The *Drosophila* Extradenticle and Homothorax selector proteins control *branchless/FGF* expression in mesodermal bridge-cells

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The stereotyped outgrowth of tubular branches of the *Drosophila* tracheal system is orchestrated by the local and highly dynamic expression profile of *branchless (bnl)*, which encodes a secreted fibroblast growth factor (FGF)-like molecule. Despite the importance of the spatial and temporal *bnl* regulation, little is known about the upstream mechanisms that establish its complex expression pattern. Here, we show that the Extradenticle and Homothorax selector proteins control *bnl* transcription in a single cell per segment, the mesodermal bridge-cell. In addition, we observed that a key determinant of bridge-cell specification, the transcription factor Hunchback, is also required for *bnl* expression. Therefore, we propose that one of the functions of the bridge-cell is to synthesize and secrete the chemoattractant Bnl. These findings provide a hitherto unknown and interesting link between combinatorial inputs of transcription factors, cell-specific ligand expression and organ morphogenesis.

Keywords: cell migration; FGF; Hox proteins; Hunchback; organogenesis

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INTRODUCTION

Many epithelial organs consist of highly branched tubular networks that transport gases or fluids. Both in the *Drosophila* tracheal system and in the mammalian lung, members of the fibroblast growth factor (FGF) family of secreted signalling molecules have a key role in the branching process, and expression of the corresponding genes is, at the same time, highly complex, yet rather stereotyped (Metzger & Krasnow, 1999; Affolter *et al*, 2003). In the tracheal system, the Branchless (Bnl) ligand (Sutherland *et al*, 1996) binds to and activates the receptor tyrosine kinase Breathless (Btl)/FGFR (FGF receptor) in nearby tracheal cells (Klamt *et al*, 1992), which in turn triggers dynamic changes in the cytoskeleton (Ribeiro *et al*, 2002) and results in

migratory behaviour of responding cells towards the ligand source. *bnl* expression is arrayed around the tracheal placode in a segmentally repeated fashion, and each expression domain is located to a specific anteroposterior (AP) and dorsoventral (DV) position within the segment (Sutherland *et al*, 1996). This suggests that the global AP and DV patterning systems of the *Drosophila* embryo specify these positions. Indeed, the *bnl* spot necessary for the outgrowth of the dorsal tracheal branch has been shown to be regulated by the BMP-like signalling molecule Decapentaplegic (Dpp), which is expressed in the dorsal-most epidermal cells (Vincent *et al*, 1997). However, no transcriptional regulators of *bnl* expression along the AP axis have been described so far.

We show that the Extradenticle (Exd), Homothorax (Hth) and Hunchback (Hb) transcription factors are required at stage 12 for the lateral expression of *bnl* in one specific cell per segment, and we identify the corresponding cell as the mesodermal bridge-cell. Bridge-cells have been proposed to serve as Bnl-independent guidance cues for elongation of the main structure of the tracheal tree, the dorsal trunk (DT), but their molecular mechanism of action has since remained elusive (Wolf & Schuh, 2000; Wolf *et al*, 2002). Here, we find that an important function of the bridge-cell is to synthesize and secrete the chemo-attractant Bnl. We also observed that Hox genes of the bithorax complex do not contribute to *bnl* activation in trunk segments of the developing embryo, which suggests a Hox-independent function of Exd and Hth in bridge-cells.

RESULTS AND DISCUSSION

The *exd* and *hth* tracheal defects are similar

We have discovered that in embryos homozygous for a null allele of the *homothorax (hth)* gene, the tracheal branches elongating along the AP axis failed to form correctly, as observed for the dorsal and lateral trunks, or the visceral branches (Fig 1C,D). *hth* encodes a homeodomain-containing transcription factor of the TALE (three-amino-acid loop extension) family that is necessary for the nuclear translocation of another TALE homeoprotein encoded by the *extradenticle (exd)* locus (Peifer & Wieschaus, 1990; Rauskolb *et al*, 1993; Rieckhof *et al*, 1997). In addition, Hth cooperates with Exd to directly control target-gene regulation (Ryoo *et al*, 1999). To test whether *exd* was also required for tracheal development, we generated embryos lacking both

