The bHLH Transcription Factor, Hairy, Refines the Terminal Cell Fate in the Drosophila Embryonic Trachea

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Abstract

Background: The pair-rule gene, hairy, encodes a basic helix-loop-helix transcription factor and is required for patterning of the early Drosophila embryo and for morphogenesis of the embryonic salivary gland. Although hairy was shown to be expressed in the tracheal primordia and in surrounding mesoderm, whether hairy plays a role in tracheal development is not known.

Principal Findings: Here, we report that hairy is required for refining the terminal cell fate in the embryonic trachea and that hairy's tracheal function is distinct from its earlier role in embryonic patterning. In hairy mutant embryos where the repressive activity of hairy is lost due to lack of its co-repressor binding site, extra terminal cells are specified in the dorsal branches. We show that hairy functions in the muscle to refine the terminal cell fate to a single cell at the tip of the dorsal branch by limiting the expression domain of branchless (bnl), encoding the FGF ligand, in surrounding muscle cells. Abnormal activation of the Bnl signaling pathway in hairy mutant tracheal cells is exemplified by increased number of dorsal branch cells expressing Bnl receptor, Breathless (Btl) and Pointed, a downstream target of the Bnl/Btl signaling pathway. We also show that hairy genetically interacts with bnl in TC fate restriction and that overexpression of bnl in a subset of the muscle surrounding tracheal cells phenocopied the hairy mutant phenotype.

Conclusions/Significance: Our studies demonstrate a novel role for Hairy in restriction of the terminal cell fate by limiting the domain of bnl expression in surrounding muscle cells such that only a single dorsal branch cell becomes specified as a terminal cell. These studies provide the first evidence for Hairy in regulation of the FGF signaling pathway during branching morphogenesis.

Introduction

Epithelial morphogenesis is a prevalent process necessary for the formation of many essential organs during embryogenesis. While some tubular organs, such as the vasculature, lung and kidney are branched structures, others, such as the gut and neural tubes are unbranched. Through the use of genetically amenable model organisms, such as Drosophila and Zebrafish, we are beginning to unravel the mechanisms of epithelial branching morphogenesis; however, it is still not clear why some tubular organs branch whereas others do not. A key signaling pathway that controls branching morphogenesis in both vertebrate and invertebrate organs is the fibroblast growth factor (FGF) pathway [1]. For example, loss of FGF signaling in the mammalian lung or the Drosophila trachea severely disrupts branching morphogenesis in these organs [2–7].

Studies in the Drosophila embryonic trachea have contributed significantly to our understanding of branching morphogenesis. The embryonic trachea is an interconnected network of branched epithelial tubes that becomes functional during the larval stage to transport oxygen and other gases throughout the organism. The pattern of the larval trachea is established during embryogenesis when cells from ten tracheal placodes on each side of the embryo invaginate into the underlying mesoderm and then migrate out in a distinct pattern to form the primary branches. During the initial outgrowth of the tracheal primary branches, tracheal cells expressing the FGF receptor, Breathless (Btl), migrate in response to the FGF ligand, Branchless (Bnl), which is expressed in discrete clusters of non-tracheal cells that surround the migrating tracheal cells [5,6,8,9]. Later in embryogenesis, bnl expression confers secondary cell fates, such as the terminal cell fate, to cells at the tip of the growing branches [5,6,10,11]. Thus, Bnl/Btl signaling is required throughout tracheal development for initial migration and outgrowth of the primary branches as well as for specification of the secondary cell fates. One mechanism by which Bnl/Btl signaling is sustained in tracheal cells is through a positive feedback loop, whereby Bnl/Btl signaling activates MAP-kinase and the ETS-domain transcription factor, Pointed, to induce late bnl expression [12].

