RESEARCH ARTICLE

The Intersection of the Extrinsic Hedgehog and WNT/Wingless Signals with the Intrinsic Hox Code Underpins Branching Pattern and Tube Shape Diversity in the Drosophila Airways

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Abstract

The tubular networks of the Drosophila respiratory system and our vasculature show distinct branching patterns and tube shapes in different body regions. These local variations are crucial for organ function and organismal fitness. Organotypic patterns and tube geometries in branched networks are typically controlled by variations of extrinsic signaling but the impact of intrinsic factors on branch patterns and shapes is not well explored. Here, we show that the intersection of extrinsic hedgehog (hh) and WNT/wingless (wg) signaling with the tube-intrinsic Hox code of distinct segments specifies the tube pattern and shape of the Drosophila airways. In the cephalic part of the airways, hh signaling induces expression of the transcription factor (TF) knirps (kni) in the anterior dorsal trunk (DTa1). kni represses the expression of another TF spalt major (salm), making DTa1 a narrow and long tube. In DTa branches of more posterior metameres, Bithorax Complex (BX-C) Hox genes autonomously divert hh signaling from inducing kni, thereby allowing DTa branches to develop as salm-dependent thick and short tubes. Moreover, the differential expression of BX-C genes is partly responsible for the anterior-to-posterior gradual increase of the DT tube diameter through regulating the expression level of Salm, a transcriptional target of WNT/wg signaling. Thus, our results highlight how tube intrinsic differential competence can diversify tube morphology without changing availabilities of extrinsic factors.

Author Summary

Tubes are common structural elements of many internal organs, facilitating fluid flow and material exchange. To meet the local needs of diverse tissues, the branching patterns and tube shapes vary regionally. Diametric tapering and specialized branch targeting to the
brain represent two common examples of variations with organismal benefits in the *Drosophila* airways and our vascular system. Several extrinsic signals instruct tube diversifications but the impact of intrinsic factors remains underexplored. Here, we show that the local, tube-intrinsic Hox code instructs the pattern and shape of the dorsal trunk (DT), the main *Drosophila* airway. In the cephalic part (DT1), where Bithorax Complex (BX-C) Hox genes are not expressed, the extrinsic Hedgehog signal is epistatic to WNT/Wingless signals. Hedgehog instructs anterior DT1 cells to take a long and narrow tube fate targeting the brain. In more posterior metameres, BX-C genes make the extrinsic WNT/Wingless signals epistatic over Hedgehog. There, WNT/Wingless instruct all DT cells to take the thick and short tube fate. Moreover, BX-C genes modulate the outputs of WNT/wingless signaling, making the DT tubes thicker in more posterior metameres. We provide a model for how intrinsic factors modify extrinsic signaling to control regional tube morphologies in a network.

**Introduction**

Branched tubular networks, like our vasculature transport and exchange vital gases and nutrients along entire organisms. The branching patterns, tube structures and dimensions in these networks show considerable regional variations to meet the different needs of target organs and ensure optimal organ function and animal fitness [1–4]. Adaptations of branch morphologies to the tissue environments can be achieved by changing the local availability of extrinsic factors like guidance molecules and/or by intrinsic regional differences in tube cell competence to respond and modify signaling outcomes. Although the prominent roles of variations in extrinsic signals in organotypic branching become widely established [3,5,6], the tube intrinsic mechanisms determining the differential responses of tube cells to signaling remain to be explored [7–10].

Despite the huge evolutionary distance of insects and mammals, the formation and maturation of the respiratory tube network in *Drosophila melanogaster* has served as a fruitful model system of branching morphogenesis [11,12]. Here, we use this system to evaluate the contribution of tube intrinsic, regionally differential competence in diversification of tube morphology.

The fly respiratory network, also called the tracheal system ramifies extensively to deliver oxygen to each cell in the body (Fig. 1A) [13,14]. It derives from 10 primordial cell clusters specified in ectodermal para-segments (PS) 4–13 on each side of the body [15]. At stage 11, the metameric cell clusters invaginate and begin to extend 6 primary branches. The tracheal metameres (Tr1-10) interconnect into a network through the activities of 5 to 6 specialized fusion cells in each branching unit [16–18]. These cells find and adhere to their ipsilateral or contralateral counterparts in neighboring metameres to form continuous tubes.

Tracheal branching morphogenesis is controlled by two conceptually different groups of extrinsic signals [6,19]. One is required in all primary branches while the other class specifies unique sets of primary branches. FGF/Branchless (Bnl) [20] is dynamically expressed in the surrounding tissues and serves as a general extrinsic branching signal by activating FGFR/Breathless (Btl) on the airway cells [21–23]. Btl activation initiates oriented cell migration and enhances branch elongation while it also organizes cell fate specification along each primary branch. The gradual specification of distinct fusion and terminal cell fates at the branch tips is essential for both branch fusion and further ramification branching [14,20].

The second class of signals includes BMP, Wnts and Hh proteins. BMP/Decapentaplegic (Dpp) is expressed in dorsal and lateral stripes along the length of the embryo [24]. Dpp
Figure 1. Developmental and genetic distinction between DTa1 and the rest of DT. Embryos are aligned with their anterior left and dorsal up unless otherwise indicated. (A), Times series of airway development visualized with trh-lacZ. DTa1 is marked with arrowheads. Schemes of the typical primary branches or the metamere 1/2 are shown on the right. See text for details. (B, C), Late stage11 embryos marked with trh-LacZ and RNA in situ hybridization against salm (B) or kni (C). Typically, DT and DB initiate salm expression while kni expression represses salm later in the DB. Note that DTa1 (arrowheads) expresses kni but not salm. (D, E) Stage 13 control and Df(kni/knrl) embryos marked with kni-(dpp)-LacZ, Salm and Trh. Schemes of their expression are shown in the bottom. In the control (D), Salm is detected in DTp1-DT10. Salm is also reproducibly detected in DB1/10 and TC1. kni-(dpp)-LacZ marks subsets of Kni positive branches (DB/TC/GB) responding to dpp signaling. kni-(dpp)-LacZ and Salm overlap only in DB1/10. In Df(kni/knrl) (E), some abdominal segments are missing or fused. Note that kni-(dpp)-LacZ positive cells co-express Salm in DB, LT or GB (arrow) and that the whole terminal metameres, presumably Tr1/10, including DTa1 (arrowhead) express Salm. (F-K) Airway branching revealed by Gasp expression at stage 15. DTa1/CB is marked with arrowheads or asterisks. Compared to the control (F), in Df(kni/knrl) (G), kni positive branches (DB, LT, GB, VB and DTa1) are stalled or missing. Branches in Tr1 and Tr10 become very thick. btl-gal4 driven UAS-kni (H) or UAS-knrl (I) significantly rescues DTa1/CB formation. btl-gal4 driven UAS-Axn-GFP (J) partially rescues DB extension without effects on dorsalward extension of CB while btl-gal4 driven UAS-salm abrogates CB formation (K). Scales bars: 50um.

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signaling specifies and licenses dorsally (dorsal branch, DB) and ventrally migrating primary branches (lateral trunk, LT and ganglionic branch, GB) by inducing the expression of the TFs kni and knirps-related (knrl) in airway cells [24–26]. Wg is expressed in a repetitive pattern of transverse ectodermal stripes and together with other WNT signaling molecules specifies dorsal trunk (DT) identity [27–29]. It upregulates the expression of the TF salm [30] in the major airways of the network. salm promotes short and thick tubes by suppressing intercalation of the tube constituent cells [31,32]. On the other hand, kni/knrl can repress salm expression in DB [25] and promotes cell intercalation in the long and narrow tubes of dorsal (DB) and ventral branches [31]. Hh is also segmentally expressed in ectodermal stripes and modulates the airway branching, both indirectly through positive regulation of bnl expression [33] and directly by acting on terminal cell specification or extension [34,35]. The functions of hh in primary branching remain to be thoroughly studied [34,36,37]. Collectively, the differential primary branch identities established by the second class of extrinsic signals are intimately linked with the distinctive branching patterns and dimensions of individual primary branches [38].