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Fig 1 | *hth* and *exd* mutant embryos show similar tracheal defects. (A,C,E,G,H) Whole-mount staining of stage-14 embryos with the 2A12 antibody. (B,D,F) Whole-mount antibody staining with anti-GFP (green fluorescent protein) of stage-14 embryos bearing the recombinant *btl-Gal4* and *UAS-actin-GFP* constructs on the second chromosome. (A,B) Wild-type (WT) embryos. (C,D) *hth*^{P2} mutant embryos. (E,F) Maternal and zygotic *exd*^{Y012} mutant embryos. (G,H) Embryos triple-stained (G) or double-stained (H) with anti-Exd (Extradenticle) and anti-Hth (Homothorax) antibodies to recognize the genetic backgrounds (not shown). (G) *hth*^{P2} mutant embryos bearing *arm-Gal4* and *UAS-nls-exd* transgenes. The constitutively nuclear-localized form of Exd cannot rescue *hth*^{P2} dorsal trunk (DT) defects. (H) *hth*^{P2} mutant embryos bearing *arm-Gal4* and *UAS-hth* transgenes show a rescue (rarely complete; see Fig 5O) of DT defects.

maternal and zygotic *exd* contributions. The tracheal phenotype of *exd*-null embryos was similar to that of *hth* mutants, with selective defects in all tracheal branches elongating along the AP axis (Fig 1E,F). To assess whether the *hth* tracheal phenotype could indirectly result from impaired nuclear localization of Exd, we ubiquitously expressed a nuclear form of Exd in *hth* mutant embryos. In such conditions, we did not observe a rescue of the *hth* tracheal phenotype (Fig 1G), which is consistent with the proposed role of Exd and Hth in transcription regulation as a heterodimeric complex. The Exd/Hth complex could be required in tracheal cells or, alternatively, in cells surrounding the developing trachea. To distinguish between these two possibilities, we expressed the Hth protein in *hth* mutant backgrounds, either ubiquitously in the embryo (Fig 1H) or specifically in the tracheal system with the *Btl-Gal4* driver (not shown). Significant rescue of

hth DT defects was only observed when Hth was expressed in the entire embryo (Fig 5O), indicating that the two selector genes have an essential role in non-tracheal cells.

Exd and Hth control *bnl* expression in bridge-cells

During the past few years, a large number of genes with essential roles in diverse aspects of tracheal development have been identified. Several mutations have been reported to affect, rather selectively, the development of the DT, and we therefore tested whether we could link the *hth* and *exd* phenotypes to any of these previously reported genes. We first tested the expression profile of *knirps* (*kni*), which leads to a lack of DT formation when ectopically expressed in DT cells (Chen *et al*, 1998), and *spalt* (*sal*), which contributes to the unique morphological aspect of the DT (Kuhnlein & Schuh, 1996; Chihara & Hayashi, 2000; Ribeiro

Fig 2 | The transcription factors Extradenticle (Exd), Homothorax (Hth) and Hunchback (Hb) are specifically required for the dorsolateral *bnl* expression sites. All embryos are in stages 12–13 and carry the recombinant *btl-Gal4* and *UAS-actin-GFP* (green fluorescent protein) transgenes on the second chromosome. The tracheal system is visualized with anti-GFP by fluorescent (in red in A,A',B,B',C,C') or enzymatic (in brown in D–G) immunostaining. (A) The expression of Knirps (Kni, in green) is unchanged in dorsal and ventral parts of tracheal metameres (white arrows in the higher magnification, A') of *hth*^{P2} mutant embryos. (B) The expression of Spalt (Sal, in green) is unchanged in the dorsal trunk (DT; a white arrow in the higher magnification, B') of *hth*^{P2} mutant embryos. (C) The expression of Hb (in green) is unchanged in bridge-cells (a white arrow in the higher magnification, C') of *hth*^{P2} mutant embryos. (D–G) The expression of *bnl* is visualized in blue by *in situ* hybridization. The dorsolateral *bnl* expression sites necessary for DT elongation are shown (dotted circles). Compared with wild-type (WT) embryos (D), these *bnl* expression sites are lost in *hb*^{FB} (E), *exd*^{mat/zyg} (F) and *hth*^{P2} (G) mutant embryos. Note that in these mutant embryos, the dorsal *bnl* expression sites are also diminished but still present, whereas the ventral *bnl* expression sites are not affected.

et al, 2004). In *hth* mutant embryos, both Kni (Fig 2A,A') and Sal (Fig 2B,B') proteins were correctly expressed in the tracheal system, and we thus concluded that the *hth* DT phenotype did not result from *kni* or *sal* deregulation. A third gene that has been reported to be essential for DT development is *hunchback* (*hb*), which is expressed in the mesodermal bridge-cell (Wolf & Schuh, 2000). Although *hb*, *hth* and *exd* mutations lead to similar DT phenotypes, we did not observe a loss of Hb localization in bridge-cells of *hth* mutant embryos (Fig 2C,C'). Therefore, we tested whether lack of the DT in *hth* and *exd* mutants could result from a loss of *bnl* expression, as this ligand is required for the migration of all tracheal branches. At stage 11, *bnl* is expressed in six stereotyped ectodermal sites around the tracheal placode. In *hth* or *exd* mutant embryos, *bnl* transcription in these sites seemed normal (data not shown). However, at later stages, *in situ* hybridization experiments clearly showed the loss of *bnl* expression between the DT anterior and posterior outgrowths, in both *hth* (Fig 2E) and *exd* (Fig 2F) mutant embryos.