During migration of primary tracheal branches, markers, such as pointed and sprouty, that define the tips of migrating branches are expressed broadly, only to become restricted to a single cell later...
This suggested that all tracheal cells are initially equivalent but then specific cell fates become restricted through regulation of gene expression. In the dorsal branch, which typically consists of five or six cells, one cell at the branch tip adopts the terminal cell fate and branches extensively to deliver oxygen to neighboring tissues whereas a second cell at the branch tip adopts a fusion cell fate and mediates fusion of tracheal branches from adjacent hemisegments and contralateral branches at the dorsal midline [13,14]. Genetic mosaic analysis showed that tracheal cells at the tips of migrating branches compete with each other such that cells with the highest Bnl/Btl signaling activity become the “lead” cell which is then specified to be the terminal cell, whereas those with less signaling activity become the “follower” stalk cells of the tube [4]. FGF signaling induces Notch (N) signaling through activation of the N ligand, Delta (Dl). Activated Dl in the tip cells then activates N in neighboring stalk cells to restrict the fusion and terminal cell fates [15–17]. Thus, FGF signaling not only regulates tracheal cell migration, but also restricts cell fates via N-mediated lateral inhibition.

hairy is a pair-rule gene whose role in early patterning of the Drosophila embryo is well established [18,19]. Hairy belongs to a small family of HHLJ transcription factors related to the HES/ HESR/HRT/HEY proteins in mammals [20–22] and Gridlock in Zebrafish [23]. Hairy and its related proteins generally function as transcriptional repressors which are expressed in various tissues and regulate key developmental events such as cardiovascular development [21,24,25]. We previously showed that loss of hairy function results in expansion and branching of the normally small family of bHLH transcription factors related to the HES/HESR/HRT/HEY proteins in mammals [20–22] and Gridlock in Zebrafish [23]. Hairy function in terminal cell specification, hACT-expressing embryos phenocopied the previously reported “finger-like structure” [29] as in wild-type embryos, whereas in other mutant embryos, one or both ends of the structure was widened indicative of uncoordinated invagination (Figure 3A–C). Thus, during early tracheal development, hairy is required for coordinated invagination.

Although all tracheal cells were internalized in h22 and h674 mutant embryos, later tracheal development was defective. In stage 15 h22 and h674 mutant embryos, the dorsal trunk (DT) was broken and did not form a continuous tube (Figure 2B and C). Discontinuity of the DT was observed in 96% of h22 mutant DTs (n = 125) and 53% of h674 mutant DTs (n = 237) analyzed. h674 mutant embryos did not show defects in formation of the primary tracheal branches (data not shown); however, in embryos trans-heterozygous for h22 and h674, the DT was broken (data not shown). h22 and h674 mutant embryos, which had no segmentation defects, formed a normal tracheal network (data not shown).

During earlier stages of tracheal migration, h22 mutant cells failed to migrate out and instead formed large clusters of internalized cells (Figure 2E and H). Tracheal migration defects were milder in h674 mutant embryos although delayed migration of DT cells was apparent (Figure 2F and I). We confirmed that DT migration defects in hairy mutant embryos were not due to a failure to specify DT identity by staining for the DT marker, split; in both h22 and h674 mutant embryos, split was expressed in the same temporal and spatial manner as heterozygous siblings (data not shown).

### Results

Hairy protein was first detected in the tracheal placodes prior to and during invagination but not in the internalized tracheal cells (Figure 1A). In addition to tracheal expression, Hairy protein was also expressed in the visceral and somatic mesoderm, as previously reported (Figure 1B and C) [18,19]. To understand hairy function in tracheal development, we analyzed an allelic series of hairy mutants, including h22, h674, h1, h1J3, h37 and h47, for tracheal defects. h22 is a lethal EMS-induced null allele encoding a truncated protein of only 42 amino acids due to a premature stop codon; h674 is a lethal EMS-induced hypomorph allele encoding a protein lacking the C-terminal 103 amino acids due to a premature stop codon and h1J3 and h1 are viable alleles with inserted transposable elements [26,27]; Flybase. Although the exact molecular lesion of h22 and h674 are not known, they are reported to have no segmentation defects and to fully complement the segmentation and lethality of strong hairy alleles [28]. Consistent with their molecular lesions, the hairy pair-rule phenotype was most severe in h22 embryos compared to h1J3 and h1 embryos which showed no patterning defect (data not shown). h674 mutant embryos showed a range of segmentation defects, though none as severe as that of h22 mutant embryos [26].

In embryos homozygous for either h22 or h674, all tracheal cells invaginated (Figure 2H and I), indicating that although hairy is expressed in invaginating tracheal cells loss of hairy function did not prevent tracheal cells from being internalized. However, hairy may still play a role in tracheal invagination since recent studies showed that tracheal cells can still be internalized in mutants where certain features of invagination are defective [29,30]. In some h674 mutant embryos, invaginating tracheal cells formed the previously reported “finger-like structure” [29] as in wild-type embryos, whereas in other mutant embryos, one or both ends of the structure was widened indicative of uncoordinated invagination (Figure 3A–C). Thus, during early tracheal development, hairy is required for coordinated invagination.