The region-specific modification of serially homologous organs and appendices is a general theme in animal development [39–42]. The evolutionary conserved Hox gene complexes are key selector genes of tissue identities along the anterior posterior (A-P) axis of animals [39,41,43–45] [46–50]. In Drosophila, 2 groups of Hox genes, the Antennapedia complex (ANTP-C) and Bithorax complex (BX-C) confer regional differences to the body plan by graded expression of their products in register with para-segmental units [39,51]. The 3 protein-coding genes of the BX-C [52] are expressed in distinct and partially overlapping domains along the A-P embryonic axis. Ultrabithorax (Ubx) expression initiates in the cells of PS5, abdominal-A (abdA) expression starts from PS7 and Abdominal-B (AbdB) from PS10 [44,53]. A connection between regional modification of airway morphology and Hox genes has been established already in the early studies of Hox-gene mutants. Upon loss of all BX-C genes the tracheal metameres Tr2-Tr10 become transformed to Tr1 [39,54]. However, the genetic and molecular mechanisms establishing the different branch morphologies along the airway tubes have been largely unexplored.

Here, we focus on the regulation of 2 distinct morphological modifications along the DT major airways. We first analyze how the most anterior part of the DT diverges its branching pattern and tube size to generate long and narrow tubes targeting the head. We show that these regional modifications in cell behaviors are controlled by a combination of hh signaling and the airway intrinsic Hox code. We further investigate the mechanism of tube tapering in the central domain of the DT airways. We find that BX-C genes modulate the anterior-to-posterior gradation of DT tube diameter partly through regulating the expression of level of salm, a target of WNT/wg signaling. Our work highlights that the intrinsic Hox code locally modifies the outcomes of extrinsic signals to establish regionally different branching patterns and to coordinate tube shapes in register with the embryo axis.

Results and Discussion

Specialized branching patterns and tube shapes in Tr1

The DT is a continuous tube running along the A-P axis of the embryo. It connects with the exterior through the narrow tube of the spiracular chamber in the posterior spiracle (PSP) [55]. The DT is constructed by the fusion of an anterior (DTa) and a posterior (DTp) branch from each tracheal metamere (Tr1-Tr10) [13,17]. The DT airway encompasses several regional variations that provide a suitable system for the study of the interplay of external signaling with intrinsic factors during tube morphogenesis [13]. First, the most anterior end of the DT extends several specialized branches (see below). Second, it shows a pronounced posterior to anterior
diameter tapering contrasting the largely cylindrical shape of other primary branches in the network. Third, its most anterior metameric unit (Tr1) lacks a DTa fusion cell, whereas the most posterior one (Tr10) does not generate a fusion cell in its posterior branch (DTp).

More generally, Tr1 is distinct from the rest of the tracheal metameres because it encompasses more cells and branches to oxygenate the specialized organs of the head and thorax. The specialized branches of Tr1 include the cerebral branch (CB) targeting the brain, the pharyngeal branch (PB) to the anterior intestine, the ventral cephalic branch (VC) extending to epidermis and muscles and the ganglionic branch GB0, which penetrates the ventral nerve cord. Among these, the CB and VC are directly linked to the anterior end of the DT airways.

Despite the pronounced differences in the final branching patterns, branching in Tr1 is comparable to the common stereotypic primary branching of Tr2-Tr9 during stage 11 (Fig. 1A). At stage 12 however, DTa1, elongates further than other DTa branches and shifts dorsally. By stage 13, visceral branch 1 (VB1) and DTa1 co-segregate from the transverse connectives (TC). Later, DTa1 extends dorsally and posteriorly and turns towards the brain, forming CB [13]. DTa1/CB develops very narrow and long tubes compared to the thick and short DT branches in posterior metameres. VB1 extends more anteriorly, forming the pharyngeal branch (PB) (Fig. 1A).

**kni** expression in DTa1/CB is essential for CB formation

The gradual morphological diversification of CB/DTa1 compared to DTa branches in other metameres prompted us to examine the expression of branch identity TFs, **salm** and **kni**. In a “typical”, central metamere, **salm** expression is upregulated by wg/WNT signaling [27–29] and is detected in DB and DT at stage 11 [30]. Later, **kni** is induced in DBs by **dpp**, where it represses **salm** expression [24–26]. **kni** and **salm** are co-expressed in DB1/10 (see below). In contrast to the DTa of other metameres, we found that **kni** expression is strongly upregulated in DTa1 from late stage 11 (Fig. 1B). Concomitantly, **salm** is not detectable in DTa1 (Fig. 1C) although it becomes upregulated in DTp1 as in other metameres (Fig. 1D) [30].

To test the significance of the differential **kni** expression in DTa1 we analyzed mutants lacking both **kni** and its paralog **knrl**. In these chromosomal deficiency mutants, the abdominal segments are missing due to the early gap gene function of **kni**, while trunk development is rather normal [56]. In the trunk region of **Df(kni/knrl)** mutants, the formation of **kni** positive primary branches [24,25] is variably affected, ranging from complete absence to branch stalling [25], while the **salm**-positive DT branches can form and fuse (see below) [57]. We noticed that in **kni/knrl** mutants, airway cells initiate branch outgrowth in the dorsal and ventral directions and respond to **dpp** by inducing the **dpp**-responsive **kni** reporter, **kni-(dpp)-lacZ** (Fig. 1E). In these embryos, **salm** is ectopically detected in **kni-(dpp)-lacZ** positive cells in either dorsal or ventral cells near the **wg** stripe (Fig. 1E). This suggests that **dpp** mediated **kni/knrl** induction suppresses **salm** induction by **wg** in both the dorsal [25,26] and ventral branches. However, in the first and the last metameres of **Df(kni/knrl)** mutants, **salm** expression additionally expands to nearly cover the entire metamere, including the putative CB/DTa1 (Fig. 1E). This suggests that **kni** functions in DTa1 to repress **salm**. Additionally, the competence of tracheal cells to induce **salm** is differentially modulated in the terminal Tr1 and Tr10 metameres compared to the central metameres.

Consistent with the notion that generalized reduction of **wg/WNT** signaling can bypass the requirement of **dpp/BMP** signaling during DB extension in Tr2-Tr10 [31], **btl-Gal4** [58] mediated overexpression of GFP fused to **Axin** (**Axn**) [59–61], a negative regulator of **wg** signaling moderately rescues dorsal extension of the residual DBs (DB1 and DB2) of **Df(kni/knrl)** mutants but does not appreciably rescue the extension of DTa1/CB (Fig. 1). In sharp contrast, **btl-
gal4 driven UAS-kni or UAS-knrl in Df(kni/knrl) mutants restores CB formation (Fig. 1F-I). Thus, we conclude that kni induction and the resultant salm repression in DTa1/CB are essential for its formation and extension. In support for this, btl-gal4 driven UAS-salm in wild-type background significantly suppresses DTa1/CB formation but has little effect on the extension of DTa in Tr2-Tr10 [28,62], which endogenously expresses salm (Fig. 1K). Collectively, the results suggest that kni induction and the resultant salm repression in DTa1/CB are essential for its formation and extension.