The lack of specific markers for cells expressing and secreting the Bnl/FGF ligand has thus far hindered the identification and further characterization of most of these cell clusters. However, as the bridge-cell and the dorsolateral Bnl spot, which is under the control of *hth* and *exd*, seemed to be in a similar position

(compare Fig 3A,A' and B,B'; see also Fig 1 in Wolf *et al*, 2002), we asked whether the bridge-cell was transcribing the *bnl* gene. Enzymatic revelation of *bnl* messenger RNA by *in situ* hybridization, and anti-Hb by immunostaining procedures, indicated that *bnl* was indeed transcribed by the cell expressing nuclear Hb protein (Fig 3C). This result was confirmed by high-resolution colocalization studies using confocal microscopy, which showed the nuclear protein Hb to be surrounded by the cytoplasmic *bnl* mRNA (Fig 3D,D'). Moreover, the observation of a specific loss of the dorsolateral *bnl* expression sites in *hb* mutants (Fig 2G), which results in apoptosis of bridge-cells (Wolf & Schuh, 2000), shows that *bnl* is indeed transcribed in these cells (in contrast to the findings of Wolf & Schuh, 2000). Loss of *bnl* expression in *hth* and *hb* mutant embryos was also inferred from the absence of filopodia formation in DT branches of living embryos (supplementary Fig S1B and C online, respectively).

In many instances, the Exd and Hth proteins act as cofactors for homeotic proteins (Mann & Morata, 2000). Therefore, we tested whether Hox proteins also participate in bridge-cell specification. As we observed that both *bnl* and Hb expressions, as well as DT elongation, were normal in *Hox* mutant embryos (supplementary Figs S1D and S2 online), we concluded that *exd* and *hth* carry out Hox-independent functions in the bridge-cell.

Fig 3 | Expression of *bnl* in mesodermal bridge-cells. (A,A',B,B') Whole-mount antibody staining with anti-GFP (green fluorescent protein; red) of stage 12–13 embryos bearing *btl-Gal4* and *UAS-actin-GFP* constructs. (A,A') Hunchback (Hb; green) is expressed in the nucleus of the bridge-cell (white arrowhead). (B,B') Fluorescent *in situ* hybridization against *bnl* RNA (green). The spot necessary for DT elongation is indicated (white arrowhead). (C) Enzymatic co-staining of Hb (immunostaining in brown) and *bnl* (*in situ* hybridization in blue) shows that *bnl* messenger RNA is produced by the bridge-cell (black arrowhead). (D) Fluorescent triple staining of *btl-Gal4/UAS-actin-GFP* embryos with anti-GFP (blue), anti-Hb (red) and *bnl* mRNA (green). (D') Highlight of the region boxed in (D). The nucleus of the bridge-cell (white arrowhead) is surrounded by the cytoplasmic *bnl* mRNA. WT, wild type.

Finally, we looked at the nuclear localization of Exd and Hth in the mesodermal bridge-cell. Although *hb* expression starts at stage 11 in a posterior lateral margin of each tracheal metamere (Wolf & Schuh, 2000), we did not observe nuclear colocalization of Exd in this first *hb*-positive epidermal cell (Fig 4A,A',A''). Nuclear localization of Exd was only observed later in the daughter bridge-cell (Fig 4B,B',B'',B'''), concomitantly with Hth (Fig 4C,C',C''). We also confirmed that nuclear colocalization of Exd and Hb occurred in mesodermal cells (Fig 4D,D',D''). The nuclear translocation of the two selector proteins at stage 12 coincides with the appearance of the bridge-cell and its guidance role for the outgrowth of DT branches. This suggests that local expression of *bnl* in the bridge-cell could result from the simultaneous presence of the three transcription factors Hb, Exd and Hth. However, the fact that the same transcription factors are also present in the ventral daughter cell of the bridge-cell precursor (Fig 4B,B',B'',B''',C,C',C''), which does not express *bnl*, indicates that more factors are required to determine the fate of the particular mesodermal bridge-cell.