Hairy function in terminal cell specification due to hairy’s well-established role in embryonic patterning, we limited our analysis of hairy function in tracheal development to h674 mutant embryos which did not exhibit any segmentation defects [28] and h674 embryos which have mild or no segmentation defects. In wild-type embryos, a single cell at the distal tip of each dorsal branch becomes specified as the terminal cell (TC) (Figure 4A). In contrast, in h22 and h674 homozygous embryos and trans-heterozygous embryos of h1J3 and h674, extra TCs were found at the tips of the DBs (Figure 4B, C and E); extra TCs were also detected in the lateral trunk branches of h22 homozygous embryos compared to heterozygous siblings (Figure 4G and H). To eliminate the possibility that specification of extra TCs observed in h674 mutant trachea could be a secondary consequence of hairy’s patterning role in the early embryo, we analyzed embryos expressing a heat shock-induced activated form of Hairy (HairyACT) where the transcriptional activation domain of the herpes simplex virus VP16 protein is fused to a truncated Hairy protein that retains the bHLH and helical/Orange domains but lacks the C-terminal WRPWM motif required for repression by hairy [31]. HairyACT (hACT) has been shown to act as an activator instead of a repressor by promoting transcription of specific target genes [31]. Heat shock-induced expression of hACT at eight hours of embryogenesis after segmentation of the early embryo is complete resulted in a properly segmented embryo with an intact tracheal network (data not shown); however, in terms of TC specification, hACT-expressing embryos phenocopied the hairy mutant phenotype with extra TCs being specified at the tips of primary tracheal branches.
DBs (Figure 4D). The extra TCs specified in h^ACT expressing embryos that were properly segmented confirmed that hairy is normally required for refining the TC fate to a single cell and that this tracheal function of hairy is independent of its earlier patterning role.

Formation of terminal branches requires the ETS domain transcription factor, Pointed (Pnt) [13]. In embryos homozygous for a null allele of pnt, pnt^{D88}, TCs were not specified unlike in wild-type embryos (Figure 5A and B). In contrast, overexpressing wild-type pnt in the entire trachea with btl-GAL4 led to specification of extra TCs in all tracheal branches, including the dorsal trunk (Figure 5C). To test whether wild-type pnt function was required for specifying extra TCs in h^{674} mutant trachea, we reduced the gene dosage of pnt in h^{674} homozygous embryos and analyzed TC specification. One copy of pnt^{D88} in h^{674} homozygous embryos prevented the specification of extra TCs by h^{674} such that no DBs contained two TCs compared to h^{674} mutants alone (Figure 5E). These data demonstrate that specification of extra TCs in h^{674} mutant embryos is dependent on normal pnt function. To test whether pnt normally acts downstream of hairy in restriction of the TC fate, we analyzed embryos mutant for both hairy and pnt. In h^{674}pnt^{D88} double homozygous embryos, no TCs were specified, like in pnt^{D88}, demonstrating that pnt likely acts downstream of hairy (Figure 5D). Since pnt function correlates with specification of the TC fate, we next performed in situ hybridization for pnt RNA to test whether pnt RNA expression was altered in h^{674} mutant trachea. In h^{674} heterozygous embryos, 80% of DBs had a single pnt-expressing TC and 20% had two pnt-expressing TCs (Figure 5F and G). In contrast, in h^{674} homozygous embryos, only approximately 30% of DBs had a single pnt-expressing TC and the remainder of DBs had between two and six pnt-expressing TCs per branch (Figure 5F and H). Similar to h^{674} homozygous embryos, DBs of embryos expressing heat shock induced-h^{ACT} contained extra pnt-expressing DB cells (Figure 5F).

Tracheal-specific overexpression of an activated form of Bdl (Bd^{ACT}) or, ubiquitous overexpression of its ligand, Branchless (Bnl) results in an increased number of TCs (Figure 6B) [5]. Therefore, we tested whether the specification of extra TCs observed in hairy mutant trachea could be due at least in part to hyper-activation of the Bdl/Bdl signaling pathway. Concomitant

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**Figure 1. Hairy protein expression during embryogenesis.** In wild-type embryos, Hairy protein is expressed in invaginating cells of the tracheal placode (TP) (A, arrow), in the visceral mesoderm (VM) (B, arrow) and somatic mesoderm (SM) (C, arrow). Scale bar A represents 20 μm.