**hh is necessary for DTa1 patterning**

The diversified expression of kni in DTa1 compared to the remaining DTa branches could be regulated by differential expression of exogenous guidance factors around DTa1 and/or by intrinsic differences of competence among the DT1 cells. Firstly, to investigate which extrinsic factors are upstream of kni induction in DTa1, we analyzed the expression or function of known, secreted airway branching regulators.

At stage 11, Tr1 like the rest of the tracheal metameres is surrounded by 6 patches of bnl expressing cells, prefiguring the stereotypic directions of the common primary branches (S1A Fig.). This general pattern diversifies in the cephalic region of stage 12 embryos, where the DTa1 migrates toward a more dorsal bnl expression spot (S1B Fig.). Despite this difference, kni induction in DTa1 is still detected in btl mutants (S1C,D Fig.), excluding a major role of bnl in kni induction in DTa1. dEGFR/faint little ball/torpedo (top) [63] encodes an RTK that is suggested to positively act on salm expression [64] upon binding the Spitz/EGF ligand [65]. In embryos mutant for rhomboid (rho), encoding a protease required for generating the active Spitz [66,67] or for dEGFR, the expression of kni in the DTa1/CB still occurs (S1E,F Fig.). This argues against a role of localized dEGFR activation in controlling kni expression in DTa1.

dpp and wg are known inducers of kni/knrl in DB [24] and salm in DT [27,28], respectively, in a “typical” metamere. Thus, variations of their expression in the Tr1 proximity might influence the specialized expression patterns of kni or salm in DTa1. However, the expression of both dpp and wg is comparable around Tr1-3 (S1G, H Fig.), arguing against an instructive role of these two factors in kni induction in DTa1. Indeed, neither mutants of tkv, encoding one of the two dpp receptor subunits [68,69] nor arm mutants lacking an essential component of wg signaling [70–72], showed major defects in kni induction and outgrowth of DTa1/CB (S1I, J Fig.).

hh is a signaling molecule that binds its receptor patched (ptc), thereby relieving ptc-mediated inhibition of the 7 transmembrane domain protein smoothened (smo) [73,74]. hh is expressed in stripes in the ectoderm, abutting the anterior edge of the airway primordia at stage 10 and overlying the anterior part of the invaginated airway cells of each metamere at stage 11 (S1K, L Fig.) [34]. Glazer and Shilo showed that hh induces marker gene expression in the anteriorly migrating branches of central metameres [34], arguing that hh signaling patterns the anterior primary branch fates of the “typical”, central metameres. We found that just after invagination of the airway primordial cells, expression of ptc, a transcriptional target of hh [75,76] is upregulated in the DTa1 precursors (Fig. 2A), suggesting that hh signaling is active there. In hh mutants the dorsalward CB extension is hardly detectable and salm expression is expanded in the entire DT1 (Fig. 2B-D). This suggests that hh signaling in DTa1 induces kni and thereby represses salm expression. Among the ectopically salm expressing cells in DTa1, some cells also express kni while others do not. We suggest that in the absence of hh signaling, hh responsive kni induction is lost while dpp signaling may take over to induce kni expression in some salm positive cells. Such an ectopic activation of kni in the absence of hh could induce ectopic kni/salm-double positive cells resembling DB1 cells. This interpretation is consistent with the
de-repression of \textit{kni-(dpp)-lacZ} in \textit{Df(kni/knrl)} mutants (Fig. 1E). Thus, we conclude that \textit{hh} signaling is required to induce \textit{kni} and to repress \textit{salm} in DTa1. The earlier function of \textit{hh} is also required for the maintenance of the striped expression of \textit{wg} [75,77], which induces \textit{salm} expression in DT. Thus, the variability of \textit{salm} expression either in DTa1 or in DTa/DTp of any metamere in \textit{hh} mutants may partly reflect a reduction or loss of epidermal \textit{wg} expression.

In \textit{hh} mutants, \textit{bnl} expression guiding DB migration in central metameres is lost but \textit{bnl} expression in surrounding cells guiding CB is still detectable at stage 12 (S1M Fig.). Nevertheless, the dorsal extension of a CB-like branch is not detected in \textit{hh} mutants at later stages (S1N Fig.). To more directly address the effect of \textit{hh} signaling in DTa1 we attempted to inactivate its components specifically in the airways. \textit{hh} signaling modifies the transcriptional activity of \textit{cubitus...
interruptus (ci) [74]. In the absence of hh, Ci is proteolytically processed and acts as a repressor [78], while upon hh pathway activation, smo mediated signaling suppresses this proteolysis and turns Ci into an activator [78–80]. The balance of loss of the repressor form and generation of the activator form of Ci determines the hh signaling outputs [79–81]. We generated embryos expressing dominant negative forms of ci, ciDN (ci<sup>REP</sup> [82,83] and/or ci<sup>75</sup> [78,84]) exclusively in the airways and assessed the expression levels of kni-(dpp)-lacZ (a DB and LT/GB marker) [25] and of salm-TSE-lacZ (a reporter of salm expression) [30] in metamere 1. Both markers are ectopically induced in the DTa1 of these embryos at stage 13 (Fig. 2E-H) although the cell number in this branch did not significantly change (20 cells, standard deviation SD = 0.707 for 5 wild type embryos at stage 13 and 19.2 cells, SD = 0.447 for 5 btl<sup>X2</sup> > ci<sup>REP</sup> embryos). We interpret that the incomplete inactivation of hh signaling in the airways by ciDN, partially transformed DTa1 cells to DTp1. These cells are still receiving enough Dpp to express kni-(dpp)-lacZ. The weaker effects of ciDN expressing embryos compared to hh mutants may reflect ineffectiveness of CiDN or the delayed btl-Gal4 mediated expression [58] of CiDN, which starts slightly later than the initiation of hh action on DTa1. Additionally, the airway-specific overexpression of CiDN or general hh inactivation in smo mutants frequently resulted in DTa1/VB1 co-segregation defects and CB misrouting at stage 16 (Fig. 2I, J and S1O Fig.). A similar CB misrouting phenotype has been described in mutants of unplugged (unpg) [54]. Indeed, the expression of an unpg enhancer trap in the CB of wild type embryos is lost upon CiDN overexpression (Fig. 2I, J).

Collectively, these results identify a selective, direct role of hh signaling in inducing the distinct cell identities of DTa1 compared to the cells of the remaining DT branches.

hh pathway overactivation transforms DB1 and DTP1 into DTa1-like branches

The transformation of DTa1 to DTP1/DB1 upon inhibition of hh signaling suggests that its overactivation may be sufficient to transform DTP1/DB1 to DTa1. To examine this, we analyzed ptc mutants, where hh signaling is inappropriately activated due to the loss of ptc-mediated inhibition of smo [73,74]. In ptc mutants, the dorsal part of metamere 1 expresses Kni but not Salm already at late stage 11 (Fig. 3A, B). Correspondingly at stage 13, expression of salm-TSE-lacZ is specifically lost from metamere 1, suggesting a defect in both DTP1 and DB1 specification (Fig. 3C and S3A Fig., note that in wild type, salm is expressed in DB1 and DTP1). Consistent with a loss of the DB1 fate in ptc mutants, kni-(dpp)-lacZ expression in the dorsal part of metamere 1 is completely lost (Fig. 3E) while Kni protein is expressed in the whole distal part of Tr1 (S2B Fig.). Although ptc mutants contain fewer airway cells [33], presumably due to an early upregulation of wg [85], a negative regulator of the airway primordia size [86], reduction of cell number in CycA mutants [38] does not significantly abrogate DB1/DT1 fates (S2D, H Fig.). This suggests that the effect of ptc on DB1/DT1 specification is more direct and not due to a general reduction in the number of airway cells. Because btl-Gal4 driven Ci<sup>REP</sup> (Fig. 3D, F) or Ci<sup>75</sup> can restore the expression of both kni-(dpp)-lacZ and salm-TSE-lacZ in the Tr1 cells of ptc mutants and because Ptc is expressed in all airway primordia including the entire Tr1 primordium (Fig. 2A), these results suggest that overactivation of hh signaling in Tr1 abolishes the DTP1/DB1 fates.

