was deleted. All sequences between EP2039 insertion site and *elB* were intact, while on the other side sequences of the transposable *yoyo* element were identified. Further Southern analysis indicated a deletion of the genomic sequence in the region upstream to EP2039 insertion. Therefore, it is possible that an inversion coupled to a deletion of sequences upstream to EP2039 has taken place. elB^{d47} was lethal over *Df(2L)noc10* and over the smaller deficiency *Df(2L)fn2*, uncovering 35A3-35B2. While elB^{d47} did not remove genomic regions containing the *elB* transcribed region, it was nevertheless allelic to the EMS-induced viable mutation elB^{1}, which displayed the typical smaller wing phenotype. It is thus possible that crucial transcriptional regulatory sequences of *elB* were removed in elB^{d47}.

Homozygous elB^{d47} embryos displayed defects in tracheal development. Below, we describe these defects and demonstrate that they can be specifically rescued by expression of *elB*. In all segments, there was no migration of the lateral trunk anterior, and the ganglionic branches were shorter. The cells, which failed to migrate into the lateral trunk remained in the transverse connective. The number of cells that form the dorsal trunk and visceral branch did not change (Fig. 5). Dorsal branch migration was also impaired, and failure of dorsal branch fusion was observed at stage 16. Cell counts of dorsal branch cells in the mutant embryos showed a considerable number of branches with only three to four cells (Fig. 1E). In some of the segments, the dorsal branch cells migrated aberrantly, and laterally adjacent branches fused (Fig. 5H,J). The same tracheal phenotype was observed in embryos homozygous for the *Df(2L)fn2* deficiency, as well as trans-heterozygotes of elB^{d47} over *Df(2L)fn2* (not shown), demonstrating that elB^{d47} is a null mutation.

As elB^{d47} may not affect only the *elB* gene, we needed to confirm that the observed tracheal phenotypes indeed result from loss of *elB*. *Btl-Gal4* was used to drive *UAS-elB* inserted on the third chromosome, in the background of homozygous elB^{d47} embryos. The most pronounced tracheal phenotype of elB^{d47} embryos was manifested in stalled anterior lateral trunk migration (Fig. 5J). As tracheal misexpression of *elB* did not give rise to defects in the LTa of wild-type embryos (Fig. 1F), it was possible to examine rescue this phenotype. All embryos misexpressing ElB were identified by anti-ElB staining and the characteristic visceral branch defects. While 25% of these embryos are homozygous for elB^{d47}, no severe LTa migration defects were observed. It was possible to identify specifically some of the rescued homozygous elB^{d47} embryos, by virtue of subtle defects in other branches such as the dorsal and ganglionic branch (Fig. 5K,L).

elbow mutants display normal Mad phosphorylation

The tracheal phenotype of *elB* mutants is reminiscent of defects

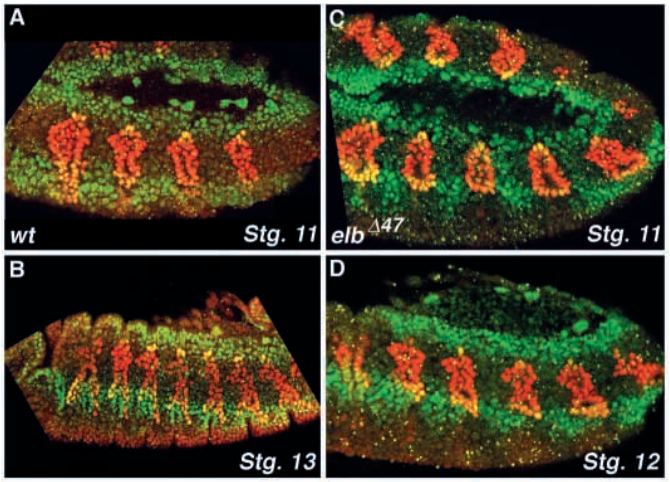

Fig. 6. Tracheal pMad patterns in wild-type and *elB* mutant embryos. (A) In wild-type embryos pMad (green) is induced at stage 11 by dorsal and lateral stripes of Dpp expression. pMad is observed in approx. five dorsal tracheal cells and 10-15 ventrolateral tracheal cells. (B) At stage 13 pMad is still detected in the same cells. (C,D) In *elB* mutants at stages 11 and 12, normal pMad is detected in the trachea. All tracheal nuclei are marked by anti-Trh (red).

