

Reviewed Preprint

v1 • May 5, 2026

Not revised

✉ For correspondence:

ryo.matsuda@su.sechristos.samakovlis@su.se

† Deceased

Competing interests: No competing interests declared**Funding:** See [page 17](#)**Reviewing editor:** Sofia J Araújo, Universitat de Barcelona, Spain

© 2026, Matsuda et al. This article is distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

Functional definition of the *Drosophila* airway progenitor field through overlapping compensatory regulators

Ryo Matsuda¹ ✉, Chie Hosono¹, Kaoru Saigo^{2,†}, Christos Samakovlis^{1,3} ✉¹ScilifeLab and Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden • ²Department of Biophysics and Biochemistry, School of Science, the University of Tokyo, Tokyo, Japan •³Cardiopulmonary Institute (CPI), Member of the German Center for Lung Research (DZL), Justus Liebig University, Giessen, Germany

eLife Assessment

In this **important** manuscript, Matsuda and colleagues present a model describing the regulation of tracheal tubulogenesis in *Drosophila melanogaster* embryos. The authors support this model using **convincing** approaches that combine novel experimental results with previously published work from their group. While some conclusions are consistent with earlier studies, the present manuscript introduces distinct molecular markers not previously reported, which reinforce the authors' prior findings. In addition, the manuscript analyses, using experimental strategies, the requirement of the Dpp and EGFR signalling pathways for the maintenance of trachealess (*trh*), one of the key transcription factors governing tracheal development.

<https://doi.org/10.7554/eLife.111290.1.sa2>

Abstract

Tubular organs present a common solution to fluid transport in multicellular organisms. They often arise by an initial bulging of flat epithelial progenitor cells, which then undergo branching morphogenesis. Here, we present 3 cooperative programs fully defining the *Drosophila* airway progenitor field and their roles in early morphogenesis linking the radial pattern of the 2-dimensional (2D) field to the proximo-distally patterning of the 3D tubes. We previously showed that extrinsic Hedgehog (Hh) and intrinsic POU-Homeobox TF Ventral-veinless (Vvl)/Drifter/U-turn dominantly drive the transcriptional program toward the distal airway cell identity at the expense of a proximal program specified by the GATA TF *grain* (*grn*). Both programs require the basic-HLH-POU TF *trachealess* (*trh*) (Matsuda et. al, 2015). Whereas *trh* is not essential for primordia invagination, we show that in *hh vvl* double mutants, the oval-shaped primordia frequently remain at the 2D plane, retaining *trh* expression in a *grn* dependent manner. Therefore, *hh* and *vvl* are the principal regulators of progenitor invagination independent of *trh*. Each of the 3 regulators, *Trh*, *Vvl* and *Grn* fulfills only complementary or compensatory functions in transcription and morphogenesis but their combinations functionally define the airway progenitor field. We further provide a comprehensive description for allocating the airway progenitors on the body coordinates, involving dorsal Decapentaplegic/BMP signaling along the dorso-ventral axis and subsequent radial EGFR signaling along the proximo-distal axis. The presence of 3 complementary, regulatory programs in early gene expression and morphogenesis of the simple *Drosophila* airways may reflect the vital needs for respiration, and their influence on the evolution of various strategies in tubular organ development.

Introduction

The reiterative tube formation and its ramification in our vasculatures, airways and lungs generate the pulmonary-vascular network to efficiently supply oxygen to the whole body. The airway tubes allow the airflow to alveolar structures, where blood cells inside the fine vascular tubes exchange carbon dioxide with oxygen to deliver it to the body (Herriges and Morrissey 2014 [↗](#); Potente and Makinen 2017 [↗](#); Kishimoto and Morimoto 2021 [↗](#)). Surprisingly, a similar pulmonary-blood cell connection is also found in the fruit fly *Drosophila*. There, reiterative ramification of the epithelial tubes (the tracheal system) allows the airflow inside the body that directly delivers oxygen to the target cells (Manning and Krasnow 1993 [↗](#); Samakovlis et al. 1996a [↗](#); Hu and Castelli-Gair 1999 [↗](#); Ghabrial et al. 2003 [↗](#)), while the blood cells in the open circulatory system transiently attach to the fine airways to boost oxygen transport (Shin et al. 2024 [↗](#)).

The lungs and the *Drosophila* airways both derive from 2D-primordial cell fields (Romero et al. 2025 [↗](#)). Lung formation initiates as tube evaginations from the foregut epithelium (Herriges and Morrissey 2014 [↗](#); Kishimoto and Morimoto 2021 [↗](#)). Distal ramification of these buds and further differentiation into distal airways and alveoli involve FGFR activation by extrinsic FGFs (Min et al. 1998 [↗](#); Weinstein et al. 1998 [↗](#); Arman et al. 1999 [↗](#); Sekine et al. 1999 [↗](#); Brownfield et al. 2022 [↗](#); Jones et al. 2022 [↗](#); Sountoulidis et al. 2023 [↗](#)). Similarly, *Drosophila* airway formation initiates from cell primordia specified on the ectodermal plane, each invaginating to form a primitive cavity (Perrimon et al. 1991 [↗](#); Manning and Krasnow 1993 [↗](#); Samakovlis et al. 1996a [↗](#); Hu and Castelli-Gair 1999 [↗](#)). Extrinsic FGF/Branchless (Bnl) activates FGFR/Breathless (Btl) to guide branch ramification and network connection (Klambt et al. 1992 [↗](#); Guillemin et al. 1996 [↗](#); Samakovlis et al. 1996a [↗](#); Samakovlis et al. 1996b [↗](#); Sutherland et al. 1996 [↗](#)) (Figure 1A [↗](#)). Accordingly, highly ramified fine tubes are generated around each branch terminus that serve for oxygen transport.

trh is the earliest TF gene marking the *Drosophila* airway primordia and the mature airways (Isaac and Andrew 1996 [↗](#); Wilk et al. 1996 [↗](#)). *trh* has been regarded as the master TF of the *Drosophila* airways because tubes as well as differentiation markers are not detected in *trh* mutants at later stages (Isaac and Andrew 1996 [↗](#); Wilk et al. 1996 [↗](#); Brodu and Casanova 2006 [↗](#); Sotillos et al. 2010 [↗](#); Chung et al. 2011 [↗](#); Matsuda et al. 2015b [↗](#)). However, in *trh* null mutants, expression of *rhomboid* (*rho*) (Bier et al. 1990 [↗](#)), a protease activating the EGFR ligand Spitz (Schweitzer et al. 1995 [↗](#)) initiates in the primordia (Ogura et al. 2018 [↗](#)) but its propagation (Matsuda et al. 2015b [↗](#)) fails (Ogura et al. 2018 [↗](#)). Consistent with the role of *rho* in promoting primordial invagination (Brodu and Casanova 2006 [↗](#); Nishimura et al. 2007 [↗](#)), *trh* mutants initiate primordia invagination but neither sustained invagination nor maintained tube structures (Kondo and Hayashi 2019 [↗](#)). The initiation of invagination and the early localized *rho* expression in *trh* mutants suggest additional regulators, other than *trh* that are critically responsible for gene expression in airway primordia and for tubulogenesis.

We had previously shown that the proximo-distal axis of the *Drosophila* airway tubes is generated from the centro-peripheral patterning of the 2D primordia (Matsuda et al. 2015b [↗](#)). One set of genes, the distalizing factors, *hh*, *vvl*, *rho* and *btl/FGFR* cooperate to realize the distal gene expression program at the expense of the proximal one, whereas another set, the proximal factors including *grn*, negative regulators of *EGFR* signaling and *hh* signaling realize the proximal gene expression program (Figure 1A [↗](#)). The two programs establish distinct proximal and distal cellular domains in the branching network (Matsuda et al. 2015b [↗](#)). Two of the distalizing factors, *rho* and *btl/FGFR* promote primordia invagination but are not essential for tubulogenesis (Brodu and Casanova 2006 [↗](#); Matsuda et al. 2015b [↗](#)), indicating that essential regulators of primordial morphogenesis have been elusive.

Here, we report that in the absence of the remaining two distalizing factors *hh* and *vvl*, the trachea primordia cells frequently fail invagination and are only detected on the 2D plane. Therefore, extrinsic Hh and intrinsic Vvl represent the missing factors that cooperatively promote primordia invagination. Although *trh*, *vvl* or *grn* alone cannot define the airway progenitors, we propose that

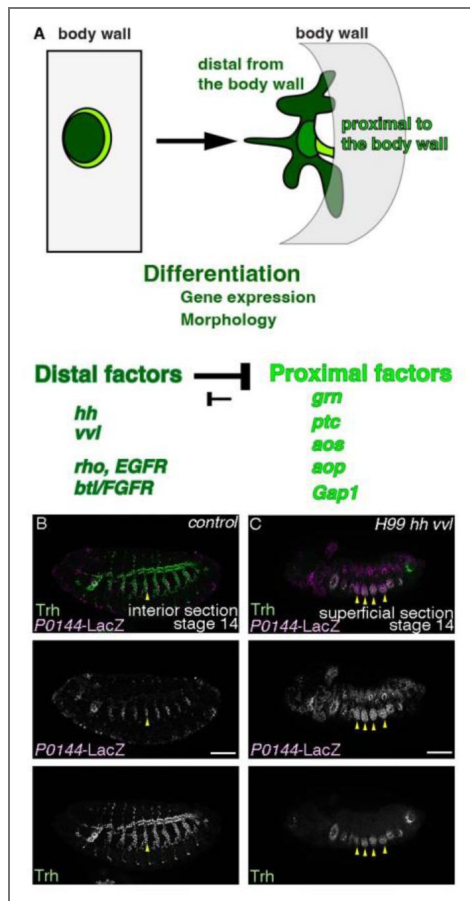


Figure 1. The distalizing factors *hh* and *vvl* dominantly promote invagination of the airway progenitors.