To further test the role of hh signaling in determining branch identities in Tr1 we analyzed the RNA expression of unpg. In control embryos at early stage 12, unpg expression is strongly detected in DTa1 [54] and weakly in the anterior part of TC1. Both of these regions correspond to hh signaling activation (Fig. 3G). At stage 13, unpg RNA is detected in CB and GB0/GB1 in Tr1 and also in the GBs of the more posterior metameres Tr2-Tr9 (Fig. 3H) [54]. Consistent
Figure 3. Overactivation of hh signaling transforms the primary branch identities in Tr1. (A, B) Expression of Salm, Kni and Trh at late stage 11. In contrast to the control (A), where Salm is upregulated in DTP1 (arrow), in ptc mutants (B), Salm upregulation in DTP1 (arrow) is not detected and Kni occupies the whole distal part of Tr1. Note that the airway primordia look smaller in ptc mutants [33]. (C-F) Effects of ptc mutations on kni-(dpp)-lacZ and salm-TSE-lacZ expression. The airway cells are labeled by Trh or Hnt. Compared to the control (Fig. 2E,G), in ptc mutants (C, E), the expression of salm-TSE-lacZ (C) and kni-(dpp)-lacZ (E) is lost from the dorsal part of Tr1 (asterisks). Overexpression of CiDN with btl-Gal4 in ptc mutants restores their expression in Tr1 (D, F, arrows). (G-M) unpg RNA expression at stage 12 (G, I, K) and stage 13 (H, J, L) in relation to the airway development visualized with trh-lacZ. A summary of phenotypes is shown in (M). (G, H) In the control, unpg RNA is strongly detected in CB (arrowheads) at stages 12 and 13. unpg expression is also strong in GB at stage 13 (arrow). (I, J) In ptc mutants, unpg expression is expanded to DB1, DTp1 and TC1 (arrowheads) while its expression in GB is barely detectable (asterisks). (K, L) In hh mutants, unpg expression is lost in CB (asterisk) while its expression is derepressed in LTa in the central metameres (arrows). (N-P) Airway branching pattern visualized by Gasp. Compared to the control (N), ptc mutants (O, P) show persistent loss of DT1/2 fusion (asterisks). In addition, duplication of CB like branches occasionally happen (P, arrowheads). Scales bars: 50um.

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with the loss of \textit{unpg-lacZ} expression in CB upon Ci\textsuperscript{DN} overexpression, \textit{unpg} RNA expression is lost in DT\textsubscript{a1}/CB of \textit{hh} mutants (Fig. 3K, L). Conversely in \textit{ptc} mutants, it is expanded posteriorly to cover the positions of DB1/DTp1/TC1 (Fig. 3I, J), indicating their transformation to CB-like fates. Consistent with the expanded \textit{unpg} expression, we often detected a duplication of CB-like branches in \textit{ptc} mutants (Fig. 3P). We additionally noted that \textit{unpg} expression in GB is lost in \textit{ptc} mutants (Fig. 3J) while \textit{unpg} is derepressed in LT\textsubscript{a} in \textit{hh} mutants (Fig. 3L), in accord with the notion that \textit{hh} confers the anterior branch identity in the central metameres [34].

At stage 16, \textit{ptc} mutants show variable branching defects including stalled GBs, DBs and DT breaks [33]. Concomitantly with the loss of DTp1 fate marker (\textit{salm-TSE-lacZ}), DT\textsubscript{1} and DT\textsubscript{2} never fuse in \textit{ptc} mutants (Fig. 3N-P). In wild type, one of DTp1 cells takes the fusion cell fate, activates \textit{dys} expression and attaches to a fusion cell in DT\textsubscript{a2} (S2E Fig.), [18]. In \textit{ptc} mutants, \textit{dys} is not activated in DTp1 while \textit{dys} expression is variably expanded in more cells of the DT branches in posterior metameres (S2E-G Fig.). This may reflect an increase of epidermal expression of \textit{wg} [85], an inducer of the fusion cell fates [27,28]. The failure of \textit{dys} activation and DT\textsubscript{1} fusion in \textit{ptc} mutants is significantly rescued by \textit{btl-Gal4} mediated overexpression of Ci\textsuperscript{rep} (S2I Fig.). Moreover, both loss of \textit{dys} expression in DT\textsubscript{1} as well as DT\textsubscript{1}/DT\textsubscript{2} fusion defects are variably observed when dominant active Ci, Ci\textsuperscript{act} [84,87] is overexpressed in the airway cells (S2J Fig.). Notably however, the Ci\textsuperscript{act} overexpression by \textit{btl-Gal4} did not diminish \textit{salm-TSE-lacZ} or \textit{kni-(dpp)-lacZ} expression.

In summary, we suggest that \textit{hh} signaling instructs the DT\textsubscript{a1} fate at the expense of DB\textsubscript{1}/DB\textsubscript{p1} fates. In DT\textsubscript{p1}, \textit{hh} signaling must be kept low to allow the proper selection of the fusion cell fate and subsequent DT\textsubscript{1}/DT\textsubscript{2} branch fusion.

**Abdominal Hox genes shunt \textit{hh} signaling from induction of \textit{kni} in DT\textsubscript{a}**

The \textit{hh} induction of \textit{kni} expression in the DT\textsubscript{a} of wild type embryos as well as the loss of DT/DB fates in \textit{ptc} mutants are confined to Tr\textsubscript{1}. However, \textit{hh} signaling outcomes are expected to be equally profound in the more posterior metameres of both wild type [34] and \textit{ptc} mutant embryos [33]. The exclusive restriction of Hh responses within Tr\textsubscript{1} implies the presence of an inhibitory mechanism preventing \textit{kni} activation in the DT\textsubscript{a} branches of posterior metameres. The BX-C genes represent obvious candidate regulators of posterior metamere identity and modulators of \textit{hh} signaling outcomes along the A-P axis of the airways. Indeed, \textit{unpg} expression is de-repressed in progressively more posterior metameres in \textit{Ubx} mutants and \textit{Ubx abdA AbdB} triple mutants [54].

BX-C gene expression is graded along the A-P axis of the airway metameres (S3A-D Fig.). \textit{Ubx} expression starts in Tr\textsubscript{2} (PS5) and peaks at Tr\textsubscript{3} (PS6) (S3A Fig.), \textit{abdA} expression starts in Tr\textsubscript{4} (PS7) and peaks in Tr\textsubscript{6} (PS9) (S3B Fig.) while \textit{AbdB} expression starts in Tr\textsubscript{7} (PS10) and peaks in Tr\textsubscript{10} (PS13) (S3C Fig.). These expression patterns are in register with the expression of BX-C genes in the ectoderm [53], which is the origin of the airway primordia.