Bridge-cells have *bnl*-dependent functions

To verify the importance of *bnl* expression in bridge-cells at the functional level, we analysed whether the DT defects in *hb* or *hth* mutant embryos could be rescued by expressing Bnl ubiquitously, or by expressing a constitutively active form of the Btl receptor in the tracheal system (Dossenbach *et al*, 2001). As a control, DT formation was also followed in wild-type or *bnl*^{P1} mutant embryos under the same conditions. To take into account the variability of the DT phenotypes in each mutant situation, we reported, for each genetic background, the proportion of embryos with the corresponding number of fused DT branches formed along the AP axis.

As shown earlier (Sutherland *et al*, 1996), activation of Bnl signalling in all tracheal cells leads to a significant rescue of the DT in *bnl* mutants (Fig 5D–F,M). In *hb* mutant backgrounds, most of the embryos (89%) have from zero to three fused DT metameres (Fig 5G), but this number can increase to four or five (for 10% and 1% of the population, respectively; Fig 5N). Activation of Bnl signalling increases the proportion of embryos with four or five fused DT metameres (around 70%; Fig 5H,I,N). As a maximum of six fused DT metameres were formed in such embryos, because of

Fig 4 | Dynamic expressions of Extradenticle (Exd) and Homothorax (Hth) in the nuclei of bridge-cells. All embryos bear the recombinant *btl-Gal4/UAS-nls-GFP* (green fluorescent protein; A,A',A'') or *btl-Gal4/UAS-actin-GFP* (B,B',C,C',C'') transgenes, and are stained with anti-GFP (blue) and anti-Hb (Hunchback; green). The red staining shows either the Exd (A,A',A'',B,B',B'',B''') or the Hth (C,C',C'',C'') proteins. White arrows indicate bridge-cells. At stage 11, the Exd protein (red) is not localized in the nucleus of the bridge-cell (A,A',A''). Nuclear translocation of Exd (red) is observed at stage 12 (B,B',B'',B'''), concomitantly with the nuclear expression of Hth (red) in bridge-cells (C,C',C''). (D,D',D'') Fluorescent immunostaining of embryos carrying the mesodermal *twist-24B-Gal4* driver and the *UAS-lacZ* reporter gene, with anti- β -galactosidase (β Gal; blue), anti-Exd (red) and anti-Hb (green). WT, wild type.

the lack of segmental anlagen (Tautz *et al*, 1987), we conclude that a significant rescue of the *hb* DT phenotype occurs in the presence of Bnl signalling. In *hth* mutant backgrounds, most of the embryos (90%) have from zero to three DT metamerites (Fig 5J,O). Activation of Bnl signalling leads to a significant, although not complete, DT rescue; a large proportion of *hth* mutant embryos (65%) showed four or five fused DT metamerites, and this number varied up to six or seven (for 10% of embryos), a frequency never observed in *hth* mutants without added Bnl signalling (Fig 5K,L,O).

The partial rescue of the *hb* and *hth* DT phenotypes can be explained by three hypotheses. First, these genes could also be required in cells other than the bridge-cell, and/or for later events during tracheal system morphogenesis, such as those implicating *Ubx* and *abdB*. Second, our rescue experiments did not rely on specific activation of Bnl signalling in bridge-cells. To bypass this limitation, we tested several EP insertions as potential Gal4 drivers (listed in the Methods), but none of them was exclusively expressed in bridge-cells (data not shown). However, as our

Fig 5 | Constitutive activation of the Branchless (Bnl) signalling pathway can partially rescue the *hb* and *hth* dorsal trunk (DT) phenotype. Bnl signalling was induced with the *arm-Gal4* and *UAS-bnl* transgenes (B,E,H,K), or with the recombinant *Btl-Gal4/UAS-actin-GFP* (green fluorescent protein) and *UAS-Btl^{tor}* transgenes (C,F,I,L). Embryos are stained with anti-2A12 (green) and anti-Blistered/DSRF (red; A,B,D,E,G,H,J,K), or with 2A12 (green) and anti-GFP (C,F,I,L). Embryos were triple stained with anti- β -galactosidase to identify homozygous mutants by the absence of the blue balancer. (A–C) Wild-type (WT) embryos. (D–F) Homozygous *bnl^{P1}* mutant embryos. (G–I) Homozygous *hb^{FB}* mutant embryos. (J–L) Homozygous *hth^{P2}* mutant embryos. (M–O) Graphical representation of the number of DT metameres observed (abscissa) per embryo (as a percentage; Y-axis), either in the mutant (red curves) or in the rescue (green and blue curves) context. In each situation, the total number (*n*) of embryos scored with the corresponding genotype is indicated. (M) Statistical representation of the *bnl^{P1}* DT phenotype compared with the two rescue situations. (N) Statistical representation of the *hb^{FB}* DT phenotype compared with the two rescue situations. (O) Statistical representation of the *hth^{P2}* DT phenotype compared with the two rescue situations. A supplementary curve (in black) illustrates the efficiency of *hth^{P2}* DT rescue obtained with the *arm-Gal4* and *UAS-hth* transgenes.