(A) A schema showing that invagination transforms the centro-peripheral patterning of the primordium into the proximo-distal patterning of the tubes. Extrinsic Hh and intrinsic Vvl dominantly promote the distal gene expression program (dark green domain) whereas *rho* and *bt/FGFR* are the distal factors that are known to distalize differentiation along both gene expression and morphology. The proximal factors including *grn* promote the proximal gene expression program (light green domain). (B-C) Single sections of lateral views of stage 14 embryos stained with Trh (a pan airway marker) and *P0144-LacZ* (a proximal marker). In the control (B), both the distal as well as the proximal airway progenitors (yellow arrowheads) invaginate to form a tube network whereas in *H99 hh vvl* mutants (*H99 hh^{13C} vvl^{utH599} P0144* homozygotes) (C), Trh⁺ cells express the proximal marker *P0144-LacZ* and many of them stay at the epidermal layer (yellow arrowheads). Scale bar is 50 μm

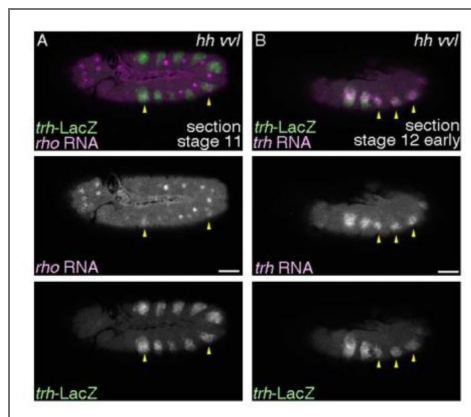


Figure 1 figure supplement 1. Expression of *rho* and *trh* in *hh vvl* double mutants, *hh^{AC} vvl^{utH599}/hh^{13C} Df(vvl)* transheterozygotes.

(A) Weak and sporadic expression of *rho* is detected at early stage 11 in *hh vvl* double mutants (yellow arrowheads). (B) The airway progenitors frequently fail invagination in *hh vvl* double mutants as well and those cells retain *trh* expression at stage 12 (yellow arrowheads). Scale bar is 50 μ m

combinations of the three TFs can intrinsically define the airway progenitors, considering their subsequent roles on gene expression and morphogenesis. Even in the simple *Drosophila* airways, combinations of multiple factors induce the organ progenitor field and subsequent tubulogenesis to cope with physiological stress of respiration in a terrestrial environment.

Results and Discussion

The distalizing factors *hh* and *vvl* drive airway primordia invagination independent of *trh*

Among the distalizing factors, *hh*, *vvl*, *rho* and *btl/FGFR* (Figure 1A [↗](#)), *hh vvl* double mutants show more extensive distal-to-proximal gene expression conversion than the loss of both *rho* and *btl/FGF-btl/FGFR* signaling (Matsuda et al. 2015b [↗](#)). As *EGFR* (also known as *torpedo/top* or *faint little ball*) and *btl/FGFR* signaling also orchestrate the distal morphogenetic processes, including primordial cell invagination (Llimargas and Casanova 1999 [↗](#); Brodu and Casanova 2006 [↗](#); Nishimura et al. 2007 [↗](#); Kondo and Hayashi 2013 [↗](#)) and subsequent branching (Glazer and Shilo 1991 [↗](#); Klambt et al. 1992 [↗](#); Sutherland et al. 1996 [↗](#); Llimargas and Casanova 1999 [↗](#); Matsuda et al. 2015b [↗](#)), *hh* and *vvl* are expected to control the distal morphogenetic program as well. In the absence of *hh* and *vvl*, *rho* expression is detected at early stages sporadically and weakly (Figure 1-figure supplement 1A [↗](#)) and *btl* expression initiates in the primordia, but both fade away shortly thereafter (Matsuda et al. 2015b [↗](#)). Despite the initial expression of *rho* and *btl/FGFR* in *hh vvl* mutants, *hh vvl* embryos show severer branching phenotypes than the *rho btl* mutants (Matsuda et al. 2015b [↗](#)).

We thus investigated potential invagination defects of *trh* positive cells in *hh vvl* double mutants and detected aberrant expression of both *trh* RNA and *trh*-LacZ persisting on the epidermal plane at stage 12 (Figure 1-figure supplement 1B [↗](#)). However, massive ectodermal apoptosis precluded analysis at later stages. In *H99 hh vvl* triple mutants, where apoptosis is suppressed by deletion of major pro-apoptotic genes (White et al. 1994 [↗](#)), we often detected failure in invagination, where all the *Trh*⁺ cells remained in a 2D plane at stages 12-13 (Figure 1A-B [↗](#)). By stage 15, small cavities are detected in most metameres of *H99 hh vvl* mutants, suggesting that proximal cell types invaginate by a mechanism independent of distal cell types differentiation. Taken together, the two distalizing factors *hh* and *vvl* define the central/distal cell identity, controlling both gene expression and cell invagination. It follows that 3 intrinsic TFs, *Trh*, *Vvl* and *Grn* cover the major functional aspects of airway progenitor differentiation.

dpp/BMP specifies the airway progenitors along the D-V axis

We further investigated upstream determinants of the primordial field defined by the expression of these 3 TFs. We first allocated their expression domains along the D-V axis of the embryo. The surface of the embryonic trunk at mid embryogenesis is largely divided into 3 sectors along the D-V axis, amnioserosa, the dorsal and the ventral ectoderm (Figure 2A [↗](#)) (Wharton et al. 1993 [↗](#); von Ohlen and Doe 2000 [↗](#)). Both the dorsal and the ventral ectoderm are further subdivided into 3 parts along the D-V axis, medial, intermediate and lateral (Figure 2A [↗](#)) (von Ohlen and Doe 2000 [↗](#)). The dorsal intermediate column marked with *caupolican* (*caup*)/*araucan* (*ara*) spans several cells located dorsally to the *trh*-LacZ positive spiracular branches (Figure 2-figure supplement 1A [↗](#)), whereas the *Grn*-GFP expression domain, which also marks the most proximal spiracular branches extends ventrally from the dorso-lateral ectoderm (Figure 2-figure supplement 1B [↗](#)). On the other hand, the ventral limits of the initial *trh* expression at stage 10 about *Dichaete*-LacZ (*D*-LacZ) expressing cells in the intermediate and medial columns of the ventral ectoderm (Figure 2B-C [↗](#)) (Zhao and Skeath 2002 [↗](#)). Thus, the initial *trh* expression straddles from the ventro-lateral column to halfway to the dorso-lateral column.

Compared to *trh* expression, *vvl* expression in the airway primordia is more restricted (Figure 2D [↗](#)). *vvl* expression straddles the boundary of the dorsal and the ventral ectoderm demarcated by *Drop* (*Dr*)/*muscle homeobox* (*msh*) whereas the ventral limit of *vvl* expression is far from the

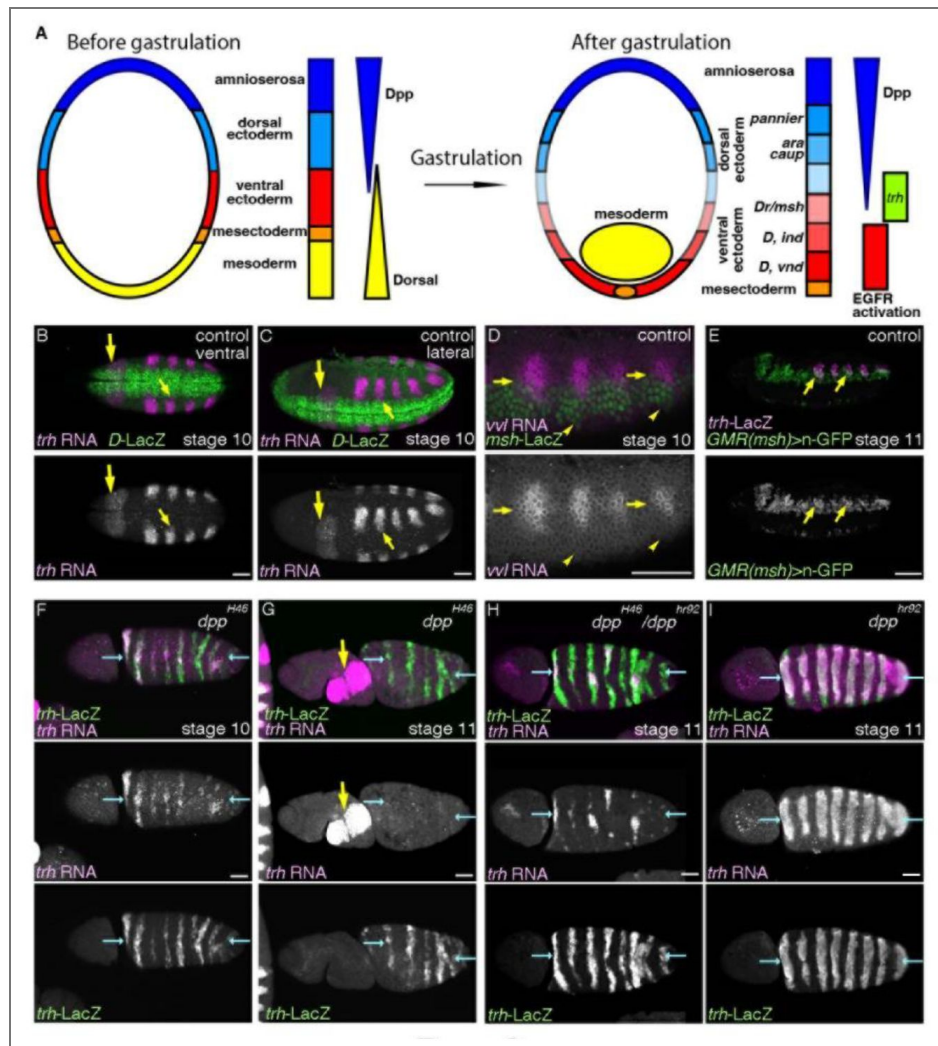


Figure 2. Medium Dpp/BMP activity promotes *trh* expression and the airway progenitors.