arising when signaling by the Dpp pathway is blocked (e.g. in *thickveins* mutant embryos) (Affolter et al., 1994; Vincent et al., 1997; Wappner et al., 1997). Most notable is the stalled migration of the lateral trunk anterior, the ganglionic branch and the dorsal branch. Dpp is expressed at stage 11 in two ectodermal stripes: the dorsal stripe is restricted to a single row of the dorsal-most ectodermal cells, and is positioned several cells away from the dorsal edge of the tracheal pit. The ventrolateral stripe of Dpp expression abuts the ventral side of the tracheal pit (Vincent et al., 1997).

Activation of the Tkv/Punt receptors by Dpp leads to the phosphorylation of the Mad protein, which forms a heterodimer with Medea and translocates to the nucleus to trigger transcription (Raftery and Sutherland, 1999). Antibodies specifically recognizing the C-terminal phosphorylated form of Smad1 were shown to also recognize the phosphorylated form of *Drosophila* Mad (Persson et al., 1998; Tanimoto et al., 2000; Dorfman and Shilo, 2001). We used these antibodies to follow Dpp signaling in the trachea in wild-type and *elB* mutant embryos.

In wild-type embryos, pMad is observed in the tracheal pits beginning at early stage 11, when Dpp is expressed in two stripes in the dorsal and ventrolateral ectoderm (Fig. 6A). Dpp activation subdivides the tracheal pit into three parts: the activated dorsal and ventral domains and the central region, which is not activated by Dpp. The number of cells in which pMad is generated in the ventral domain of the pit is significantly larger than the number of tracheal cells displaying pMad in the dorsal domain. As the dorsal stripe of Dpp is positioned several cell diameters above the tracheal pit, the diffusion of Dpp reaches and activates only approx. five tracheal cells. This corresponds to the number of cells that will be recruited to the dorsal branch.

The ventral Dpp stripe abuts the tracheal pit, and pMad is observed in more cells in the ventral aspect of the pit, in accordance with the larger number of lateral trunk cells influenced by Dpp signaling. It is interesting to note that despite the higher levels of Tkv expression in the tracheal pits (Affolter et al., 1994), the amount of pMad in the tracheal cells versus the adjacent ectodermal cells is comparable. As migration of the tracheal branches continues, the lateral trunk cells migrate ventrally beyond the stripe of Dpp. Consequently, only the tracheal cells lying under this stripe retain pMad activation (Fig. 6B).

The pMad patterns allowed us to determine directly whether the reception of Dpp signaling is compromised in *elB* mutants. *elB*^{d47} embryos were stained with the pMad antibody, and identified by the stalled migration of the lateral trunk anterior. The number of dorsal and ventral cells displaying pMad in the pit at stage 11/12 is comparable with wild-type embryos (Fig. 6C,D). ElB is thus not required for normal signaling by the Dpp pathway.

ElB functions in parallel to Dpp in the trachea

ElB could be required downstream of pMad, for the induction of Dpp-target genes. Expression of *kni*, which encodes a zinc-finger transcription factor, is induced by the activated Tkv/Punt receptors (Vincent et al., 1997; Chen et al., 1998). We used a *kni-lacZ* transgene to follow the dependence of *kni* expression on ElB. Overexpression of ElB did not alter the expression pattern of *kni* (Fig. 2D). Likewise, in *elB* mutants, the expected number of dorsal branch cells retained *kni* expression (Fig. 7D). However, while normally the *kni*-expressing cells segregate completely from the dorsal trunk into the dorsal branch, in the *elB* mutant, several cells were retained within the dorsal trunk. This may be a reflection of failure of the cells destined to form the dorsal branch to lose their identity completely as dorsal trunk cells. Indeed, when expression of the dorsal trunk marker Sal was examined, we observed residual Sal in all the *kni*-expressing cells that remained within the dorsal trunk, and in some of the cells forming the dorsal branch (Fig. 7C,D). Thus, Kni is not sufficient to repress Sal expression, and requires cooperation with ElB for complete repression.