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reduction of hairy gene dosage in wild-type trachea already expressing BtlACT resulted in the specification of more TCs than with BtlACT alone (Figure 6B, C and D). Moreover, in h674 mutant DBs, btl RNA expression was not restricted to the distal most two cells as normally occurred in wild-type DBs and instead was expressed in more proximal cells of the DB (Figure 6E and F). Thus, by reducing hairy function, domain of btl RNA expression was expanded to include not only the tip cells but also the stalk cells. Altogether, these data suggest that hairy normally limits btl expression domain and terminal cell specification.

Figure 2. Tracheal development is defective in hairy mutant embryos. In h22 heterozygous embryos, the dorsal trunk (DT) is a continuous tube (A, arrow) whereas in h22 (B) and hh4 (C) homozygous embryos, the DT is broken (B and C, arrows). In h22 heterozygous embryos (D and G), tracheal cells migrate to form six primary branches (D and G, arrows). In h22 (E and H) and hh4 (F and I) homozygous embryos, tracheal cells fail to migrate out (E, F, H and I, arrows). Embryos in panels A, B and C were double stained for 2A12 and β-galactosidase (β-gal) to distinguish heterozygous from homozygous siblings. Embryos in panels D to F were stained for Crumbs (Crb) to label the lumen and β-gal (not shown), and embryos in panels G to I were stained for Crb (white), β-gal (not shown) and Tracheal+ (Trh) (red) to label tracheal nuclei. Panels in D to I are projections of confocal sections of laterally viewed embryos. Scale bars in D and G represent 10 μm.

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Figure 3. Hairy is required for proper invagination. In wild-type embryos (A), invaginating tracheal cells form a finger-like structure. In some hh4 mutant embryos (B), invaginating tracheal cells form a finger-like structure, whereas in other mutant embryos (C), one end of the structure was widened (C, arrowhead). Embryos in A–C were double stained for Crb and β-gal (not shown). All panels of embryos are lateral views. Scale bar in A represents 5 μm.

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hairy also genetically interacts with bnl. Bnl is known to be haploinsufficient for tracheal branch migration [6]; however, whether it is also haploinsufficient for TC specification has previously not been tested. In embryos with one copy of either bnlP1 or bnlP2, about 40% and 12% of DBs had no TCs, respectively, compared to embryos homozygous for bnlP1, where 85% had no TCs and embryos homozygous for bnlP2, where 25% of DBs had no TCs (Figure 7). Thus, bnl is haploinsufficient for TC specification. In bnlP2 heterozygous embryos that were also heterozygous for h674 (h674bnlP2/WT), the percentage of DBs with no TCs was reduced to 3% from 15% in bnlP2 heterozygotes alone suggesting that one copy of h674 is sufficient to partially alleviate the bnlP2 haploinsufficient phenotype. Reduction of bnl gene dosage in h674 homozygous embryos reduced the number of DBs with two TCs from 15% in h674 homozygotes alone to 7% in h674 homozygotes with one copy of bnlP2 and 9% in h674 homozygotes with one copy of bnlP1 (Figure 7). Furthermore, in embryos double homozygous for h674 and bnlP2, 83% had no TCs, a phenotype more like bnl than hairy mutants. These data demonstrate that hairy and bnl genetically interact to refine TC specification and that bnl promotes TC specification whereas hairy normally acts to restrict it.