To explore the function of BX-C genes in DT\textsubscript{a} fates, we first monitored \textit{dys} expression in various BX-C mutants. In \textit{Ubx} mutants, single fusion cells are detected in Tr\textsubscript{1}, Tr\textsubscript{2} and Tr\textsubscript{3} (S3E, F Fig.) suggesting that DT\textsubscript{a2} and 3 are transformed to become DT\textsubscript{a1}/CB. \textit{abdA} single mutants do not show \textit{dys} expression defects in the DT (S3G Fig.) while a superfluous fusion cell in DT\textsubscript{p10} is detected in both \textit{AbdB} single and \textit{abdA AbdB} double mutants (S3H, I Fig.). This suggests that DT\textsubscript{10}, which normally contains only a single fusion cell in its DT\textsubscript{a} branch, is transformed into a more anterior identity. Compared to \textit{Ubx} single mutants, \textit{Ubx abdA} double mutants have single fusion cells in Tr\textsubscript{1–8} and often in Tr\textsubscript{9} (S3J Fig.). In \textit{Ubx abdA AbdB} triple mutants, the DT stumps of all metameres contain single fusion cells (S3K Fig.). This implies
that DTa branches in progressively more posterior metameres are transformed to become DTa1 upon progressive loss of BX-C genes [39]. Any single gene of the BX-C is sufficient to suppress the DTa1 fate. Consistently, we detected expansion of kni expression and a loss of salm in the transformed DTa in Ubx single, Ubx abdA double and Ubx abdA AbdB triple mutants (Fig. 4A-C). These phenotypes are often accompanied with the appearance of dorsally extending branches that are positive for Kni but negative for kni-(dpp)-LacZ, Salm and salm-TSE-LacZ, resembling the CBs of wild type embryos (S3K Fig.) [54]. The marker expression analysis in BX-C mutants suggests that in the posterior metameres, Ubx, abdA and AbdB interfere with the outcomes of hh signaling.

If the antagonistic role of BX-C on hh-mediated kni induction reflects an essential function of the BX-C in posterior metameres, one might expect some rescue of the branching defects of BX-C mutants upon simultaneous loss of hh or kni/knrl. Indeed, salm expression is de-repressed in DTa1-9 of Ubx abdA hh and of Ubx abdA kni/knrl mutants (Fig. 4D, E). Additionally, DT fusion is weakly restored in both the triple and quadruple mutants (S3L, M Fig.).

Taken together, we suggest that BX-C genes antagonize hh-mediated induction of kni in DTa branches.

**Airway-specific expression of abdominal Hox genes diverts hh signaling from kni activation**

In addition to the airways, BX-C genes are expressed in many embryonic tissues. Where do they act to divert hh signaling from kni induction in the DT branches? In lack of reagents for the reliable conditional inactivation of the BX-C genes in the airways, we monitored the effects of airway-specific ectopic expression of BX-C genes on DT1 cell specification. The first metamere does not express the BX-C genes (S3A-D Fig.) and thereby may provide a naïve environment for assessing the effects of their overexpression on marker gene activation [41]. btl-Gal4 mediated overexpression of any of the BX-C genes in wild type background, variably decreases kni expression in the DTa1 and concomitantly leads to increased salm levels at stage 13/14 (Fig. 4F-J). At later stages, DTa1 branches are thick, resembling typical DTa branches of posterior metameres (see below) in agreement with the previously reported loss of CBs upon abdA overexpression [88].

Similarly, btl-Gal4 mediated overexpression of either Ubx or abdA restores the fusion defects of DT1 and DT2 in ptc mutants (S3N, O Fig.). We detected that expression of both salm-TSE-lacZ and kni-(dpp)-lacZ is restored in the dorsal part of Tr1 of ptc mutants upon abdA overexpression (S3P, Q Fig.). These results argue that the Hox code in the airway cells autonomously changes hh-signaling outputs in Tr1 both in wild type and in ptc mutants, where the hh pathway is hyper activated. Finally, we asked if transgenic expression of abdA or Ubx in the airways could restore the branch fusion defects along the entire DT of Ubx abdA double mutants. Again, this manipulation rescues the branch fusion phenotypes (S3R-T Fig.) arguing that BX-C genes autonomously shunt hh signaling from inducing kni in the DTa branches of all metameres to promote continuous DT formation.

**BX-C genes control tube tapering**

A common characteristic of biological tubes is the tapering of tube diameter along their length. The *Drosophila* larval airways receive air only from the PSP and distribute it anteriorly. Correspondingly, the tubes show a posterior to anterior tapering [38], which presumably gradually increases the flow rates to the anterior and facilitates air diffusion from the PSP to the most distant anterior organs (http://hyperphysics.phy-astr.gsu.edu/hbase/pfric.html).
salm is a master selector gene for DT identity. Intriguingly, its expression levels in the DT tubes at stage 13/14 show a largely proportional decrease from posterior to anterior metameres matching the tapering of the airways (Fig. 4F)[30]. The graded diameter along the airway
length also coincides with the graded expression of BX-C proteins along the A-P axis. To explore the potential regulatory roles of BX-C factors and salm in tube shaping, we first analyzed airway shapes in BX-C mutants and detected 2 kinds of effects of BX-C genes on tube diameter, metamere-autonomous and systemic (see below).

Consistent with graded AbdB expression in PS 10–13, in AbdB mutants, tube diameter in DT7-10 lost its tapering and became narrower suggesting that the amount of AbdB proportionally controls the tube diameter. (Fig. 5A, C, I and S1 Table). Similarly, in abdA mutants, the gradient of tube diameter in DT4-6 was lost and DT4-9 became narrower (Fig. 5B, I and S1 Table) suggesting again that AbdA levels proportionally control DT tube diameter. In abdA AbdB double mutants the shape of the airways is changed further (Fig. 5D, I, J and S1 Table). The airways of Tr4-10 acquire a more cylindrical shape compared to the conically shaped tubes of wild type embryos. We suggest that in wild type embryos the gradient of abdA and AbdB activities would superimpose on a weak but clear, abdA and AbdB-independent gradient of DT tube thickness (Fig. 5I and S1 Table). This may explain why abdA mutants, where PS7-9 (DT4-6) are expected to transform to PS6 (DT3) still show a clear difference in tube diameter between DT3 and DT4 and why AbdB mutants, where PS10-13 (DT7-10) are expected to transform to PS9 (DT6) show a distinct tube caliber in DT6 and DT7 (Fig. 5I and S1 Table). Thus, in accord with their known functions in determining cell fates and morphogenesis in the embryonic ectoderm [39,44,53], the BX-C genes control the tapering of airways along the A-P axis autonomously. We note however that there is also a systemic effect of BX-C mutations along the entire DT. In either abdA, AbdB single or in abdA AbdB double mutants, the diameter of the more anterior metameres, where corresponding BX-C genes are not expressed also show a slight reduction of tube diameter (Fig. 5I and S1 Table). Among different possibilities, these results may suggest that the activities of abdA or AbdB control the hydrostatic pressure in the lumen to non-autonomously assure proportional growth of all the DT tubes [89] (see below). The residual tapering of abdA AbdB double mutants implies a mechanism of A-to-P gradient formation independent of abdA and AbdB. Ubx could exert such a function in the absence of abdA and AbdB. We analyzed bxd113 or bxd100 mutants, where Ubx expression levels in PS5 become similar to that of PS4 but its ectodermal expression is lost from PS7 onwards [90] (S4A Fig.). In these embryos, DT3 tube diameter approaches that of DT2 and there is also an overall reduction of tube diameter in more posterior metameres (Fig. 5I, S4C-E Fig. and S1 Table), suggesting that the endogenous Ubx level controls DT diametric tube expansion. However, in abdA AbdB double mutants, Ubx levels are largely uniform in DT4-10, which correspond to PS7-13 [91] (S5B Fig.). This suggests the presence of an additional, BX-C-independent cue in DT tube shaping. In Ubx abdA AbdB triple mutants, the transformed Salm positive residual DT/TC branches in posterior metameres are slightly thicker than those in anterior metameres, further arguing for the existence of the postulated BX-C-independent mechanism in tube shaping (S3K, S4F Figs.).