The normal reception of Dpp signaling and *kni* expression in *elB* mutants still left open the possibility that ElB is a downstream component of the Dpp pathway. We also tested whether expression of *elB* is regulated by the Dpp pathway. Overexpression of activated Tkv with the *btl-Gal4* driver did not alter the expression pattern of *elB* RNA (Fig. 7E). In addition, ectopic *kni* or *knrl* did not alter the *elB* expression pattern in the trachea, in agreement with the above results with activated Tkv (not shown). These results imply that while ElB and the Dpp pathway affect the same tracheal branches, they function in a parallel manner.

The combined effects of ElB misexpression and uniform activation of Tkv in the trachea could be manifested in cell fate changes leading to excess LTa cells. Following misexpression of only ElB or activated Tkv, the number of LTa cells was unchanged, while the typical visceral branch and dorsal branch abnormalities were observed (Fig. 7G,H, respectively). However, misexpression of both constructs also lead to changes in LTa cell fates (Fig. 7G), as extra cells were recruited into this branch.

noc mutants display similar tracheal phenotypes

The similarity between ElB and Noc proteins prompted us to

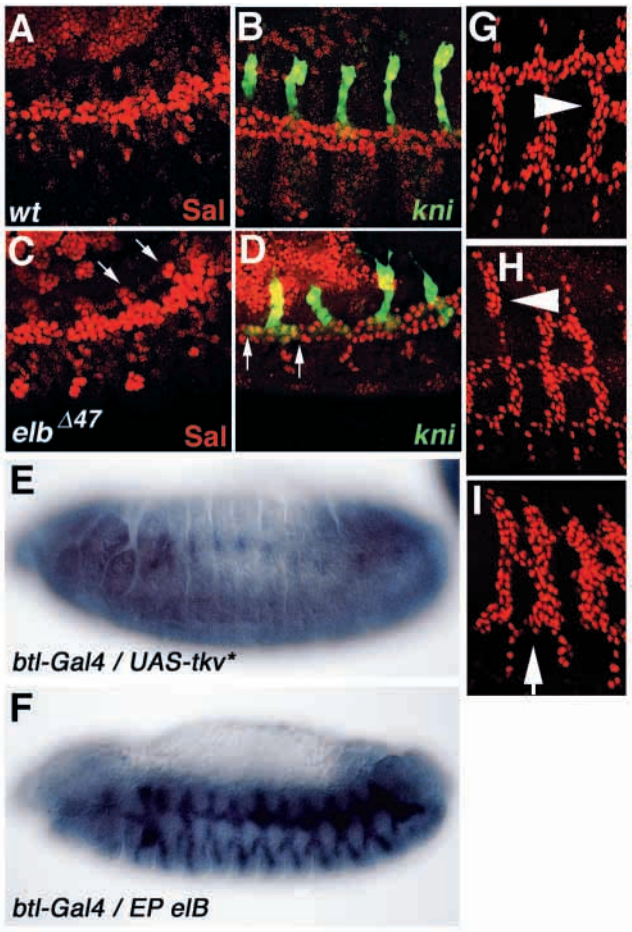

Fig. 7. Parallel activities of ElB and Dpp pathway. (A,B) In wild-type embryos, at stage 14 Sal expression (red) is restricted to the dorsal trunk, and *kni-lacZ* (green), a Dpp-target gene, is expressed in several tracheal branches including the dorsal branches shown here. (C,D) In *elB* mutant embryos, expression of *kni* was retained. Note, however, that the number of *kni*-expressing cells in the dorsal branches was reduced. The remaining *kni* cells were located within the dorsal trunk (arrows in D). In addition, in contrast to wild-type embryos, some of the dorsal branch cells continued to express Sal (arrows in C). Thus, while ElB is not necessary for *kni* expression, it is required in conjunction with Kni for repression of Sal expression in the dorsal branches, and for the capacity of all putative dorsal branch cells to detach from the dorsal trunk. (E) Additional experiments demonstrated that ElB and Dpp function in parallel. Expression of *elB* RNA is not elevated when activated Tkv is induced in all tracheal cells by *btl-Gal4*. (F) The same driver can induce uniform tracheal expression of *elB* when crossed to the EP2039 element upstream to *elB*. (G) In *btl-Gal4/EP2039* embryos, defects in visceral branch were observed (arrowhead) while the LTa appeared normal. (H) In *btl-Gal4/UAS-tkv** embryos, an excess of dorsal branch cells was detected (arrowhead) while again the LTa was normal. (I) In embryos misexpressing both constructs in the trachea, an excess of LTa cells was observed (arrow).

examine the role of Noc in tracheal development. In situ hybridization shows that *noc* is expressed from stage 4 in the embryonic termini, and at stage 5/6 in ectodermal stripes. At stage 11, *noc* is expressed in the invaginating tracheal pits, while from stage 13, *noc* is expressed ubiquitously (Fig. 8A-D) (Cheah et al., 1994). As *noc* expression is not spatially restricted, it is not surprising that no tracheal phenotypes were observed after Noc misexpression by *btl-Gal4/EP2000* (Fig. 8E). Higher levels of induction of *noc* expression (using *UAS-noc*), did not abolish the visceral branches, but their direction of migration was occasionally misrouted (not shown). In addition, misexpression of both ElB and Noc in the trachea did