Since TC specification occurs after Hairy protein expression disappears in the tracheal cells, we sought to determine whether Hairy could regulate tracheal TC specification in a non cell-autonomous manner. Late expression of btl at the tips of growing primary branches is known to be dependent on bnl expression in surrounding non-tracheal cells [12]. Since Hairy remains expressed in the somatic and visceral muscle at the stage when TCs are specified (Figure 1), we tested the hypothesis that Hairy may normally regulate TC specification through restriction of bnl expression in non-tracheal cells. In wild-type and h674 heterozygous embryos (Figure 8A and E), bnl in non-tracheal cells at mid-embryogenesis was expressed in clusters close to the migrating tracheal dorsal branch, visceral branch, lateral trunk and ganglionic branches, as previously reported [6,12]. We observed expanded domains of bnl RNA expression in select groups of non-tracheal cells that were in close proximity to migrating tracheal dorsal branch, visceral branch, lateral trunk and ganglionic branches, as previously reported [6,12]. In hACT embryos, we observed expanded bnl RNA expression in the visceral and ganglionic clusters (Figure 8F and G). The proximity
of these bnl expressing non-tracheal cells to the tracheal branch tips of h674, h47 and hACT mutant embryos likely accounts for the extra TCs observed. Using the bnlP2 bnl-lacZ enhancer trap, Bnl was previously reported to be expressed in the visceral muscle and ectodermal clusters [12]; however, the ectodermal expression of Bnl was not confirmed with an ectoderm-specific marker. To better understand the dynamic pattern of Bnl expression during mid-embryogenesis when the TC fate is refined, we analyzed β-galactosidase expression conferred by the bnl-lacZ enhancer trap together with an ectoderm-specific marker, E-cadherin (E-cad) and a muscle-specific marker, β3 tubulin (β3t). Bnl expression was detected in the most dorsal row of branch cells as well as in single cells within the somatic muscle which corresponded to the visceral and ganglionic groups of bnl RNA expressing cells (Figure 9A-C). We did not detect Bnl in the dorsal epidermis; however, Bnl was robustly expressed in epidermal cells that lined the segmental grooves (Figure 9D and E). Thus, the cells expressing ectopic bnl RNA observed in hairy mutant embryos corresponded to areas of normal bnl expression in the lateral body wall muscle, the same tissue that hairy is expressed in.

As previously reported [18,19] and shown here in Figure 1, Hairy is expressed in the somatic and visceral muscle during mid-embryogenesis. To test whether Hairy refines the TC fate non cell-autonomously, we tested whether hairy function was required in the muscle. Expression of wild-type hairy in the somatic and visceral muscle did not affect TC specification (Figure 7). Expression of wild-type hairy in the somatic and visceral muscle of h674 homozygous embryos reduced the percentage of dorsal branches with two TCs from 45% to 20% and increased the percentage of DBs with one TC from 55% to 80% (Figure 7). Moreover, expression of wild-type hairy specifically in the muscle of bnlP2 heterozygous embryos enhanced the haploinsufficient phenotype of bnlP2 by forming more DBs with no TCs compared to bnlP2 heterozygotes alone. These data demonstrate that hairy functions at least in part in the muscle to refine the number of TCs specified in the trachea.

Since our data demonstrated a role for hairy in the muscle, we next tested whether overexpression of bnl in the muscle can phenocopy the h674, h47 and hACT mutant phenotype of extra TC specification. We expressed wild-type bnl in the somatic and visceral muscle with twi-GALA mef2-GALA, or in a subset of the...
**Figure 7.** *hairy* functions in the muscle and genetically interacts with *bnl* to refine the terminal cell fate. Graph shows number of DSRF-labeled terminal cells per dorsal branch in metameres two to nine of wild-type, *hairy* and *bnl* mutant embryos. Between 36 and 106 terminal branches were scored for the various genotypes. doi:10.1371/journal.pone.0014134.g007

**Figure 8.** Ectopic expression of *bnl* in *hairy* mutant embryos. In *h674* heterozygous embryos (A), *bnl* RNA is expressed in clusters close to the tracheal visceral branch (vis) (A, large arrows), in dorsal clusters (dor) (A, small arrowheads) and in lateral clusters (lat) (A, large arrowhead). In *h674* homozygous embryos (B), the dorsal clusters of *bnl* RNA are expanded (B, small arrowheads). In *h47* homozygous embryos (C and D), clusters of *bnl* RNA close to the tracheal visceral branch (vis) (C, large arrow) and dorsal clusters (dor) (D, small arrowheads) are expanded. In wild-type embryos (E and E'), a cluster of *bnl* expression is found close to the tracheal ganglionic branch (gan) (E and E', small arrows). In *hACT* embryos (F and G), *bnl* RNA close to the tracheal visceral branch (vis) (F, large arrows) and close to the ganglionic branch (gan) (G, small arrows) are expanded. All embryos shown are at stage 13 and were processed for ISH for *bnl* RNA and lac-Z to distinguish *h674* heterozygous from homozygous embryos. Embryos in panels A and B are lateral views whereas embryos in panels C–G are ventral-lateral views. Panel E' is a more internal focal plane than that shown in panel E. doi:10.1371/journal.pone.0014134.g008
visceral muscle and the ventral longitudinal muscle 1 with 5053-GAL4 (Figure 10A–D). Overexpression of bnl in the muscle led to formation of DBs that consisted almost entirely of TCs (Figure 10F); extra TCs were also specified in the transverse connective and the lateral trunk (Figure 10I). Overexpression of bnl with 5053-GAL4 phenocopied the hairy mutant phenotype more closely where a few extra TCs were specified in the dorsal, lateral trunk and ganglionic branches (Figure 10G and J). These data demonstrate that overexpression of bnl in the somatic and visceral muscle is sufficient to phenocopy the hairy mutant phenotype.