(A) A schema showing that the embryonic trunk is divided into discrete domains along the D-V axis. Out of the 5 domains (amnioserosa, dorsal ectoderm, ventral ectoderm, mesectoderm and mesoderm), mesoderm invaginates during gastrulation. Graded activities of a TF *dorsal* orchestrates the ventral domains whereas Dpp/BMP signaling orchestrates the dorsal domains. Differential expression of TFs subdivides both the dorsal and the ventral ectoderm into medial, intermediate and lateral columns. EGFR is dynamically activated to establish the ventro-intermediate column (Yagi et al. 1998). Expression of TFs marks the column of dorso-medial (*pannier*), dorso-intermediate (*araucan/ara*, *caupolican/caup*), ventro-lateral (*Dr/msh*), ventro-intermediate (*Dicheaete/D*, *intermediate neuroblasts defective/ind*), ventro-medial (*D*, *ventral nervous system defective/vnd*). (B-E) Distribution of *trh* and *vvl* transcripts relative to the ectodermal subdivision along the D-V axis. (B-C) Ventral and lateral views stage 10 embryos. The ventral limit of *trh* expression abuts the dorsal limit of *D-LacZ* expression (small yellow arrows) which marks the ventro-medial and the ventro-intermediate columns. Large arrows show *trh* expression in the salivary gland primordia. (D-E) *vvl* expression at stage 10 straddles the border between the dorsal ectoderm and the *Dr/msh-LacZ* positive ventro-lateral ectoderm (D, yellow arrows) whereas the ventral limit of *vvl* expression is some cells away from the ventral limit of *Dr/msh-LacZ* expression (D, yellow arrowheads). An enhancer fragment of *Dr/msh* active in the ventro-lateral column marks the ventral parts of the airway tubes at stage 11 (E, yellow arrows). (F-I) Expression of *trh-LacZ* and *trh* transcripts in allelic series of *dpp* mutants. Dorsal views of embryos where the presumptive dorsal midline are marked with blue arrows. In *dpp* null mutants (F, G), *trh* expression detected with *trh-LacZ* or *trh* RNA expands to the dorsal midline (blue arrows). However at later stages, *trh* RNA is not maintained though *trh-LacZ* positive cells remain (G). Note that in *dpp* null mutants the body is twisted. In the milder condition (H), *trh* RNA is sporadically maintained near the dorsal midline whereas in *dpp* hypomorph homozygotes (I), *trh* RNA is detected in many of the expanded progenitor areas. Scale bar is 50 μ m

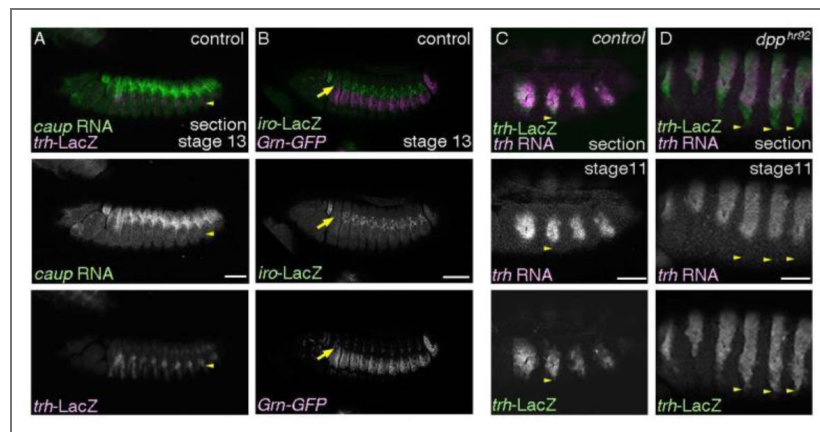


Figure 2 figure supplement 1. The proximal airways and the ectodermal subdivision.

(A-B) Lateral views of stage 13 embryos. The spiracular branch cells (*trh-LacZ*, yellow arrowheads) are several cells ventrally to the *caup* RNA expressing cells (A). Grn-GFP expresses not only in the spiracular branches but also in the dorso-lateral ectodermal cells that reside ventrally to the *iroquois-LacZ* (*iro-LacZ*)/*araucan-LacZ* positive dorso-intermediate column (B, yellow arrows). (C-D) Lateral views of stage 11 embryos. *trh-LacZ* positive cells that lose *trh* RNA are pronounced in the ventral parts of the control (C). This becomes more pronounced in the *dpp* hypomorphs (D). Yellow arrowheads mark the ventral limits of *trh-LacZ* expression. A, C and D are single sections. Scale bar is 50 μ m

ventro-intermediate column (Figure 2D) (von Ohlen and Doe 2000). Consistently, the ventro-lateral ectoderm enhancer of *Dr/msh* (Pfeiffer et al. 2008; Manning et al. 2012) marks the ventral parts of the invaginated airway progenitors (Figure 2E).

Medium level of Dpp/BMP signaling positively regulates the expression of *vvl* and *grn* (Matsuda et al. 2015b) but its function of *trh* regulation is only partially investigated. In the absence of Dpp/BMP, the dorsal 2 sectors, amnioserosa and the dorsal ectoderm take the cell differentiation program of the ventro-lateral ectoderm (von Ohlen and Doe 2000). Consistent with that, the initial *trh* expression straddles the ventro-lateral ectoderm (Figure 2B-C), *trh* expression expands to the dorsal midline in *dpp* null mutants (Figure 2F) (Isaac and Andrew 1996). However, *trh* expression is extinguished by stage 12 in *dpp* null mutants (Figure 2G), consistent with the Dpp/BMP's role on expression of *vvl* and *grn*, which in turn maintain *trh* expression (Matsuda et al. 2015b). In milder inactivation conditions of *dpp/BMP* hypomorphic mutants (*dpp^{H46}/dpp^{hr92}* trans-heterozygotes) (Wharton et al. 1993), *trh* maintenance, if any, occurs only around the dorsal midline, where reduced Dpp/BMP activity levels are presumed to be sporadically present (Figure 2H). In even milder conditions of *dpp/BMP* inactivation in *dpp^{hr92}* hypomorphs (Wharton et al. 1993), *trh* maintenance occurs in the more ventral cells as well (Figure 2I). However, we note that concomitant with reduction of Dpp/BMP activities, more cells in the ventral part of the initial *trh* expression domain fail to maintain *trh* expression (Figure 2F-I, Figure 2-figure supplement 1C-D). Loss of the ventral *trh* expression appears to occur also in wild type embryos (Figure 2-figure supplement 1C), possibly reflecting that the most ventral cells are farthest from the Dpp/BMP source.

The distal and the proximal progenitors are specified in *CycA* mutants (Matsuda et al. 2015b), where the last progenitor mitosis does not occur (Beitel and Krasnow 2000). As the distal cells are double positive for *trh* and *vvl* and occupy 90% of the mature airways, we speculate that *Trh*⁺ cells that are in and near the *Vvl*⁺ areas are the airway progenitors whereas the remaining *Trh*⁺ cells would become epidermal. These airway progenitors are specified straddling the canonical dorsal-ventral ectoderm boundary. We conclude that an optimal Dpp/BMP activity specifies the airway progenitors along the D-V axis.

The radial EGFR signaling primes the airway progenitors and realizes airway differentiation along the P-D axis

Airway cells expressing late differentiation markers are reduced in number in *H99 EGFR btl/FGFR* mutants, where anti-apoptotic functions of *btl/FGFR* and *EGFR* are compensated by *H99* deficiency (Matsuda et al. 2015b). These missing cells prompted us to investigate the potential roles of the two RTKs on *trh* expression earlier.

trh expression is comparable in the distal and the proximal cells at stage 12 in *H99 btl/FGFR* mutants (Figure 3A). In contrast, *trh* expression in the proximal area becomes reduced or extinguished at stage 12 in *H99 EGFR* mutants (Figure 3B). Moreover, *trh* expression in the main airways is very much reduced in *H99 EGFR btl/FGFR* mutants, leaving only residual expression in a subset of the invaginated cells (Figure 3C). Thus, EGFR signaling is the predominant factor promoting maintenance of *trh* expression. In its absence, this function can be compensated by *Btl/FGFR* signaling (Figure 3D).

In contrast to a model, where maintenance of *trh* expression correlates with the transition of primordial cells from a planar 2D into a 3D tubular tissue architecture (Kondo and Hayashi 2019), we show that *trh* expression is maintained in the cells on the 2D ectodermal plane in the *hh vvl* double mutants (Figure 1C, Figure 1-figure supplement 1B). Additionally, *trh* expression is very much reduced in the invaginated cells of *H99 EGFR btl/FGFR* mutants (Figure 3C), uncoupling *trh* gene expression from the morphogenetic process of invagination.

To further test if tissue architecture contributes to the maintenance of *trh* expression, we made use of mutations in *wingless (wg)/WNT*, which is expressed in longitudinal stripes and represses expression of *trh* and *vvl* along the anterior-posterior (A-P) axis (Figure 3E-H) (de Celis et al. 1995; Wilk et al. 1996). In *wg* mutants, some of the progenitors fail to internalize so that cell

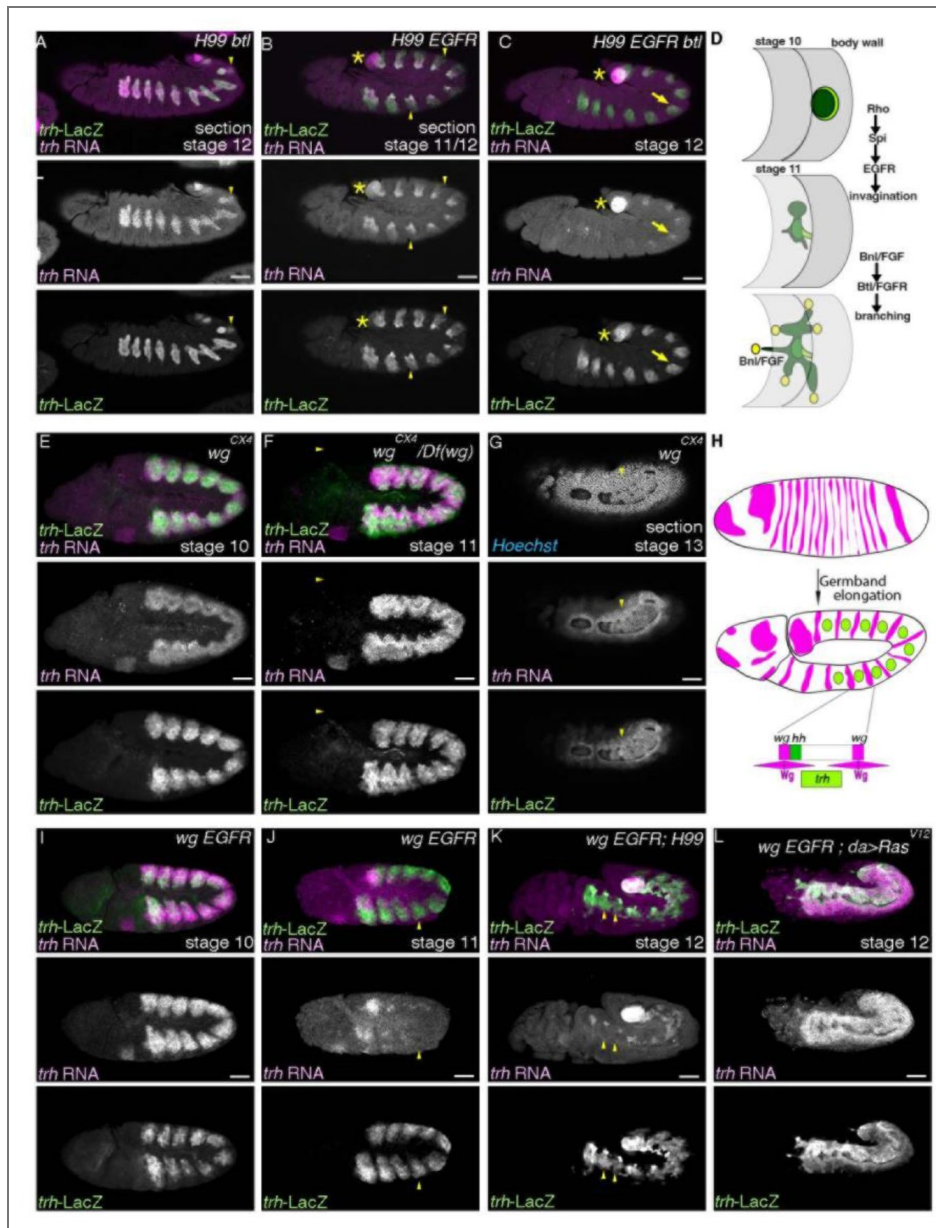


Figure 3. Maintenance of *trh* expression does not follow changes in tissue architecture Lateral views of embryos stained with *trh-LacZ* and *trh* transcripts.