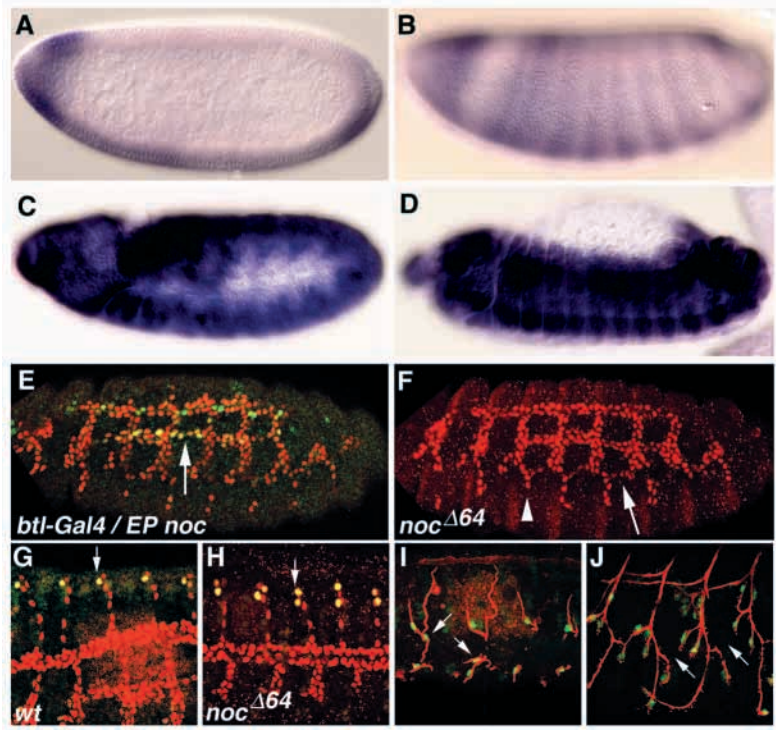

Fig. 8. Expression and mutant phenotype of *noc*. (A) *noc* RNA is first seen at stage 5 in the anterior region of the embryo. (B) At stage 6 a striped pattern is observed. (C) At stage 11, prominent expression is seen in the tracheal pits. (D) Expression is broad and uniform from stage 13. (E) Uniform tracheal expression of a *noc* EP2000 element does not give rise to tracheal phenotypes or the elimination of expression of the visceral enhancer trap marker *l(2)01351* (arrow, green). (F) *noc* mutant embryos show tracheal defects that are similar to but weaker than the defects seen in *elB* mutants. Note the reduced lateral trunk anterior (arrow) and the shortened ganglionic branch (arrowhead). Tracheal nuclei are stained with anti-Trh (red). (G) In wild-type embryos, only the terminal cell of each dorsal branch expresses SRF (green, arrow). (H,I) In *noc* mutant embryos, the fusion cell also expressed SRF (arrows). This may account for the lack of dorsal trunk fusion in *noc* and *elB* mutants. It correlates with the specific late expression of ElB in the fusion cells, and implies a role for ElB/Noc in repressing expression of terminal cell markers in the fusion cell. (J) Fusion of lateral branches was also disrupted in *noc* mutants (arrows).

not generate a phenotype that is more severe than the one produced by misexpression of ElB alone.

In order to examine the normal role of Noc in tracheal development, imprecise excisions were generated for the EP(2)2173 element, located 518 bp upstream of the 5′UTR. A lethal excision termed *noc^{d64}* was characterized by Southern blotting and inverse PCR. It represents a deletion of 848 bp, removing all residues between the insertion site of EP2173 and *noc* transcription start site, as well as 330 bp of the 5′UTR, thus defining a null mutation in *noc*. Homozygous *noc^{d64}* mutants show a weaker mutant phenotype, but similar to that of *elB* in terms of the affected tracheal branches (Fig. 8F). Fewer cells are observed in the lateral anterior trunk and ganglionic branch, and lateral branch fusion is occasionally missing (Fig. 8J). In addition, while all dorsal branches are formed, some branches have only up to three cells. The basis for partial lack of branch fusion in *noc* mutants was examined. Normally, in the dorsal branch only one terminal cell expresses SRF (Fig. 8G), while the preceding cell becomes the fusion cell and expresses specific markers. In *noc* mutants, repression of SRF expression in the fusion cell failed, giving rise to duplicated terminal cells at the expense of fusion cells (Fig. 8H,I).