Hairy controls terminal branch lumen length in a bnl-dependent manner

In addition to refinement of the TC fate, Hairy also restricts terminal branch lumen length. Quantification of terminal branch (TB) lumen length (see Materials and Methods) showed longer lumens in h^674 and h^47 homozygous embryos compared to wild-type embryos (Figure 11). TB lumen length of h^674 and h^47 homozygous embryos measured 15±4.5 µm and 17±6 µm, respectively, compared to 12±3 µm in wild-type embryos. Extension of TB lumen length is dependent on bnl expression since loss of one copy of bnlP2 in otherwise wild-type embryos or in h^674 homozygous embryos reduced TB lumen length to less than that of wild-type embryos (Figure 11).

Discussion

Hairy plays a well characterized role in patterning of the early Drosophila embryo; however, our knowledge of hairy function in epithelial morphogenesis is limited to our previous study on hairy's
role in the regulation of apical membrane growth during embryonic salivary gland development. In this study, we demonstrate a novel function for hairy in refinement of the terminal cell fate to a single cell at the tip of the dorsal branch through restriction of bnl expression domains in muscle cells surrounding tracheal cells. Due to the strong requirement for hairy in early embryonic patterning, it was necessary to distinguish hairy function in tracheal development from its earlier patterning role. Thus, we focused our analysis of hairy function in the trachea to mutations that did not perturb segmentation of the embryo. We showed that as previously reported, h47 mutant embryos had no patterning defect, and yet, extra TCs were specified.

hACT, which not only lacks the WRPW motif but also contains the transcriptional activation domain of VP16, was previously shown to induce ectopic expression of target genes when expressed in the late blastoderm stage, the time when endogenous Hairy is expressed and is active [31]. We showed that expression of hACT in mid-embryogenesis, prior to specification of the terminal cell fate led to ectopic expression of bnl in muscle cells and specification of extra TCs. Since hACT was induced after patterning of the early embryo was complete, there were no segmentation defects in hACT-expressing embryos and yet, extra TCs were specified. Similar to hACT, in h674 mutant embryos with no segmentation defect, bnl expression domain was expanded and extra TCs were specified. Since, h674, like hACT, lacks the C-terminal co-repressor binding WRPW tetrapeptide [26,31] it is possible that the Hairy mutant protein of h674 embryos also acts as an activator and induces expression of downstream target genes. Thus, the h47, h674 and hACT mutant embryos which are segmented properly and yet show specification of extra TCs in the tracheal dorsal branches provide evidence that hairy's role in early patterning and in tracheal development are indeed distinct.

In addition to a role for hairy in refinement of the terminal cell fate through regulation of bnl expression in muscle cells, we also
provide evidence that hairy and bnl act antagonistically to regulate terminal branch lumen length. Our studies provide the first evidence for a role for bnl in tracheal lumen size control. Although hairy mutant tracheal cells invaginated completely, they did so in an uncoordinated manner compared to wild-type. Thus, hairy function is required at multiple stages of tracheal development.

Our data support a model where Hairy in the somatic muscle, normally refines the spatial expression of bnl in the muscle cells that are in close proximity to the migrating tracheal branches, such that only a single cell at the tip of each dorsal branch becomes specified as the terminal cell. Upon loss of Hairy’s repressive activity, bnl expression expands in the muscle cells and abnormally activates the bnl/bnl signaling pathway, such that extra TCs become specified. Our data do not suggest whether bnl is a direct or indirect transcriptional target of Hairy; future studies will distinguish between these two possibilities. It is also possible that Hairy regulates bnl/bnl signaling and TC specification via mechanisms other than control of bnl expression. For example, it was recently shown that in the developing tracheal air sac of Drosophila larvae, metalloprotease Mmp2 spatially restricts FGF signaling [32]. Thus, hairy may modulate the extent of bnl/bnl signaling in tracheal cells in a pretranslational manner as well.