Is there any causative link between the BX-C mediated DT expansion control and the A-to-P gradual increase of salm expression levels in the DT (Fig. 4F) [30]? We noticed that Salm levels are reduced in central and posterior metameres of abdA, AbdB or abdA AbdB double mutants (S4G-J Fig.). This reduction is largely consistent with the changes in shape and DT tube diameters in the corresponding mutants. Conversely, upon overexpression of AbdB, higher Salm amounts are detected in the DT of all metameres at stage 13 (Fig. 4I). The DT branches of these embryos often stall and fail to fuse making evaluation of tube diameter difficult. Nevertheless, the DT diameter in all metameres appears comparable to the diameter of the most posterior DT branches (Fig. 5G). Overexpression of Ubx or abdA renders Salm expression levels uniform in the anterior metameres (Fig. 4G, H). Correspondingly, the DT diameter in the anterior metameres becomes thicker (Fig. 5E, F, I, J and S1 Table).
How does the Salm gradient along the DT A-P axis correlate to the graded DT tube expansion? salm overexpression confers DT identity to other primary branches [28,62]. We noted that salm overexpression also generally expands tube diameter not only in the transformed branches [28] but also in the DT, which endogenously expresses salm (Fig. 5H, I, J and S1 Table). The programmed secretion of luminal and apical proteins has been proposed to drive tube dilation of the Drosophila airways [92–95]. Tenectin (Tnc) is a luminal glycoprotein accumulating in the DT and hindgut tubes during diametric expansion [96]. Tnc overexpression in the airways drives DT tube dilation in a dose-dependent manner potentially through increasing hydrostatic pressure [89] and the tnc mRNA levels increase in a characteristic graded fashion along the A-P axis of the DT tubes in wild type embryos [89]. We found that the diametric increase caused by salm overexpression in the airways is accompanied by an increase in the luminal levels of Tnc and conversely, Tnc becomes undetectable in the tracheal tubes of salm mutants (S4K-M Fig.). This suggests that Salm adjusts the graded expression levels of Tnc and presumably other proteins during tube dilation.

Additionally, the diameter increase in the branches of salm over expressing embryos is still most pronounced in the posterior metameres. (Fig. 5H, I, J and S1 Table). The accentuated tube enlargement in posterior metameres upon salm overexpression suggests a salm-independent control mode of A-to-P gradient of DT tube diameter. This is consistent with the observation that tube diameter of the remaining branches in salm mutants are still thickest in Tr10 (S4N Fig.).

In conclusion, our work suggests two interdependent mechanisms by which the tube intrinsic Hox code controls branch identity and tube shape in the Drosophila respiratory network (Fig. 6A, B). Firstly, Hox genes autonomously divert extrinsic hh signaling from kni induction in the DT thereby generating continuous, salm positive DT airways. We suggest that this allows a second tier of DT tube shape regulation, where the Hox activity gradient both locally and systematically organizes graded tube dilation via salm dependent and independent modes. The Ci/BX-C circuit may control tube morphology directly by binding to the regulatory regions of kni and salm, as recent genome-wide TF binding studies suggest that kni is a direct target of ci [37] and that salm is a direct target of Ubx [97]. In our model, tube tapering and thereby luminal fluid flow are calibrated by the balance between extrinsic signals and the intrinsic Hox code. Since Hox selector genes are regionally expressed in other developing tubular organs like the mammalian lung [98] and vasculature [9,10], a similar regulatory logic of tube branching and shaping may apply to other systems.

Materials and Methods

Fly genetics

Flies kept over balancer chromosomes [99] were grown in standard medium. We obtained the appropriate genotypes by standard genetic crosses. For overexpression of genes, we used the Gal4/UAS system [100]. Mutant embryos were identified by the expression of twi-lacZ, ftz-

Figure 5. BX-C genes control DT tube tapering. (A–H) BX-C control of DT tube tapering visualized by luminal Gasp and the apical protein PTP10D. In the control (A), DT tube diameter shows posterior-to-anterior tapering. In abdA mutants, tube diameter of DT4-6 approaches that of DT3 while in AbdB mutants, tube diameter of DT7-10 resembles that of DT6. In abdA AbdB double mutants, tube diameter of DT4-10 resembles that of DT3. Note that there is still a weak tapering of the tube. Upon overexpression of Ubx (E) or abdA (F), tube diameter of DT2/3 becomes thicker than the control. We noticed that DT diameter in the posterior metameres becomes adversely reduced. This could be attributed to competition between the overexpressed proteins and endogenous abdA or AbdB [46], leading to a systemic decrease of tube diameter. Upon overexpression of AbdB (G), DT fusion becomes frequently defective. However, it is apparent that DT tube diameter of the anterior metameres resembles that of DT10. Upon overexpression of salm (H), DT tube diameter becomes thicker than the control. In addition, tube diameter of other branches also becomes thicker [28]. (I,J) Quantification (I) and cartoons (J) of diameter phenotypes of this figure and other figures are shown. Raw data used for quantification and some statistics are shown in S1 Table. See text for details. Scales bars: 50um.
lacZ, Ubx-lacZ or dfd-GFP [101] constructs inserted on balancer chromosomes. We identified mutants harboring Ubx or AbdB mutations by selecting embryos with previously reported phenotypes in the anterior spiracle or PSP. For collection of large amounts of virgins, we used a Y chromosome harboring hs-hid construct developed by R. Lehmann and M. Van Doren [102]. See Flybase [103] for details of strains described below.

**Mutant strains.** abdAM1 (a gift from B. Gebelein) [104], AbdBM1, AbdBM5 and Df(3R)P115 as Df(Ubx abdA AbdB) (gifts from I. Lohmann) [105], btlΔoh10 and btlΔoh24-1 [106], Df(5) as Df(salm/salr) (a gift from M. Llimargas) [28,107], hh13C [108], kni early rescue fragment; Df(3L)riXT (a gift from R. Schuh) [25], rho7M (a gift from J. Skeath) [111], top24 (a gift from K. Moses) [112], arm4 and CycAβ were obtained from National Institute of Genetics (NIG), Mishima, Japan. Ubx1, abdAΔM1, abdAΔD18, Ubx1 abdAΔD18 AbdBΔD18 [113], Df(109) as Df(Ubx,abdA), CycAΔLR1, Df(3L)Exel6115 as Df(CycA), hhΔAC, Df(3L)BSC448 homozygous or its transheterozygote over Df(3L)riXT as Df(kni/knrl), ptc9, Df(2R)Exel7098 as Df(ptc), smo9, tkv7 and Df(2R)Exel6076 as Df(top) were obtained from Bloomington stock center (BDSC), Indiana, USA.

**Enhancer trap strains.** 1-eve-1 as trh-lacZ (a gift from N. Perrimon) [114] and unp93 (a gift from F. J. Diaz-Benjumea, R. Urbach and G. M. Technau) [115,116]. hhP30 [117] was obtained from BDSC.