Association of ElB and Noc

In view of the overlapping tracheal expression patterns and the previously reported genetic interactions (Davis et al., 1997), it is possible that the ElB and Noc proteins form a heterodimeric complex. As each protein contains only a single zinc finger, a heterodimer may provide the appropriate number of zinc fingers for DNA binding. GST pull-down experiments were carried out using GST fusions to full-length constructs of the ElB protein, as well as to fragments of the ElB protein. Association with in vitro translated full-length ElB or Noc was examined. Indeed, GST-ElB was capable of associating with Noc. In addition, GST-ElB was also capable of associating with ElB, demonstrating that ElB can form homodimers as well as heterodimers with Noc (Fig. 9). Truncated constructs of ElB show that its C-terminal region, which contains the Cysteine-rich and zinc-finger domains, is sufficient for these interactions. The specificity of these interactions was demonstrated by the inability of GST alone, or of GST fused to an irrelevant 60 kDa human protein to associate with labeled ElB.

ElB associates with Groucho

The biological activities of ElB and Noc are consistent with repression of transcription. Overexpression of ElB in the trachea repressed the expression of specific genes in the dorsal trunk and visceral branch (Fig. 2). Conversely, absence of ElB resulted in expanded expression of Sal to the dorsal branch, and *noc* mutants displayed failure to repress SRF expression in the fusion cells of the dorsal branch (Figs 7, 8). We tested the notion that the complex may posses repressive activity by virtue of its association with known inhibitors of transcription. Two such proteins are known, CtBP and Groucho.

The Groucho protein is known to mediate long-range transcriptional repression, and to associate with DNA-binding proteins bearing a number of motifs, including FKPY. This sequence is conserved in ElB, Noc and the two human homologs. We thus tested the capacity of ElB to associate with Groucho. Indeed, specific association was detected (Fig. 9).