(A-D) In *H99 bt1/FGFR* mutants (*H99 bt1^{Δoh10}/H99 bt1^{Δoh24-1}*) (A), *trh* expression is detected both in the distal and the proximal regions whereas in *H99 EGFR* mutants (*top^{f2}/top^{f24}; H99*) (B), *trh* expression in the proximal regions is significantly reduced (yellow arrowheads). In *H99 EGFR bt1/FGFR* mutants (*top^{f2}/top^{f24}; H99 bt1^{Δoh10}/H99 bt1^{Δoh24-1}*) (C), residual *trh* expression is detected in parts of the invaginated cells (yellow arrows). Asterisks mark *trh* expression in the posterior spiracle primordia. A schema in D shows stages and functions of RTK activation in the airway progenitors. (E-L) In the absence of Wg/WNT (E-G), which is expressed in stripes along the A-P axis (H), the airway progenitor areas expand along the A-P axis. *trh-LacZ* and *trh* RNA are largely co-expressed since before invagination. Arrowheads in G show that *trh* is expressed in cells that take the 2D planar configuration. In *EGFR wg* double mutants (*top^{f24} wg^{CX4}* homozygotes) (I, J), maintenance of *trh* RNA becomes defective at around the stage of invagination (compare I and J). *trh* maintenance is restored not by suppression of apoptosis (*top^{f24} wg^{CX4}; H99*) (K) but by *daughterless* (*da*)-Gal4 driven overexpression of Ras^{V12} (*top^{f24} wg^{CX4}; da-Gal4/UAS-Ras^{V12}*) (L). Note that *trh* RNA is not detected in cells positive for *trh-LacZ* in J and K (yellow arrowheads). Scale bar is 50 μm

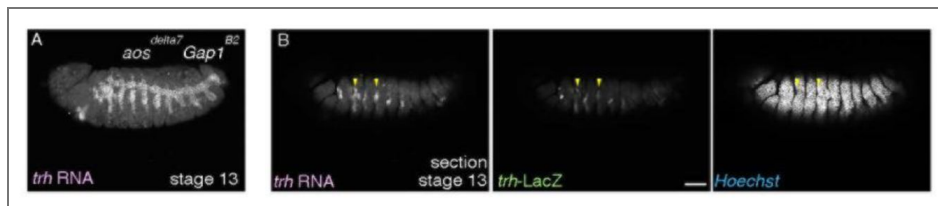


Figure 3 figure supplement 1. *trh* expression in *aos Gap1* double mutants do not respect tissue architecture.

(A-B) Lateral views of *aos Gap1* mutant embryo stained with *trh-LacZ* and *trh* transcripts. A projection (A) or a single section (B). *trh* expression is often detected in planes at the embryo surface (yellow arrows). Scale bar is 50 μ m

expressing either distal or proximal markers are detected at the 2D planar embryo surface (Oda et al. 1994 [↗](#); Matsuda et al. 2015b [↗](#)). Correspondingly, *trh* RNA and *trh*-LacZ are detected on the embryo surface in *wg* mutants at stage 13 and later (Figure 3G [↗](#)).

Similar to *wg* mutants, the initial *trh* expression expands along the A-P axis in *wg EGFR* double mutants (Figure 3I [↗](#)). However, *trh* RNA expression becomes very weak when most cells still reside on the 2D planes around the stage of primordia invagination (Figure 3I [↗](#)). This significant reduction of *trh* expression domain is still evident even in *H99 wg EGFR* mutants where *H99* deficiency suppresses apoptosis (Figure 3K [↗](#)). Together, we conclude that tissue architecture is dispensable for *trh* maintenance.

We suggest that *trh* maintenance is stimulated concurrently with the robust EGFR activation that occurs and propagates in the 2D airway primordia (Gabay et al. 1997a [↗](#); Gabay et al. 1997b [↗](#); Wappner et al. 1997 [↗](#); Matsuda et al. 2015b [↗](#)). EGFR activation also distalizes gene expression (Matsuda et al. 2015b [↗](#)) and initiates morphogenesis (Brodu and Casanova 2006 [↗](#); Nishimura et al. 2007 [↗](#)). In this scenario, rather than indirectly through tubulogenesis (Kondo and Hayashi 2019 [↗](#)), RTK signaling directly promotes *trh* maintenance irrespective of tissue geometry. We note that in double mutants of *aos* and *Gap1*, which are both negative regulators of EGFR signaling, there is expression of airway distal marker on the embryo surface (Matsuda et al. 2015b [↗](#)). This is accompanied by detection of *trh* expression on the embryo surface (Figure 3-figure supplement 3A-B).

Thus, subsequent to the Dpp/BMP mediated specification of the airway progenitors along the D-V axis, the radial EGFR signaling sustains *trh* expression in the progenitors and initiates airway cell differentiation along the P-D axis.

DRaf/MAPKKK and Dsor1/MAPKK are required for EGFR mediated priming of the airway progenitors

Trh activity can be boosted by the PI3kinase-PKB pathway through phosphorylation of Serine 665 (Jin et al. 2001 [↗](#)). However, it is not clear how far this pathway is required *in vivo* for Trh activity and *trh* autoregulation. We thus investigated if EGFR mediated priming and *trh* expression maintenance in the airway progenitors require the Ras-MAPK pathway (Figure 4A [↗](#)) (Hou et al. 1995 [↗](#); Mishra et al. 2005 [↗](#)). When MAPKK-kinase/Draf or MAPK-kinase/Dsor1 is depleted from the embryos with the *ovo-FRT* germline clone technique (Hou et al. 1995 [↗](#); Chou and Perrimon 1996 [↗](#)), *trh* expression initiates, as detected with *trh*-LacZ enhancer trap (Figure 4B-C [↗](#)). However, around the stage of primordial invagination, *trh* expression is very much reduced, in both non-invaginated or invaginated cells (Figure 4B-C [↗](#)).

Ras^{V12} boosts Trh activity

Ras (*Ras85D* or *Ras1* in *Drosophila*) is one of the major signaling mediators of RTKs through Draf/MAPKKK-Dsor1/MAPKK-MAPK (Hou et al. 1995 [↗](#); Mishra et al. 2005 [↗](#)) and through PI3kinase- PKB (Figure 4A [↗](#)) (Orme et al. 2006 [↗](#)). Consistent with the major role of *Ras85D/Ras1* in RTK signaling, comparable defects in maintenance of *trh* expression are detected in *EGFR btl/FGFR* double mutants and upon complete loss of *Ras85D/Ras1*, when anti-apoptotic functions are compensated by the *H99* chromosomal deletion (Figure 3C [↗](#), 4D [↗](#)). Conversely, *trh* maintenance in *EGFR wg/WNT* double mutants is significantly rescued resembling *wg/WNT* single mutants alone upon overexpression of the gain of function form of *Ras85D*, Ras^{V12} (Fortini et al. 1992 [↗](#)) (Figure 3L [↗](#)). Thus, *Ras* serves as the major signaling transducer to promote *trh* maintenance downstream of EGFR and Btl/FGFR.

The mechanisms by which, *Ras* promotes *trh* expression may involve boosting Trh activity because *trh* auto-regulates its own expression (Figure 4A [↗](#)) (Wilk et al. 1996 [↗](#); Sotillos et al. 2010 [↗](#); Chung et al. 2011 [↗](#)). Boosting of Trh activity may involve PKB mediated phosphorylation of Trh (Jin et al. 2001 [↗](#)). Alternatively, inferred from the positive roles of MAPK mediated phosphorylation of Hif1, a *trh* homologue (Mylonis et al. 2006 [↗](#)), MAPK may directly upregulate Trh activity. Consistently, overexpression of the phosphomimetic form of Trh, Trh^{S665D} or

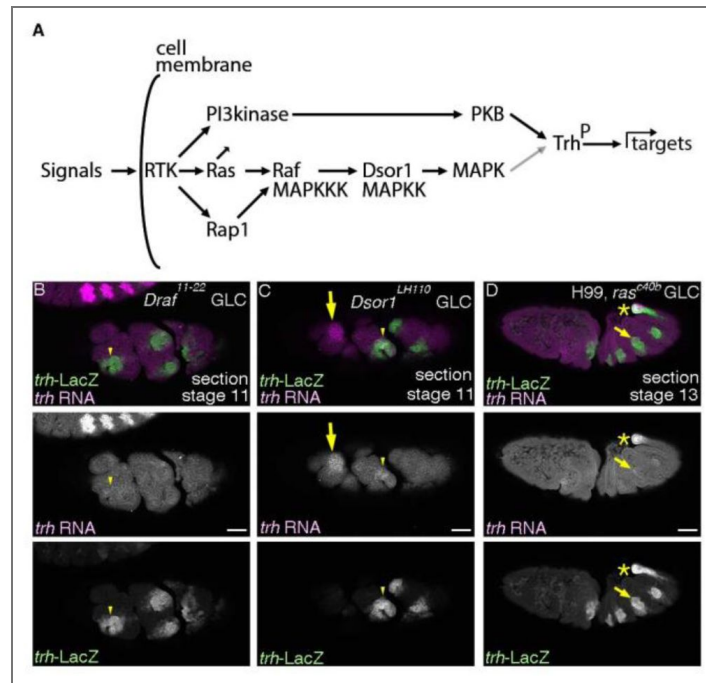


Figure 4. RTKs require the Ras-Raf/MAPKKK-Dsor1/MAPKK pathway for maintenance of *trh* expression.