**Enhancer reporter strains.** kni-(dpp)-lacZ [25] and salm-TSE-lacZ [30] (gifts from R. Schuh).

**Gal4 and UAS strains.** btl-gal4 on 2nd and 3rd chromosomes (gifts from S. Hayashi) [58], UAS-abdA (a gift from F. J. Diaz-Benjumea) [115], UAS-c75 and UAS-cH3 (gifts from S. Ishii) [84], UAS-ci75 (a gift from A. Moore) [83], UAS-kni and UAS-knrl (gifts from R. Schuh) [25]. UAS-AbdB, UAS-Axn-GFP, UAS-salm and UAS-Ubx were obtained from BDSC.

Figure 6. Models for the BX-C dependent control of branching patterns and of DT tube diameter. (A) In all metameres, expression of hh and wg in cells surrounding the tracheal primordia are comparable. In metamere 1, where there is no input from BX-C, hh signaling induces kni in DTa1 in wild type, and additionally in DTP1 in ptc mutants. kni represses wg/WNT mediated upregulation of salm, making DTa1 branches thinner. In the posterior metameres, BX-C shunts hh signaling from induction of kni, allowing salm upregulation by wg/WNT. BX-C additionally enhances salm expression in the DT. (B) BX-C genes coordinate the posterior-to-anterior tube tapering through salm dependent and independent modes. DT tube tapering may also be regulated independently of BX-C genes through inputs from the maternal, gap or segmentation gene cascades.

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Egg collection was done with apple/grape juice plate at 25°C. Embryos were bleached and fixed as previously described [118] for 15–30 minutes with a 1:1 mixture of heptane and a fix solution (3.7% formaldehyde, 0.1M Hepes pH6.9, 2mM MgSO4). Embryos were dechorionated with methanol and incubated in 0.1% PBT supplemented with 0.5% BSA. Staging of embryos was done as previously described [119].

For immunostaining the following primary antibodies were used:

Guinea-pig anti-AbdA (1:500, a gift from B. Gebelein) [104], rabbit anti-Dys (1:500, a gift from L. Jiang) [18], Guinea-pig anti-Gasp (1:1000) [95], mouse anti-Ubx (1:10, a gift from R. White) [120], Guinea-pig anti-Kni (1:300), (developed by J. Reinitz and distributed by Y. Hiromi, East Asian Segmentation Antibody Center, Mishima, Japan) [121], rabbit anti-Salm (1:200, gifts from R. Barrio and T. Cook) [107,122], rabbit anti-Tnc C-terminal (1:1000, a gift from Z. A. Syed and A. Uv) [89], rat anti-Trh (1:200, a gift from D. Andrew) [123] and rabbit anti-Trh (1:50). Mouse anti-Abd-B (1:10, donated by S. Celniker) [124], mouse anti-Cut (1:10, donated by G. M. Rubin) [125], mouse anti-Hnt (1:10, donated by H. D. Lipshitz) [126], mouse mab2A12 (anti-Gasp) (1:5, donated by M. Krasnow, N. Patel and C. Goodman) [17,95], mouse anti-Ptc (1:10, donated by I. Guerrero) [127] and mouse anti-PTP10D (1:10, donated by K. Zinn) [128] were obtained from Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. Commercially available antibodies were anti-LacZ (E.coli β-Galactosidase) antibodies made in goat (1:500, Biogenesis) or rabbit (1:1000, Capel) and anti-GFP antibodies made in rabbit (1:500, JL-8 Clontech), mouse (1:1000, GFP20 Sigma) or goat (1:500, ab6673 Abcam).

Donkey or goat biotin- or fluorescently labeled secondary antibodies made against the host species of primary antibodies were purchased from Jackson Laboratories. Streptavidin coupled with AMCA, FITC or Cy5 were used when necessary. For mab2A12 detection TSA amplification (PerkinElmer) was used.

Double fluorescent labeling with RNA probe and antibody was carried out as described [129]. The following cDNA clones were used to make hybridization probes; bnl (a gift from M. Krasnow) [20], dpp [108] and unpg (a gift from P. A. Beachy) [54]. wg, salm and kni clones were obtained from Drosophila Genomics Resource Center (DGRC), Indiana, USA.

Confocal images were taken by Biorad MRC1024, Olympus Fluoview 1000 or Zeiss LSM780. Images of controls and mutants taken by the same confocal microscopes were used for comparison. Images were processed by ImageJ and figures were prepared with Photoshop and Illustrator.

Quantification of Trh positive cell number of CB was done for stage 13 embryos stained with antibodies against Trh and DE-cad, scanned at 40× magnification with 0.6 um intervals.

For quantification of DT tube thickness, mid-late stage 16 embryos stained with antibodies against PTP10D, Gasp and DE-cad were scanned at 40× magnification with 0.6 um intervals. Using ImageJ, 3 points next to the bases of DB were selected from each Z-stacked image for each metamere to measure the maximal distance of PTP10D positive apical membranes perpendicular to the longitudinal tube axis. 3 embryos for each genotype were measured. Average, SD and p-value of student t-test were calculated by Excel.

Supporting Information

S1 Fig. Expression or potential functions of dFGF/bnl, dEGFR, wg, dpp and hh for DTa1/CB formation. DTa1 is marked with arrowhead or asterisks in the different panels.

(A-D) bnl signaling.

(A, B) Expression of bnl.

(A) At late stage 11, corresponding to the 6 primary branches of the airway (marked with trh-lacZ), 6 patches of surrounding cells express bnl for Tr1.
At stage 12, DTa1 appears to extend toward bnl expression corresponding to the DB0 position.

Expression of kni RNA. Compared to the control (C), in btl mutants (D) at early stage 12, kni induction in DB is variably abolished while its DTa1 expression is comparable. Different magnification was used to image the same embryo in Fig. 1B and S1C Fig.

dEGFR signaling. In either rho mutant (E) or dEGFR mutant (F), Kni expression in DTa1/CB at stages 13–14 is comparable to the control (Fig. 1D).

wg RNA distribution (G) relative to DT or dpp RNA expression (H) in the dorsal and the lateral ectodermal stripes at stages 11/12 are comparable in all metameres. In arm mutants at stage 13, expression of Kni as well as kni-(dpp)-LacZ in DB/LT/GB are comparable to the control (Fig. 1D). In tkv mutants at stage 13, kni-(dpp)-LacZ expression in DB/LT/GB is variably abolished while Kni expression in DTa1/CB is comparable to the control (Fig. 1D).

hh signaling. In hh mutants at stage 12, bnl expression corresponding to DB extension is significantly decreased (cross). The ectodermal patches of bnl expression corresponding to DT extension become continuous.

Gasp positive airway branching in hh or smo mutants. hh mutants have variable defects including loss or stalling of branches (DB, DT, VB or CB) or loss of metameres. CB does not form dorsal extension. smo mutants often show CB misrouting phenotypes.

Scales bars: 50um.

S2 Fig. Effects of hh signaling overactivation on airway branching. (A-D) Effects of ptc or CycA mutations on expression of kni or salm. A summary of ptc mutant phenotypes is shown in (C).

(A, B) In ptc/Df(ptc) mutants, expression of salm-TSE-lacZ (A) and kni-(dpp)-lacZ (B) is lost in the dorsal part of the metamere 1 (asterisks) while Kni protein is expressed in the whole distal trachea in Tr1 (B, bottom panel).

(D) In CycA mutants, where the cell number in the airways is reduced nearly to half [38], DBs in all metameres express kni-(dpp)-lacZ comparably (arrows) and CB formation can occur (arrowheads).