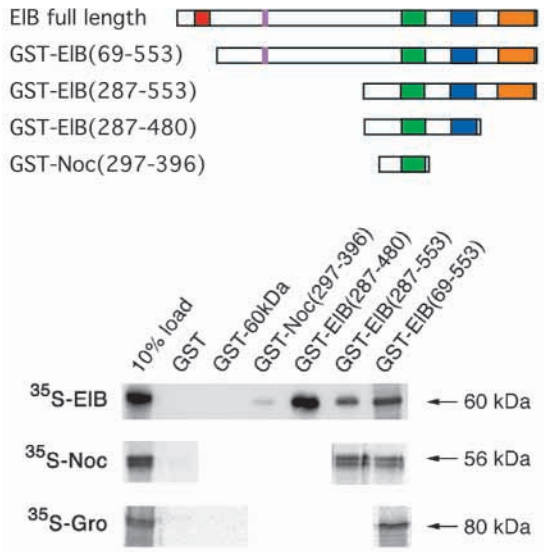

Fig. 9. Interactions between ElB, Noc and Groucho proteins. GST pull-down experiments were carried out, using GST fusions of ElB constructs, Noc, or an unrelated human 60 kDa protein. The scheme shows the fragments of ElB and Noc used for GST fusion. Domains are marked by the same color code as Fig. 3A. In vitro translated, labeled ElB, Noc or Groucho proteins were incubated with the GST constructs. ElB can associate with itself, with Noc and with Groucho.

CtBP promotes short-range repression and is known to associate with DNA-binding proteins containing the PxDLSxR/K/H motif (Zhang et al., 2001). Such a motif is not found in ElB or Noc, and the GST-ElB fusion protein shows only negligible precipitation of labeled CtBP (not shown). This result strongly suggests that the ElB/Noc complex represses transcription of target genes directly, by recruiting Groucho to these sites.

DISCUSSION

The ElB/Noc complex

In a misexpression screen for genes that disrupt tracheal morphogenesis we identified *elB*, a gene encoding a nuclear protein. ElB is a member of a new family of proteins containing a single zinc finger and additional conserved motifs. ElB is a nuclear protein, but it is not yet known whether it binds DNA, or if it functions as a monomer or multimer. The similarity between *elB* and *noc* mutant phenotypes, the genetic interactions between them (Davis et al., 1997), and the ability of ElB and Noc proteins to associate with each other (Figs 5, 8, 9), suggest that ElB/Noc heterodimers are the functional complex.

ElB overexpression was shown to repress expression of genes such as the visceral branch marker and *sal* (Fig. 2). Conversely, absence of ElB resulted in expanded expression of Sal to the dorsal branch, and *noc* mutants displayed failure to repress SRF expression in the fusion cells of the dorsal branch (Figs 7, 8), suggesting that ElB functions as a repressor of gene expression. One piece of evidence strongly indicates that the ElB/Noc complex indeed functions directly to repress the expression of target genes. Both *Drosophila* proteins, as well

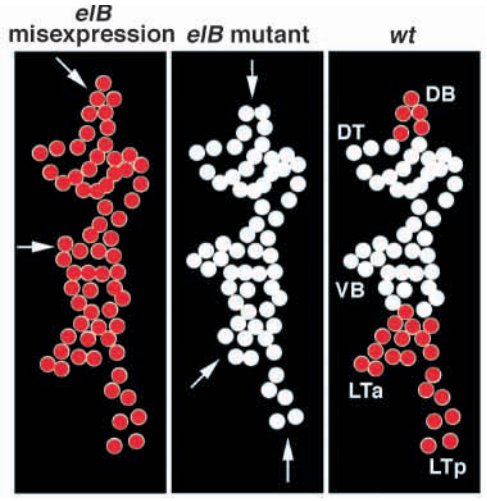

Fig. 10. Model for the activity of ElB in the trachea. Noc is an essential partner in the dimer. It is broadly expressed and thus does not give rise to a phenotype upon overexpression. Conversely, *elB* expression in the trachea is restricted to the dorsal branch and lateral trunks from stage 12 (red). This restricted expression is crucial for the correct determination of branch-specific cell fates. Overexpression of ElB represses the expression of genes in the visceral branch (VB) and dorsal trunk (arrows), while *elB* mutant embryos exhibit defects in the migration of the lateral trunks and dorsal branch (arrows). We suggest that normally ElB/Noc repress transcription of visceral-branch genes in the lateral trunks, and dorsal-trunk genes in the dorsal branch, thus contributing to the definition of branch-specific cell fates. Subsequently, ElB expression is refined to the fusion cells of the dorsal branch, where ElB/Noc are required for repression of terminal cell markers.