(A) A schema showing that RTK activation involves several downstream signaling branches including the Ras-MAPK pathway and the PI3kinase-PKB pathway. PKB is known to phosphorylate Trh and to upregulate Trh transcriptional activity (black arrow). MAPK may act the same (gray arrow). (B-D) Lateral section views of embryos stained with *trh-LacZ* and *trh* transcripts. In the complete absence of *Draf*/MAPKKK (B) or *Dsor1*/MAPKK (C), *trh* RNA is hardly maintained in the cells positive for *trh-LacZ*, either invaginated or non-invaginated. In the complete absence of *Drosophila Ras1/Ras85D*, introducing *H99* deficiency suppressing apoptosis allows visualization of airway development till later stages (D), demonstrating residual *trh* expression in parts of the invaginated cells. Yellow arrows in C show *trh* expression in the salivary gland. Yellow asterisks in D mark *trh* expression in the posterior spiracle. Scale bar is 50 μ m

simultaneous double overexpression of Ras^{V12} and Trh^{WT} significantly induced ectopic expression of *trh*-LacZ, whereas single overexpression of Ras^{V12} or Trh^{WT} did not (Figure 5C). The Ras^{V12} induced increase of Trh activity is also detected on the expression of both the distal (*btl* and *Gasp/2A12*) and the proximal marker (*upd* and *P0144-LacZ*) as well (Figure 5A-F). We conclude that Ras activation downstream of RTKs is sufficient to upregulate Trh activity toward its downstream targets (Figure 4A).

Multiple redundant regulators cooperate during early *Drosophila* airway tubulogenesis

Tubes are common structures in multicellular organisms and tubular organs fulfill essential functions for life (Romero et al. 2025). An active field in stem cell biology engages in artificial organ generation *in vitro* to complement tubular organ dysfunction (Lancaster and Knoblich 2014). *In vivo* studies of tubulogenesis may aid such *in vitro* organoid development. In line with the revised view that a simple master regulator does not dictate all the steps of the *Drosophila* airway differentiation (de Celis et al. 1995; Llimargas and Casanova 1997; Chen et al. 1998; Boube et al. 2000; Matsuda et al. 2015b; Ogura et al. 2018; Kondo and Hayashi 2019), we characterize that the cooperation of both extrinsic and intrinsic, partly-redundant regulators ensures their robust morphogenesis.

The airway progenitor specification and their subsequent differentiation is associated with continuous expression of *trh* (Isaac and Andrew 1996; Wilk et al. 1996). *trh* is necessary for expression of all airway differentiation markers and for establishment of the airway tubes (Isaac and Andrew 1996; Wilk et al. 1996; Sotillos et al. 2010; Chung et al. 2011; Matsuda et al. 2015b), assigning it as a master TF of *Drosophila* airway tubulogenesis. Surprisingly, however, *trh* mutant cells invaginate to form tubes, but later revert to a planar configuration (Kondo and Hayashi 2019). Resolving the issue of what promotes progenitor invagination other than Trh, we identified extrinsic Hh and intrinsic Vvl as the dominant regulators of invagination, leaving *trh* expression intact.

The airway progenitors are intrinsically defined with at least 3 TFs, *trh*, *vvl* and *grn*. *vvl* defines the central/distal progenitors that invaginate first, whereas *grn* defines the peripheral/proximal progenitors that invaginate later, reflecting the proximo-distal differences of the airways (Matsuda et al. 2015b). Maintenance of *trh* expression is promoted significantly by auto-regulation (Wilk et al. 1996; Sotillos et al. 2010; Chung et al. 2011). However, regulation of *trh* expression is far more complex, requiring discrete regulations along the A-P, the D-V and the radial/proximo-distal (P-D) axis (Figure 6).

Along the D-V axis, our results show that intermediate Dpp/BMP activity promotes maintenance of *trh* expression whereas high level Dpp/BMP activity is known to be repressive on initiation of *trh* expression (Isaac and Andrew 1996; Wilk et al. 1996). Therefore, Dpp/BMP has two opposing effects on *trh* expression.

Along the A-P axis, segmentally repeated expression of Unpaired ligands and the resultant activation of Domeless-JAK-STAT signaling precedes and may set where expression of *trh* and *vvl* initiates (Brown et al. 2001; Sotillos et al. 2010). The airway field is repressed in the embryonic head and the tail by a zinc finger TF *spalt* (Boube et al. 2000) whereas it is segmentally repressed in the trunk by Wg/WNT (de Celis et al. 1995; Wilk et al. 1996). It is not known what initiates the remaining expression of *trh* and *vvl* in the absence of Unpaired family ligands-Domeless-JAK-STAT signaling (Brown et al. 2001).

Along the radial/P-D axis, the centro-peripheral spreading of Rho-mediated EGFR activation in the 2D progenitor fields promotes maintenance of *trh* expression. Co-expression of Trh and Ras^{V12} potentiates ectopic Trh activity toward downstream genes. Therefore, we suggest that EGFR mediated boosting of TFs like Trh or Vvl may underly *trh* maintenance (Wilk et al. 1996) and the sequential spreading of *rho* expression (Matsuda et al. 2015b), generating a wave of feed-forward loops for progenitor priming and airway differentiation.

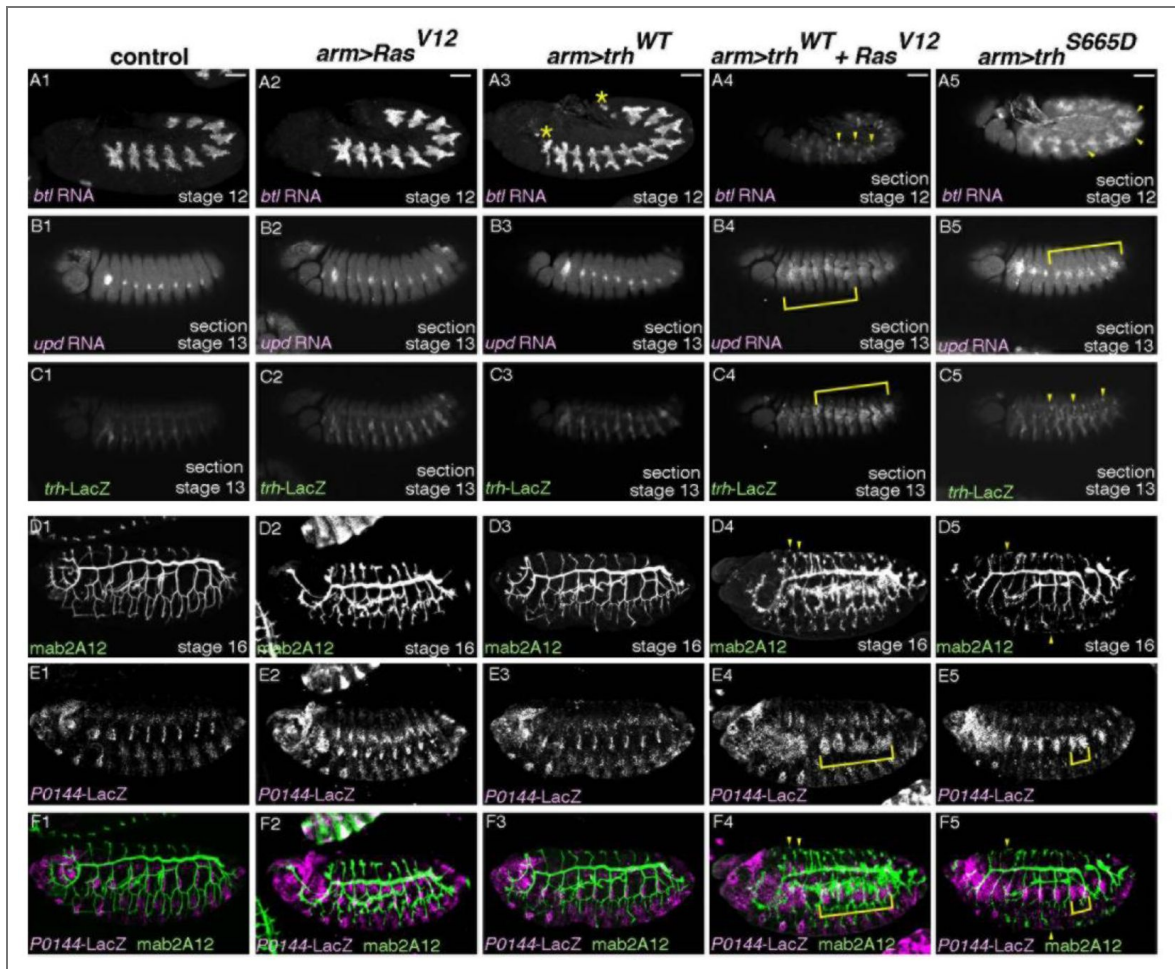


Figure 5. Ras^{V12} boosts Trh activity toward downstream genes.

(A-F) Lateral views of embryos stained as indicated. Compared to the control (first column), single overexpression of Ras^{V12} (second column) or Trh^{WT} (third column), simultaneous overexpression of Ras^{V12} and Trh^{WT} (fourth column) or single overexpression of Trh^{S665D} (fifth column) induces ectopic expression of *btl* (A), *upd* (B), *trh-LacZ* (C), *mab2A12* (Gasp) (D, F) or *P0144-LacZ* (E, F), which are marked by yellow arrowhead or yellow blankets. Asterisks in A3 marks ectopic progenitors in the anterior and the posterior segments. Scale bar is 50 μ m.

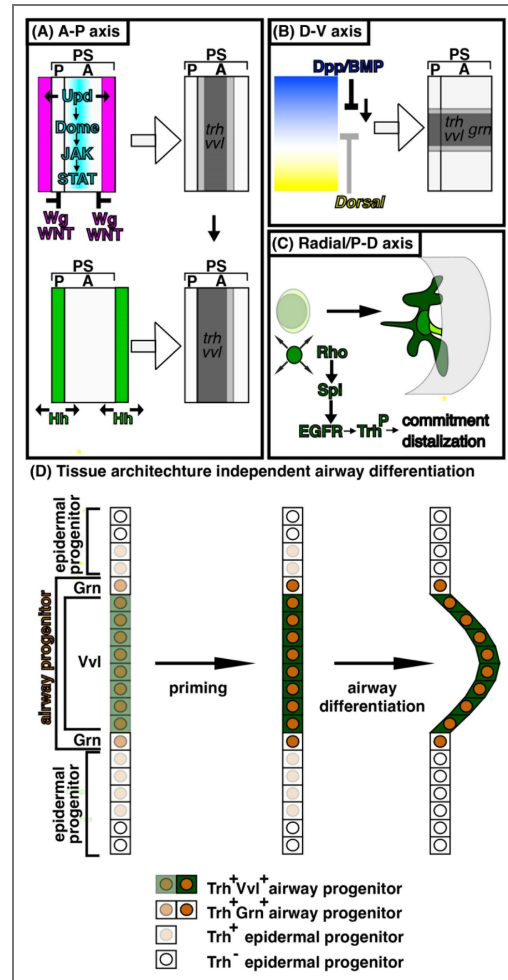


Figure 6. A model for specification and priming of the airway progenitors along the three body axes Inputs along the A-P axis and the D-V axis generate radial patterning of the airway progenitors to realize the proximo-distal differences of gene expression and morphology.