genetic approach allowed a significant rescue of the DT phenotype of *bnl* mutant embryos, we think that this hypothesis is unlikely to be correct. Third, the bridge-cell could provide other functions as a substrate for migration and/or as a regulator of the fusion process. If these *bnl*-independent processes are also controlled by *hb*, *hth* and *exd*, constitutive Bnl signalling alone would not be sufficient to completely rescue the DT trunk phenotype in the absence of these transcriptional regulators.

In conclusion, we identify the selector genes *exd* and *hth* as important factors controlling the *bnl*/FGF expression site necessary for DT elongation. We identify this *bnl* expression site as the

previously described mesodermal bridge-cell, which expresses a third transcription factor, Hb. These results link the cell-specific expression of three transcriptional factors to the three-dimensional architecture of an organ system, the trachea. Future studies will have to identify such factors involved in the determination of bridge-cell fate, and to determine whether the regulation of *bnl* by *Exd*, *Hth* and *Hb* is direct.

METHODS

Fly stocks. The following fly strains and alleles were used in this study: *hb^{FB}* (Wolf & Schuh, 2000), *bnl^{P1}* (Sutherland et al, 1996)

and *hth*^{P2} (Casares & Mann, 1998); the *exd*^{Y012} allele was used to generate female germline mosaics, as described previously (Rauskolb et al, 1993). To drive Gal4 either ubiquitously in the embryo or specifically in the tracheal system, we used *arm-Gal4* and *btl-Gal4* (recombined on second chromosome with *UAS-actin-GFP* (green fluorescent protein)) driver lines, respectively. *UAS-actin-GFP* (Verkhusha et al, 1999) was used to detect cytoskeletal dynamics in tracheal cells. We also used *UAS-bnl* (Sutherland et al, 1996), *UAS-btl^{lor}* (Dossenbach et al, 2001), *UAS-nls-exd* (Abu-Shaar et al, 1999) and *UAS-hth* (Casares & Mann, 1998). For the rescue experiments shown in Fig 5, the *bnl*^{P1}, *hb*^{FB} and *hth*^{P2} mutant chromosomes were balanced over a blue-balancer, and the transgenes were either homozygous (*UAS-bnl*, *UAS-Btl^{lor}*, *arm-Gal4*) or heterozygous (the recombined *Btl-Gal4/UAS-actin-GFP*) on the second chromosome. The fly strains tested as potential bridge-cell-specific Gal4 drivers were *l(3)PL00790*, *l(3)PL00759* and *EP(3)3557*.

Immunostainings and whole-mount *in situ* hybridization. The following primary antibodies were used: monoclonal antibody 2A12 (DSHB, Iowa City, IA, USA) diluted 1:5, monoclonal anti-Crumbs (kindly provided by E. Knust) diluted 1:20, guinea-pig anti-Hunchback (kindly provided by R. Schuh) diluted 1:400, rabbit anti-Spalt (Kuhnlein et al, 1994) diluted 1:50, mouse anti-Knirps (Chen et al, 1998) diluted 1:20, mouse anti-DSRF (Guillemin et al, 1996) diluted 1:20, rabbit anti-Exd (kindly provided by I. Duncan) diluted 1:500, and mouse or rabbit anti-GFP and anti-β-galactosidase (Promega or Cappel, respectively) diluted 1:500. Embryos were fixed and immunostained according to standard procedures, with minor modifications. Whole-mount *in situ* hybridizations on embryos were performed with digoxigenin-labelled antisense RNA probes of *bnl* (Sutherland et al, 1996), as described (Tautz & Pfeifle, 1989).

Affinity-purified secondary antibodies were coupled to alkaline phosphatase, biotin or peroxidase (Jackson Immuno Research Laboratories, West Grove, PA, USA), or were Alexa-488, Alexa-594 or Alexa-Cy5 conjugated (Molecular Probes Inc., Eugene, OR, USA). For some fluorescent staining, the signal was amplified with the aid of a Tyramide Signal Amplification kit (NEN Life Sciences, Boston, MA, USA). The fluorescent-stained embryos were then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) for further observation under a confocal microscope (Leica TCS SP2 or LSM 510 Zeiss). Images were processed with the Leica TSC NT 1.6, Zeiss LSM5 Image Browser, Imaris 5 and Adobe Photoshop 7.0 programs.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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