as the human homologs, contain the FKPY motif (Fig. 3), which was shown to be sufficient for interactions with Groucho (Zhang et al., 2001; Hasson et al., 2001). Indeed, GST pull-down experiments demonstrated that ElB can associate with Groucho (Fig. 9). It is interesting to note that another Sp1 homolog, Huckebein (Hkb), was previously shown to recruit Groucho through the FRPW motif (Goldstein et al., 1999). The ElB/Noc complex may thus serve to recruit the Groucho protein to specific target sites on the DNA, and repress the expression of distinct genes. Future identification of target genes will determine if ElB/Noc can also facilitate induction of certain genes.

Roles of ElB/Noc in the trachea

Expression of ElB is confined to distinct tracheal branches from stage 12, namely the dorsal branch and lateral trunks (Fig. 4). This restricted expression is instructive for the future fate and migration pattern of these branches. When misexpressed in other branches, ElB abolishes the migration of the visceral branch and eliminates the expression of a visceral branch marker. It also represses expression of Sal, a protein that defines the dorsal trunk identity (Figs 1, 2). ElB was also able to divert several cells from the dorsal trunk fate into a dorsal branch.

In the tracheal branches where *elB* is normally expressed, it has an essential role. In *elB* null mutants, fewer cells join the dorsal branch, and these branches migrate abnormally. In addition, the cells normally forming the lateral trunk anterior remain in the transverse connective, and the ganglionic branches are stalled. We can try to interpret these phenotypes with the repressive activity of the ElB/Noc complex in mind.

It is possible that in the dorsal branch, the complex represses expression of genes that confer dorsal trunk identity such as *sal*. A similar paradigm was previously shown for the Dpp pathway. *kni* expression in the dorsal branch is induced by the Dpp pathway. Kni can bind the *sal* promoter and repress expression of the gene (Chen et al., 1998). In *elB* mutants expression of dorsal trunk genes extends to the dorsal branch and partially converts the identity of these cells to a dorsal trunk fate. Similarly, in the lateral trunk the ElB/Noc complex may be required to repress the expression of genes conferring visceral branch identity. In *elB* mutants, the expanded expression of visceral branch- and dorsal trunk-specific genes into the lateral trunk may thus abolish or stall the migration of the lateral trunk and ganglionic branches. Subsequently, ElB expression is confined to a specific cell within the dorsal branch, namely the fusion cell. In *noc* mutants the expression of SRF in the dorsal branch expanded to the fusion cell (Fig. 8H). One way to interpret the expanded expression of SRF is by loss of direct repressive activity of ElB/Noc in the fusion cell. A model for the role of ElB in determination of branch fates is shown in Fig. 10.

Why is the *elB* mutant tracheal phenotype more severe than that of *noc*, if the two proteins form a functional complex? As *noc* does not have an early maternal RNA, the zygotic *noc* mutant phenotype should reflect the null situation. We have shown that ElB can associate not only with Noc, but also with another ElB protein. It is thus possible that in *noc* mutant embryos, ElB/ElB homodimers partially substitute for the ElB/Noc heterodimers.

Parallel activity of ElB/Noc and the Dpp pathway

The tracheal defects observed in *elB* mutants are reminiscent of tracheal defects in *tkv* mutant embryos, where signaling of the Dpp pathway is blocked (Affolter et al., 1994). However, ElB appears to function in parallel to the Dpp pathway. Phosphorylation of Mad and induction of *kni* expression, which mark the activity of the Dpp pathway, are normal in *elB* mutants. Conversely, ElB expression is independent of Dpp/Tkv activation (Figs 6, 7). The possibility that Dpp signaling directs a post-translational modification of ElB/Noc has not been ruled out. Nevertheless, the available data suggests that generation of the dorsal branch, and migration of the lateral trunk anterior and ganglionic branch, require both the input from Dpp signaling and the expression of ElB/Noc.