(A) A-P axis; Segments and para-segments (PS) are units of *Drosophila* ectoderm segmentation along the A-P axis. One PS is composed of the posterior of a segment and the anterior of its posterior neighboring segment. Wg/WNT (coloured pink) represses *trh* and *vvl* whereas Upd ligands signal through Domeless-JAK-STAT (coloured blue) to induce them, which together generate a graded airway progenitor field along the A-P axis (coloured gray). The *upd* expression domain is a guess. *upd* expression is dynamic (Harrison et al. 1998; Sotillos et al. 2010) and it is not known which *upd* expression is required for inducing the airway primordia. *trh* expression initiates already at stage 8 (Isaac and Andrew 1996) whereas *vvl* expression starts at stage 10. Hh (coloured green) positively regulates expression of *vvl*, thereby distalizes the progenitor field. (B) D-V axis; The ventrally active TF Dorsal (coloured yellow) restricts *dpp/BMP* expression to the dorsal domain. Dpp/BMP (coloured blue) in turn orchestrates the dorsal parts of the embryos. Medium Dpp/BMP activities promote expression of *trh*, *vvl* and *grn* to generate a graded airway progenitor field along the D-V axis. (C) P-D axis; The radial patterning is realized by Rho mediated activation of EGFR. EGFR activation primes the airway progenitors to commit to the airway differentiation program. At the same time, EGFR distalizes the airway differentiation along both gene expression and morphology to establish the P-D axis of the invaginated tubes. Spreading of *rho* expression and promotion of *trh* maintenance may involve EGFR mediated activation of TFs like Trh and Vvl, which boosts their transcriptional activities toward downstream genes including *rho* and *trh*. (D) A model of tissue-architecture-independent regulation of *trh* expression. *trh* expressing cells (brown coloured nucleus) are composed of the epidermal progenitors (pale brown) and the airway progenitors (brown), the latter of which is further classified into *vvl* expressing central/distal cells (green cytoplasm) and *grn* expressing peripheral/proximal cells. Each progenitor type is specified on the 2D cell fields based on the cues along the A-P and the D-V axes (left). Radial EGFR signaling primes the airway progenitors (center, dark brown), to realize morphogenetic and transcriptional differentiation along the P-D axis (right). *trh* expression in the epidermal progenitors ceases based on the epidermal differentiation programs. Airway progenitor invagination may be aided by segregation of the 2 different cell types whereas invagination processes may finetune the cell type determination processes. Note that *grn* expression in the epidermal progenitors is omitted.

Also, we previously showed that *grn* potentiates *trh* maintenance in the peripheral/proximal cells whereas *vvl* circumvents the *trh* expression dependency on *grn* in the central/distal cells (Matsuda et al. 2015b [↗](#)), arguing for region-specific requirements for *trh* maintenance in the primordium.

In our model, it is pre-determined which cells in the *trh* expressing 2D fields contribute to airways or epidermis before invagination (Figure 6D [↗](#)). Cells not receiving proper amounts of positive inputs from like optimal Dpp/BMP activities or enough EGFR activities or that receive too much negative inputs like Wg/WNT, would cease *trh* transcription. The timing of transcriptional silencing and degradation of the remaining transcripts and the proteins would determine when the cells lose *trh* products. Recently, it was discovered that micro-peptides encoded by *polished rice/tarsal-less* promote *trh* expression by suppressing the repressor activity of a zinc finger TF Ovo/Shaven-baby (Mizuno et al. 2026 [↗](#)). It is intriguing, where this circuit fits into the regulatory modes of *trh* expression we described above.

Multiple partially redundant systems on initiation and maintenance of gene expression and morphology sustain development of even simple organs like the *Drosophila* airways. The crucial roles of tubular organs for survival could be the driving force in evolving multiple overlying regulatory schemes, which may secure development of essential organs even when one regulatory scheme becomes dysfunctional.

Materials and methods

Fly genetics and histochemistry of embryos were done as previously described (Matsuda et al. 2015a [↗](#); Matsuda et al. 2015b [↗](#)). Marker genes were typically introduced as a single copy, unless otherwise noted.

Fly strains used in this study are:

aos^{Δ7} (BDSC 1004)
arm-Gal4 (BDSC 1560)
btl-Gal4 (a gift from Dr. S. Hayashi)
D-lacZ (a gift from Dr. J. Nambu and Dr. S. Russel)
*Draf*¹¹⁻²² *FRT101* (a gift from Dr. N. Perrimon)
Dsor1^{LH110} *FRT101* (a gift from Dr. N. Perrimon)
btl^{Δoh10} (Ohshiro and Saigo 1997 [↗](#))
btl^{Δoh24-1} (Ohshiro and Saigo 1997 [↗](#))
da-Gal4 (BDSC 8641)
dpp^{H46} (BDSC 2061)
dpp^{hr92} (BDSC 2069)
Dr/msh-lacZ (a gift from Dr. A. Nose)
Df(2L)Exel6017=Df(wg) (BDSC 7503)
Df(3L)Exel6109=Df(vvl) (BDSC 7588)
Gap1^{B2} (a gift from Dr. N. Perrimon)
GMR19D03 (BDSC 48846)
grn-GFP (BDSC 58483)
H99=Df(3L)H99 (BDSC 1576)
hh^{13C} (Hosono et al. 2003 [↗](#))
hh^{AC} (BDSC 1749)
hs-FLP (BDSC)
iroquois-lacZ (a gift from Dr. S. Campuzano)
ovoD1 FRT101; hs-FLP (BDSC 1813)
ovoD1 FRT82B (BDSC 2149)
P0144-lacZ (a gift from Dr. W. Janning, Flyview)
Ras85D^{Δc40b} (a gift from Dr. N. Perrimon and Dr. C. A. Berg)
top^{f2} (BDSC 2768)
top^{f24} (a gift from Dr. K. Moses)
trh-lacZ=1-eve-1 (a gift from Dr. N. Perrimon)

UAS-GFP.nls (BDSC 4776)
UAS-trh^{WT}/CyO (a gift from Dr. A. S. Manoukian)
UAS-trh^{S665D} (a gift from Dr. A. S. Manoukian)
UAS-Ras^{V12}/CyO (a gift from Dr. G. M. Rubin)
UAS-Ras^{V12}/TM3 (a gift from Dr. N. Perrimon)
wl^{utH599} (a gift from Dr. A. Salzberg)
wg^{CX4} (BDSC 2980)

Antibodies used for immunohistochemistry are;

Rabbit anti-GFP (invitrogen)

Rabbit anti-LacZ (Capel)

Rabbit anti-Trh (this study)

Double fluorescent labeling with RNA probes and antibodies was carried out as described (Goto and Hayashi 1997 [↗](#)).

DNA clones used for in situ RNA detection are;

caup (Drosophila Genomics Resource Center, DGRC)

trh (Drosophila Genomics Resource Center, DGRC)

wl (a gift from Dr. J. Casanova)

Confocal images were taken by Bio-Rad (Hercules, CA) MRC1024, Olympus (Japan) Fluoview 1000 or Zeiss (Germany) LSM800. Images were processed by ImageJ and figures were prepared with Adobe Photoshop and Illustrator.

Data availability

All the data are available.

Acknowledgements

We thank the members of the fly community who isolated, characterized or distributed fly strains, antibodies or DNA clones. Especially, we thank Drs. C. A. Berg, S. Campuzano, J. Casanova, S. Hayashi, W. Janning, A. S. Manoukian, K. Moses, J. Nambu, A. Nose, N. Perrimon, G.M. Rubin, S. Russel, A. Salzberg, DGRC and BDSC for directly sharing fly strains and DNA clones. We thank Flybase for the Drosophila genomic resources. We thank the Stockholm University Imaging Facility and MBW fly services. We thank V. Tsarouhas for microscope help. Special thanks to Y. Emori and F. Ui-Tei for help in maintaining fly strains after the retirement of K. Saigo.

This work was funded by the Ministry of Education, Culture, Sport, Science and Technology of Japan to K.S. and the Swedish Research Council, the Swedish Cancer Society and German Research Foundation to C.S.

Additional information

Author contribution

RM, conceived the project, designed the experiments, performed experiments, interpreted data, drafted the manuscript with inputs from CH, writing, figure preparation

CH, performed experiments, figure preparation, writing

KS, funding acquisition, provided experiment and analysis tools

CS, funding acquisition, provided experiment and analysis tools, writing

Funding

Funder	Grant reference number	Author
Cancerfonden (Swedish Cancer Society)		Christos Samkovlis
Vetenskapsrådet (VR)		Christos Samkovlis
Deutsche Forschungsgemeinschaft (DFG)		Christos Samkovlis