(E-J) Stage 14–15 embryos stained with Gasp and Dys. In the control (E), the fusion point of DT1/2 is occupied by two fusion cells originating from DTp1 and DTa2 (arrow). In ptc mutants (F, G), dys expression is not detected in the positions of DT1p (and LT1p) (asterisks) while CycA mutant embryos (H) are comparable to the control (arrow). btl-Gal4 driven Ci<sup> Caps </sup>(I) restores Dys positive fusion cells in DTp1 as well as fusion of DT1/2 (arrow). Overexpression of Ci<sup> act </sup> with btl-Gal4 (J) can mimic the phenotypes of ptc mutants. Dys expression in DTp1 position is lost and is accompanied by fusion defects of DT1/2 (asterisk).
S3 Fig. Expression and functions of BX-C Hox genes in relation to airway development.

(A-D) Expression of BX-C genes at stage 13 visualized by the respective antibodies. A summary of the expression patterns is shown in (D).

(A) *Ubx* expression starts from metamere 2 and peaks at metamere 3. From metamere 3 to 10, *Ubx* expression forms a gradient.

(B) *abdA* expression starts from metamere 4 and peaks at metamere 6. From metamere 7 to 10, *abdA* expression forms a gradient.

Only the anterior half of metamere 10 expresses *Ubx* and *abdA*.

(C) *AbdB* expression starts in metamere 7 and peaks at metamere 10, forming a single gradient.

(E-K) Airway branching phenotypes of BX-C mutants assessed at stage 15 with expression of Gasp, Dys and Cut.

In the control (E), DT is continuous with a pair of Dys positive fusion cells at each fusion point (arrowhead for DT1/2). The terminal parts of the airway are plugged with Cut positive ASP and PSP (arrows) [125].

In *Ubx* mutants (E), metameres 2/3 are transformed to metamere 1 as judged by the loss of fusion cells at the position of DTa (asterisks) and gain of Cut positive ASP (marked with +).

While *abdA* mutants (F) are largely normal, both *AbdB* single mutants (H) and *abdA AbdB* double mutants (I) have an ectopic fusion cell in DTp10 (asterisks), in addition to loss of Cut positive PSP (crosses).

In *Df(Ubx abdA)* mutants (J), in addition to metameres 2/3, metameres 4–6 are transformed to Tr1 while transformation is variable in Tr7-9. Namely, Dys-positive fusion cells at the position of DTa are lost in Tr2-8 while Tr 9 has 1–2 fusion cell. Fusion of DT9/10 is frequently observed. Cut positive ectopic ASPs are detected in Tr2-9 (marked by +). DTp diameter in Tr7-10 looks thicker compared to the more anterior metameres.

In *Ubx abdA AbdB* triple mutants (K), all metameres are transformed to Tr1. Cut-positive PSP is lost (cross). Instead, Cut positive ASP (+) as well as loss of fusion cell fates at the position of DTa (asterisks) is observed in all metameres. Note that in *Ubx, Df(Ubx abdA)* and *Ubx abdA AbdB* mutants, CB-like branches are variably detected in the transformed metameres [54].

(L, M) Effects of *hh* or *kni/knrl* mutations on DT of *Df(Ubx abdA)* mutants.

In *hh Df(Ubx abd)* mutants (L) or in *Df(kni/knrl) Df(Ubx abdA)* mutants, DT fusion is partially restored (arrows). Note that this rescue is not due to a fusion of the airway primordia because many of the ectopic Cut positive ASPs at the proximal part of the airway are separate.

(N-Q) Effects of overexpression of *Ubx* or *abdA* on *ptc* mutant phenotypes in Tr1.

Compared to *ptc* mutants (S2 Fig.), ectopically supplied *Ubx* (N) or *abdA* (O) in the airway cells significantly restores both fusion cell fates in DTa1 and subsequent fusion of DT1/2 (arrowheads). Similarly, *abdA* overexpression in *ptc* mutants restores expression of *kni-(dpp)-lacZ* (P) and *salm-TSE-lacZ* (Q) to metamere 1.

(R-T) Effects of overexpression of *Ubx* or *abdA* to the airway branching of *Ubx abdA* double mutants.

In *Df(Ubx abdA)/Df(Ubx abdA AbdB)* transheterozygote (R), Cut positive ASPs are detected in metameres 1–9. And frequent DT fusion occurs only in DT9/10. The airway specific overexpression of *Ubx* (S) or *abdA* (T) partially restores DT fusion (arrows). Note that *bnl* expression guiding DT branches are intact in the absence of BX-C complex [130].

Scales bars: 50um.

(TIF)
S4 Fig. *salm* dependent and independent airway tube tapering by BX-C and a potential *salm* target for tube tapering. (A-C, F) DT tube tapering visualized by Gasp and PTP10D. Compared to the control (A) where DT3 is slightly thicker than DT2, in *bxd*<sup>113</sup> (B) or *bxd*<sup>100</sup> (C) mutants, DT3 becomes comparable or slightly narrower than DT2. In BX-C triple mutants (F), the posterior metameres tend to show slightly thicker DT tubes (arrows).

(D, E) Ubx expression in BX-C mutants a stage 13/14. In *bxd*<sup>113</sup> mutants (D), Ubx expression in DT3 becomes comparable to the level in DT2 while in *abda AbdB* double mutants, Ubx expression in DT4-10 becomes comparable to the level in DT3.

(G-J) Effects of *abda* and/or *AbdB* mutations on Salm expression in DT. In the control (G), Salm expression level forms a posterior-to-anterior gradient. In *abda* mutants (H), Salm expression in the central metameres becomes comparable to the more anterior metameres while in *AbdB* mutants (I) Salm expression in the posterior metameres becomes comparable to the central metameres. In *abda AbdB* double mutants (J), the Salm gradient becomes more flat, but a weak gradient is still detected.

(K-M) Effects of *salm* on Tnc expression in DT. In the control (K) at stage 16, Tnc is secreted into the lumen but is locally detected in DT and TC1, both of which express *salm*. Expression of both Tnc and Salm appears slightly stronger in fusion points. Note that Tnc is also expressed in hindgut and the proximal part of PSP. Upon overexpression of *salm* (L) Tnc is detected in lumens of additional branches like TC (arrow). In Df(*salm/salr*) mutants where both *salm* and the neighboring *spalt-related (salr)* are deleted, Tnc expression in the airway is lost (M, asterisk), although its expression in PSP and hindgut remains.

(N) In Df(*salm/salr*) mutants, TC branches in the posterior metameres are thicker than in the anterior. Scales bars: 50um.

(TIF)

**S1 Table. DT thickness of various tube diameter mutants.** Genotypes are; Wild type = Canton S, *bxd* = *bxd*<sup>113</sup>, *abda* = *abda*<sup>MX</sup>, *AbdB* = *AbdB*<sup>M1</sup> / *AbdB*<sup>M5</sup>, *abda AbdB* = *abda*<sup>D24</sup> *AbdB*<sup>D18</sup>. Table A. Raw data of DT thickness in micrometers. For each genotype, 3 embryos were measured for DT thickness. 3 points of each DT near the base of DB were measured.

Table B. Statistics of the raw data. Average thickness, standard deviation (SD) and p-value of student t-test are shown for each DT. For p-value, comparison between WT and each mutant is shown. For wt, *bxd*, *abda*, *AbdB* single or *abda AbdB* double mutants, additional comparison is shown between different metameres or between mutants. p<0.05 is judged to be significant.

(XLSX)

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Author Contributions
Conceived and designed the experiments: RM. Performed the experiments: RM CH. Analyzed the data: RM. Contributed reagents/materials/analysis tools: KS CS. Wrote the paper: RM CS. Supported RM to drive the project: CS. Collected data for quantification: CH. Collected data for the other experiments: RM. Drafted the manuscript: RM. Contributed to finalize writing: CH KS.

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