It is not known yet how activation of distinct tracheal cells in the dorsal and ventral region of the tracheal pit by Dpp, as visualized by pMad accumulation, contributes to the capacity of these cells to form the dorsal and lateral branches, respectively. Activation by Dpp induces expression of target genes such as *kni* in these compartments. Kni in turn was shown to repress expression of dorsal-trunk genes like *sal*.

How are the ElB/Noc and Dpp signals integrated in the trachea? One possibility is that they impinge on different target genes. ElB/Noc repress expression of visceral branch or dorsal trunk genes, while the Dpp signal induces the expression of target genes in the same cells. The combined activity of the two pathways will determine the set of branch-specific genes expressed by these cells. The final identity of each branch is

likely to be a result of inputs from different pathways, which contribute to the expression of branch-specific genes and to the repression of other genes. This is exemplified most clearly when monitoring *kni* expression in the dorsal branch of *elB* mutant embryos. While the correct number of cells are induced by the Dpp pathway and express *kni*, some of these cells are stalled in the dorsal trunk in the absence of ElB (Fig. 7D). It is also demonstrated by the fact that only the combined activity of ElB and activated Tkv was capable of inducing an excess of LTa cells (Fig. 7I). ElB/Noc and Kni also cooperate in the repression of common target genes. Complete repression of *sal* in the dorsal branch cells requires both complexes, as evidenced by the expanded expression of Sal in *elB*-mutant embryos (Fig. 7C).

Future knowledge regarding the nature of these branch-specific target genes should provide insights into the mechanism that regulate branch-specific fates. These genes may encode adhesion molecules or membrane receptors that allow responses to different sets of external guiding cues. This system could provide further migrational specificity, superimposed on the common Branchless signal guiding the migration of all tracheal branches. Furthermore, it may determine the stereotyped number of cells recruited into each tracheal branch.

Regulation of ElB/Noc

The similarity in the phenotypes of *elB* and *noc* mutants, the genetic interaction between the mutants, and the complex formed between the two proteins, strongly suggest that these proteins carry out their biological roles as a complex. However, the two genes are regulated differently in the embryo. *noc* is broadly expressed and overexpression of the protein does not give rise to a tracheal phenotype, suggesting that spatial and temporal regulation of activity relies on *elB* expression. The restricted expression of *elB* is essential, as *elB* misexpression gives rise to deleterious phenotypes in the trachea. We do not know if additional tiers of regulation, such as inputs from signaling pathways or phosphorylation, also impinge on the complex.

Expression of *elB* is initially observed in all tracheal cells, suggesting that it is under the control of Trachealess and Drifter, which confer tracheal identity. However, at stage 12 expression of *elB* becomes excluded from the central part of the pit. It is possible that the restricted pattern of *elB* expression is thus a combination of induction by general tracheal transcription factors, and repression of expression in the future dorsal trunk and visceral branch. The signals leading to this repression are not known. The EGF receptor pathway is activated in the central domain of the tracheal placodes (Wappner et al., 1997). However, when the activity of this pathway was abolished in *Star* mutants, *elB* expression remained unchanged (not shown).

It will be interesting to find out the function the ElB/Noc complex in other tissues. In *noc* mutant embryos, defects in migration of cells from the procephalic lobe were observed (Cheah et al., 1994). Expression of *elB* is not restricted to the trachea, and is also observed in the wing imaginal disc and the adult photoreceptors (S. Cohen and C. Desplan, personal communication). In accordance with the roles of the ElB/Noc complex in the trachea, it is likely that in the above tissues the same complex will be essential for determination of cell fates, by repressing and possibly also inducing critical target genes.

In conclusion, using a tracheal misexpression screen we have identified two proteins that form a complex, and participate in the determination of specific tracheal branch fates. ElB/Noc define a parallel input to Dpp signaling, demonstrating that convergence of several signals contributes to the robust determination of branch-specific cell fates, and to the refinement of these fates.

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