References

- Arman E**, Haffner-Krausz R, Gorivodsky M, Lonai P (1999) Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **96**:11895-11899 <https://doi.org/10.1073/pnas.96.21.11895> | [PubMed](#)
- Beitel GJ**, Krasnow MA (2000) Genetic control of epithelial tube size in the Drosophila tracheal system. *Development* **127**:3271-3282 <https://doi.org/10.1242/dev.127.15.3271> | [PubMed](#)
- Bier E**, Jan LY, Jan YN (1990) rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster. *Genes & development* **4**:190-203 <https://doi.org/10.1101/gad.4.2.190> | [PubMed](#)
- Boube M**, Llimargas M, Casanova J (2000) Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the Drosophila embryo. *Mechanisms of development* **91**:271-278 [https://doi.org/10.1016/s0925-4773\(99\)00315-9](https://doi.org/10.1016/s0925-4773(99)00315-9) | [PubMed](#)
- Brodu V**, Casanova J (2006) The RhoGAP crossveinless-c links trachealess and EGFR signaling to cell shape remodeling in Drosophila tracheal invagination. *Genes & development* **20**:1817-1828 <https://doi.org/10.1101/gad.375706> | [PubMed](#)
- Brown S**, Hu N, Hombria JC (2001) Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. *Current biology : CB* **11**:1700-1705 [https://doi.org/10.1016/s0960-9822\(01\)00524-3](https://doi.org/10.1016/s0960-9822(01)00524-3) | [PubMed](#)
- Brownfield DG**, de Arce AD, Ghelfi E, Gillich A, Desai TJ, Krasnow MA (2022) Alveolar cell fate selection and lifelong maintenance of AT2 cells by FGF signaling. *Nat Commun* **13**:7137 <https://doi.org/10.1038/s41467-022-34059-1> | [PubMed](#)
- Chen CK**, Kuhnlein RP, Eulenberg KG, Vincent S, Affolter M, Schuh R (1998) The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during Drosophila tracheal development. *Development* **125**:4959-4968 <https://doi.org/10.1242/dev.125.24.4959> | [PubMed](#)
- Chou TB**, Perrimon N (1996) The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. *Genetics* **144**:1673-1679 <https://doi.org/10.1093/genetics/144.4.1673> | [PubMed](#)
- Chung S**, Chavez C, Andrew DJ (2011) Trachealess (Trh) regulates all tracheal genes during Drosophila embryogenesis. *Developmental biology* **360**:160-172 <https://doi.org/10.1016/j.ydbio.2011.09.014> | [PubMed](#)
- de Celis JF**, Llimargas M, Casanova J. (1995) Ventral veinless, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in Drosophila melanogaster. *Development* **121**:3405-3416 <https://doi.org/10.1242/dev.121.10.3405> | [PubMed](#)
- Fortini ME**, Simon MA, Rubin GM (1992) Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. *Nature* **355**:559-561 <https://doi.org/10.1038/355559a0> | [PubMed](#)
- Gabay L**, Seger R, Shilo BZ (1997a) In situ activation pattern of Drosophila EGF receptor pathway during development. *Science* **277**:1103-1106 <https://doi.org/10.1126/science.277.5329.1103> | [PubMed](#)
- Gabay L**, Seger R, Shilo BZ (1997b) MAP kinase in situ activation atlas during Drosophila embryogenesis. *Development* **124**:3535-3541 <https://doi.org/10.1242/dev.124.18.3535> | [PubMed](#)
- Ghabrial A**, Luschnig S, Metzstein MM, Krasnow MA (2003) Branching morphogenesis of the Drosophila tracheal system. *Annual review of cell and developmental biology* **19**:623-647 <https://doi.org/10.1146/annurev.cellbio.19.031403.160043> | [PubMed](#)

- Glazer L, Shilo BZ** (1991) The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes & development* **5**:697-705 <https://doi.org/10.1101/gad.5.4.697> | [PubMed](#)
- Goto S, Hayashi S** (1997) Specification of the embryonic limb primordium by graded activity of Decapentaplegic. *Development* **124**:125-132 <https://doi.org/10.1242/dev.124.1.125> | [PubMed](#)
- Guillemin K, Groppe J, Ducker K, Treisman R, Hafen E, Affolter M, Krasnow MA** (1996) The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**:1353-1362 <https://doi.org/10.1242/dev.122.5.1353> | [PubMed](#)
- Harrison DA, McCoon PE, Binari R, Gilman M, Perrimon N** (1998) Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes & development* **12**:3252-3263 <https://doi.org/10.1101/gad.12.20.3252> | [PubMed](#)
- Herriges M, Morrisey EE** (2014) Lung development: orchestrating the generation and regeneration of a complex organ. *Development* **141**:502-513 <https://doi.org/10.1242/dev.098186> | [PubMed](#)
- Hosono C, Takaira K, Matsuda R, Saigo K** (2003) Functional subdivision of trunk visceral mesoderm parasegments in Drosophila is required for gut and trachea development. *Development* **130**:439-449 <https://doi.org/10.1242/dev.00242> | [PubMed](#)
- Hou XS, Chou TB, Melnick MB, Perrimon N** (1995) The torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell* **81**:63-71 [https://doi.org/10.1016/0092-8674\(95\)90371-2](https://doi.org/10.1016/0092-8674(95)90371-2) | [PubMed](#)
- Hu N, Castelli-Gair J** (1999) Study of the posterior spiracles of Drosophila as a model to understand the genetic and cellular mechanisms controlling morphogenesis. *Developmental biology* **214**:197-210 <https://doi.org/10.1006/dbio.1999.9391> | [PubMed](#)
- Isaac DD, Andrew DJ** (1996) Tubulogenesis in Drosophila: a requirement for the trachealess gene product. *Genes & development* **10**:103-117 <https://doi.org/10.1101/gad.10.1.103> | [PubMed](#)
- Jin J, Anthopoulos N, Wetsch B, Binari RC, Isaac DD, Andrew DJ, Woodgett JR, Manoukian AS** (2001) Regulation of Drosophila tracheal system development by protein kinase B. *Developmental cell* **1**:817-827 [https://doi.org/10.1016/s1534-5807\(01\)00090-9](https://doi.org/10.1016/s1534-5807(01)00090-9) | [PubMed](#)
- Jones MR, Lingampally A, Ahmadvand N, Chong L, Wu J, Wilhem J, Vazquez-Armendariz AI, Ansari M, Herold S, Ornitz DM, et al.** (2022) FGFR2b signalling restricts lineage-flexible alveolar progenitors during mouse lung development and converges in mature alveolar type 2 cells. *Cell Mol Life Sci* **79**:609 <https://doi.org/10.1007/s00018-022-04626-2> | [PubMed](#)
- Kishimoto K, Morimoto M** (2021) Mammalian tracheal development and reconstruction: insights from in vivo and in vitro studies. *Development* **148** <https://doi.org/10.1242/dev.198192> | [PubMed](#)
- Klambt C, Glazer L, Shilo BZ** (1992) . breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. *Genes & development* **6**:1668-1678 <https://doi.org/10.1101/gad.6.9.1668> | [PubMed](#)
- Kondo T, Hayashi S** (2013) Mitotic cell rounding accelerates epithelial invagination. *Nature* **494**:125-129 <https://doi.org/10.1038/nature11792> | [PubMed](#)
- Kondo T, Hayashi S** (2019) Two-step regulation of trachealess ensures tight coupling of cell fate with morphogenesis in the Drosophila trachea. *eLife* **8** <https://doi.org/10.7554/eLife.45145> | [PubMed](#)
- Lancaster MA, Knoblich JA** (2014) Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* **345**:1247-125 <https://doi.org/10.1126/science.1247125> | [PubMed](#)
- Llimargas M, Casanova J** (1997) . ventral veinless, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in Drosophila. *Development* **124**:3273-3281 <https://doi.org/10.1242/dev.124.17.3273> | [PubMed](#)
- Llimargas M, Casanova J** (1999) EGF signalling regulates cell invagination as well as cell migration during formation of tracheal system in Drosophila. *Development genes and evolution* **209**:174-179 <https://doi.org/10.1007/s004270050241> | [PubMed](#)

- Manning G**, Krasnow MA (1993) *The Development of Drosophila melanogaster* Plainview, N.Y: Cold Spring Harbor Laboratory Press.
- Manning L**, Heckscher ES, Purice MD, Roberts J, Bennett AL, Kroll JR, Pollard JL, Strader ME, Lupton JR, Dyukareva AV, *et al.* (2012) A resource for manipulating gene expression and analyzing cis-regulatory modules in the Drosophila CNS. *Cell reports* **2**:1002-1013
<https://doi.org/10.1016/j.celrep.2012.09.009> | PubMed
- Matsuda R**, Hosono C, Saigo K, Samakovlis C (2015a) 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. *PLoS genetics* **11**:e1004929
<https://doi.org/10.1371/journal.pgen.1004929> | PubMed
- Matsuda R**, Hosono C, Samakovlis C, Saigo K (2015b) Multipotent versus differentiated cell fate selection in the developing Drosophila airways. *eLife* **4** <https://doi.org/10.7554/elife.09646> | PubMed
- Min H**, Danilenko DM, Scully SA, Bolon B, Ring BD, Tarpley JE, DeRose M, Simonet WS (1998) Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes & development* **12**:3156-3161 <https://doi.org/10.1101/gad.12.20.3156> | PubMed
- Mishra S**, Smolik SM, Forte MA, Stork PJ (2005) Ras-independent activation of ERK signaling via the torso receptor tyrosine kinase is mediated by Rap1. *Current biology : CB* **15**:366-370
<https://doi.org/10.1016/j.cub.2005.02.022> | PubMed
- Mizuno S**, Uemura T, Kondo T (2026) Pri micropeptide functions as a cell-intrinsic timer controlling the transient phase of cell fate induction. *Proceedings of the National Academy of Sciences of the United States of America* **123**:e2511138123 <https://doi.org/10.1073/pnas.2511138123> | PubMed
- Mylonis I**, Chachami G, Samiotaki M, Panayotou G, Paraskeva E, Kalousi A, Georgatsou E, Bonanou S, Simos G (2006) Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. *The Journal of biological chemistry* **281**:33095-33106
<https://doi.org/10.1074/jbc.m605058200> | PubMed
- Nishimura M**, Inoue Y, Hayashi S (2007) A wave of EGFR signaling determines cell alignment and intercalation in the Drosophila tracheal placode. *Development* **134**:4273-4282
<https://doi.org/10.1242/dev.010397> | PubMed
- Oda H**, Uemura T, Harada Y, Iwai Y, Takeichi M (1994) A Drosophila homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Developmental biology* **165**:716-726
<https://doi.org/10.1006/dbio.1994.1287> | PubMed
- Ogura Y**, Wen FL, Sami MM, Shibata T, Hayashi S (2018) A Switch-like Activation Relay of EGFR-ERK Signaling Regulates a Wave of Cellular Contractility for Epithelial Invagination. *Developmental cell* **46**:162-172. <https://doi.org/10.1016/j.devcel.2018.06.004> | PubMed
- Ohshiro T**, Saigo K (1997) Transcriptional regulation of breathless FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in Drosophila developing trachea. *Development* **124**:3975-3986 <https://doi.org/10.1242/dev.124.20.3975> | PubMed
- Orme MH**, Alrubaie S, Bradley GL, Walker CD, Leever SJ (2006) Input from Ras is required for maximal PI(3)K signalling in Drosophila. *Nature cell biology* **8**:1298-1302 <https://doi.org/10.1038/ncb1493> | PubMed
- Perrimon N**, Noll E, McCall K, Brand A (1991) Generating lineage-specific markers to study Drosophila development. *Developmental genetics* **12**:238-252 <https://doi.org/10.1002/dvg.1020120309> | PubMed
- Pfeiffer BD**, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Lavery TR, *et al.* (2008) Tools for neuroanatomy and neurogenetics in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **105**:9715-9720
<https://doi.org/10.1073/pnas.0803697105> | PubMed
- Potente M**, Makinen T (2017) Vascular heterogeneity and specialization in development and disease. *Nat Rev Mol Cell Biol* **18**:477-494 <https://doi.org/10.1038/nrm.2017.36> | PubMed