Materials and Methods

Drosophila Strains and Genetics

Canton-S (CS) flies were used as wild-type controls. The following fly lines were obtained from the Bloomington Stock Center and are described in FlyBase (http://flybase.bio.indiana.edu/): k2, k2, k2, pm3, pm3, tux-GAL4, mgf2-GAL4 and 5053-GAL4. k2 was generated by standard EMS mutagenesis [26]. k2, k2, k2 and UAS-hairy were generous gifts of M. Wainwright and D. Ish-Horowicz. For the UAS-GAL4 expression system [33], bbl-GAL4 was used to drive tracheal specific expression of wild-type hairy (UAS-hairy) or activated bnl (UAS-bnlACT) and tux-GAL4 mgf2-
GAL4 and 5053-GAL4 were used to drive expression of wild-type bnl (UAS-bnl) or wild-type hairy.

Antibody Staining of Embryos

Embryos fixation and antibody staining were performed as previously described [34]. The following antisera were used at the indicated dilutions: mouse 2A12 antiserum (Developmental Studies Hybridoma Bank, DSHB; Iowa City, IA) at 1:5 for DAB staining and 1:2 for fluorescence; mouse Crumbs antiserum (DSHB) at 1:10; rabbit Tracheless antiserum (a gift from M. Limargas) at 1:30; mouse DSRF antiserum (Active Motif, Carlsbad, CA) at 1:100; mouse β-galactosidase (β-gal) antiserum (Promega, Madison, WI) at 1:10,000 for DAB staining and 1:500 for fluorescence; rat Hairy antiserum (a gift from S. Small) at 1:1; rabbit β3 tubulin (β3) antiserum (a gift from R. Renkawitz-Pohl) at 1:10,000 and rat E-cadherin (E-cad) antiserum (DSHB) at 1:20. Appropriate biotinylated- Jackson Immunoresearch Laboratories, Westgrove, PA), AlexaFluor488-, 647- or Rhodamine- (Molecular Probes, Eugene, OR) conjugated secondary antibodies were used at a dilution of 1:500. Whole-mount DAB stained embryos were mounted in methyl salicylate (Sigma, St. Louis, MO) and embryos were visualized on a Zeiss Axioplan 2 microscope with Axiovision Rel 4.2 software (Carl Zeiss, Thornwood, NY). Whole-mount immunofluorescent stained embryos were mounted in Aqua Poly mount (Polysciences, Inc., Warrington, PA) and thick (1 μm) fluorescence images were acquired on a Zeiss Axioplan microscope (Carl Zeiss) equipped with LSM 510 for laser scanning confocal microscopy at the Rockefeller University Bio-imaging Resources Center (New York, NY) and the Weil Medical College imaging research core facility.

RNA In Situ Hybridization

In situ hybridization (ISH) with antisense digoxigenin-labeled RNA probes for hairy (h), spalt (sal), breathless (bl), branchless (btl), pointed (ptd) and β-galactosidase (β-gal) were performed as previously described [35]. h, sal, btl, bnl, pnt and β-gal cDNAs were used as templates for generating antisense digoxigenin-labeled RNA probes as previously described [26]. Embryos were mounted in 70% glycerol before visualization as described above for whole mount antibody staining.

Heat shock induction of HairyACT

Embryos were collected for 8 hours on apple juice agar plates and then subjected to heat shock at 37°C for 10 minutes in a dry incubator. The embryos were then aged for a further 8 hours at room temperature (25°C or 16 hours at 18°C). Stage 15 embryos were prepared for immunocytochemistry or whole mount in situ hybridization as described above.

Quantification of terminal branch lumen length

Terminal branch lumen length was measured as the distance between center of terminal cell nucleus and tip of 2A12-labeled lumen and was measured with LSM 510 software. N represents the total number of TCs scored. Statistical analysis was completed using Microsoft Excel (Microsoft, Redmond, WA). P-values were calculated using Student’s two-tailed, unpaired t-tests.

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Author Contributions

Conceived and designed the experiments: MMM. Performed the experiments: YZ SM BS MMM. Analyzed the data: YZ SM MMM. Wrote the paper: MMM.
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