- Romero A**, Walker BL, Krneta-Stankic V, Gerner-Mauro K, Youmans L, Miller RK (2025) The dynamics of tubulogenesis in development and disease. *Development* **152** <https://doi.org/10.1242/dev.202820> | PubMed
- Samakovlis C**, Hacoheh N, Manning G, Sutherland DC, Guillemin K, Krasnow MA (1996a) Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**:1395-1407 <https://doi.org/10.1242/dev.122.5.1395> | PubMed
- Samakovlis C**, Manning G, Steneberg P, Hacoheh N, Cantera R, Krasnow MA (1996b) Genetic control of epithelial tube fusion during *Drosophila* tracheal development. *Development* **122**:3531-3536 <https://doi.org/10.1242/dev.122.11.3531> | PubMed
- Schweitzer R**, Shaharabany M, Seger R, Shilo BZ (1995) Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes & development* **9**:1518-1529 <https://doi.org/10.1101/gad.9.12.1518> | PubMed
- Sekine K**, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, *et al.* (1999) Fgf10 is essential for limb and lung formation. *Nature genetics* **21**:138-141 <https://doi.org/10.1038/5096> | PubMed
- Shin M**, Chang E, Lee D, Kim N, Cho B, Cha N, Koranteng F, Song JJ, Shim J (2024) *Drosophila* immune cells transport oxygen through PPO2 protein phase transition. *Nature* **631**:350-359 <https://doi.org/10.1038/s41586-024-07583-x> | PubMed
- Sotillos S**, Espinosa-Vazquez JM, Foglia F, Hu N, Hombria JC (2010) An efficient approach to isolate STAT regulated enhancers uncovers STAT92E fundamental role in *Drosophila* tracheal development. *Developmental biology* **340**:571-582 <https://doi.org/10.1016/j.ydbio.2010.02.015> | PubMed
- Sountoulidis A**, Firsova AB, Lontos A, Theelke J, Koepke J, Millar-Büchner P, Mannerås-Holm L, Björklund Å, Fysikopoulos A, Gaengel K, *et al.* (2023) Postnatal FGFR-signaling establishes gradients of secretory cell identities along the proximal-distal axis of the lung airways. *bioRxiv* 2023.2012.2011.571142 <https://doi.org/10.1101/2023.12.11.571142>
- Sutherland D**, Samakovlis C, Krasnow MA (1996) . branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**:1091-1101 [https://doi.org/10.1016/s0092-8674\(00\)81803-6](https://doi.org/10.1016/s0092-8674(00)81803-6) | PubMed
- von Ohlen T**, Doe CQ (2000) Convergence of dorsal, dpp, and egfr signaling pathways subdivides the *drosophila* neuroectoderm into three dorsal-ventral columns. *Developmental biology* **224**:362-372 <https://doi.org/10.1006/dbio.2000.9789> | PubMed
- Wappner P**, Gabay L, Shilo BZ (1997) Interactions between the EGF receptor and DPP pathways establish distinct cell fates in the tracheal placodes. *Development* **124**:4707-4716 <https://doi.org/10.1242/dev.124.22.4707> | PubMed
- Weinstein M**, Xu X, Ohyama K, Deng CX (1998) FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125**:3615-3623 <https://doi.org/10.1242/dev.125.18.3615> | PubMed
- Wharton KA**, Ray RP, Gelbart WM (1993) An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* **117**:807-822 <https://doi.org/10.1242/dev.117.2.807> | PubMed
- White K**, Grether ME, Abrams JM, Young L, Farrell K, Steller H (1994) Genetic control of programmed cell death in *Drosophila*. *Science* **264**:677-683 <https://doi.org/10.1126/science.8171319> | PubMed
- Wilk R**, Weizman I, Shilo BZ (1996) . trachealess encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes & development* **10**:93-102 <https://doi.org/10.1101/gad.10.1.93> | PubMed
- Yagi Y**, Suzuki T, Hayashi S (1998) Interaction between *Drosophila* EGF receptor and vnd determines three dorsoventral domains of the neuroectoderm. *Development* **125**:3625-3633 <https://doi.org/10.1242/dev.125.18.3625> | PubMed

Zhao G, Skeath JB (2002) The Sox-domain containing gene *Dichaete/fish-hook* acts in concert with *vnd* and *ind* to regulate cell fate in the *Drosophila* neuroectoderm. *Development* **129**:1165-1174
<https://doi.org/10.1242/dev.129.5.1165> | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

In this manuscript, Matsuda and collaborators present a model of how tracheal tubulogenesis is controlled in *Drosophila* embryos. Some of the results backing the model are new, but others are based on information already published by the authors. However, the results in this manuscript present different molecular markers not published before, which agree with previous conclusions. The manuscript also analyses the requirement of the *dpp* and EGFR signalling pathways for tracheless (*trh*) maintenance, one of the main tracheal transcription factors.

Strengths:

The two most interesting novel points of the manuscript are:

(1) Its contribution to the analysis of how the *dpp* and EGFR pathways contribute to the maintenance of *trh* expression.

(2) The experimental evidence showing that mechanical invagination is not a requirement for *trh* maintenance in the tracheal cells, an intriguing hypothesis previously suggested by (Kondo Hayashi 2019 eLife 8:e45145) that can now be discarded by the data presented in this work.

Weaknesses:

Because of the mixture of new and already published data, this manuscript can be considered as a review/experimental paper.

Already known data:

- The results showing that *hh* and *vvl* drive tracheal invagination independently of *trh* are reported in Figure 5 of (Matsuda et al. 2015 eLife 4:e09646).

- The results showing *dpp* requirement for *trh* maintenance are partially reported in Figure 6 of (Matsuda 2015 eLife 4:e09646).

<https://doi.org/10.7554/eLife.111290.1.sa1>

Reviewer #2 (Public review):

Summary:

Matsuda et al. investigate the regulatory mechanisms controlling gene expression and morphogenesis in the *Drosophila* embryonic trachea. Building on previous findings that tracheal invagination can occur independently of *trh*, they identify extrinsic *hh* and intrinsic *vvl* as key regulators that cooperatively promote this process. The study also integrates major signaling pathways (*Dpp/BMP* and EGFR) in defining tracheal cell identity and demonstrates that Ras activation can upregulate *trh*. Overall, the work supports a model in which multiple transcription factors and signaling inputs coordinate airway progenitor specification.

Strengths:

This study uses genetic analysis of various mutants to dissect regulatory relationships underlying tracheal development. While the uncoupling of tracheal invagination from *trh* function has been previously recognized, this work advances the field by identifying *hh* and *vvl* as key regulators of invagination independent of *trh*. The study also integrates multiple signaling pathways, such as Dpp/BMP and EGFR, into a coherent framework for tracheal cell specification. In addition, the demonstration that Ras activation can upregulate *trh* provides a clear mechanistic link between RTK signaling and transcriptional regulation. Overall, the work offers important and broadly relevant insights into how gene expression and morphogenesis are coordinated during development.

Weaknesses:

Data presentation and clarity of interpretation could be improved. Many images primarily show lateral views of whole embryos, which can make it difficult to fully assess some phenotypes; higher-magnification or sectional views would enhance clarity. There are also some minor inconsistencies in the description of invagination phenotypes, particularly regarding whether all *trh*⁺ cells remain in a 2D plane versus indications of partial invagination in *hh vvl* double mutants blocking apoptosis, which would benefit from further clarification. Finally, some statements in the abstract, especially regarding the role of *grn*, are not directly supported by data in this study and could be better aligned with the scope of the presented results.

<https://doi.org/10.7554/eLife.111290.1.sa0>

Author response:**Reviewer #1**

*- The results showing that *hh* and *vvl* drive tracheal invagination independently of *trh* are reported in Figure 5 of (Matsuda et al. 2015 eLife 4:e09646).*

Reviewer #2

*Many images primarily show lateral views of whole embryos, which can make it difficult to fully assess some phenotypes; higher-magnification or sectional views would enhance clarity. There are also some minor inconsistencies in the description of invagination phenotypes, particularly regarding whether all *trh*⁺ cells remain in a 2D plane versus indications of partial invagination in *hh vvl* double mutants blocking apoptosis, which would benefit from further clarification.*

The data in our previous eLife publication (DOI: 10.7554/eLife.09646)1 were mostly projection views. Therefore, it is hard to conclude if the airway progenitors of *hh vvl* double mutants failed to invaginate or they invaginated to form sacs. We will provide magnified views of the progenitor invagination in *hh vvl* double mutants and describe the degrees of their invagination phenotypes.

Reviewer #1

*The results showing *dpp* requirement for *trh* maintenance are partially reported in Figure 6 of (Matsuda 2015 eLife 4:e09646).*

Reviewer #2

*Finally, some statements in the abstract, especially regarding the role of *grn*, are not directly supported by data in this study and could be better aligned with the scope of the*

| presented results.

trh-lacZ (*1-eve-1*) has been used as the earliest and the strongest enhancer trap line to mark the airway primordia and the airway progenitors. Perdurance of beta-galactocidase proteins makes it difficult to conclude if the marker signals result from the active transcriptional state of the *trh* locus. In our previous eLife publication we showed that Trh proteins and *trh* transcripts are not detectable in *H99 grn hh vvl* quadruple mutants and in *grn hh vvl* triple mutants (Figure 5H and Figure 5-figure supplement 2A of DOI: 10.7554/eLife.09646, respectively)¹, although *trh*-LacZ signals are detected in *grn hh vvl* triple mutants.

Similarly, although we previously showed *trh*-LacZ expression in *dpp* mutant combinations, Figure 2 in the current manuscript, shows that even strong *trh*-LacZ signals do not always correlate with *trh* transcripts in *dpp* mutants. Therefore, in the current manuscript we included the data of *dpp*-driven positive regulation of *trh* transcripts at later stages since they have not been shown before.

Assessments and advices of the Editors and the Reviewers are indispensable for improving the manuscript. We will address all the Reviewers comments (Weakness of Public review, major and minor issues of Recommendations for the authors) both experimentally and in the text.

Sincerely yours,

Christos Samakovlis on behalf of all authors

- (1) Matsuda, R., Hosono, C., Samakovlis, C. & Saigo, K. Multipotent versus differentiated cell fate selection in the developing *Drosophila* airways. eLife 4 (2015).

<https://doi.org/10.7554/eLife.111290.1.sa3>