Escargot controls the sequential specification of two tracheal tip cell types by suppressing FGF signaling in Drosophila

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Summary Statement

The migratory behavior of tracheal fusion cells in *Drosophila* is controlled by the FGF-induced expression of the transcription factor Escargot, which subsequently suppresses ERK signaling.
Abstract

Extrinsic branching factors promote the elongation and migration of tubular organs. In the *Drosophila* tracheal system, Branchless/Fibroblast Growth Factor (FGF) stimulates the branching program by specifying tip cells that acquire motility and lead branch migration to a specific destination. Tip cells have two alternative cell fates: the terminal cell (TC), which elongates the cytoplasmic extension with intracellular lumen, and the fusion cell (FC), which mediates branch connections to form tubular networks. How Branchless/FGF controls this specification of cells with distinct shapes and behaviors is unknown. Here we report that this cell-type diversification involves the modulation of FGF signaling by the zinc-finger protein Escargot (Esg), which is expressed in the FC and is essential for its specification. The dorsal branch begins elongation with a pair of tip cells with high FGF signaling. When the branch tip reaches its final destination, one of the tip cells become an FC and expresses Esg. FCs and TCs differ in their response to FGF: TCs are attracted by FGF, while FCs are repelled. Esg suppresses ERK signaling in FCs to control this differential migratory behavior.
Introduction

Tubular organs allow for circulation through blood vessels and promote the exchange of gases in the mammalian lung and insect trachea, thereby increasing the oxygen-uptake efficiency in large animals that have a high volume/surface ratio and are otherwise incapable of effective respiration. The development of tubular organs is triggered by the expression of secreted signaling molecules. The expression pattern of these molecules, either singly or in combination, acts as a branching signal by instructing signal-receiving tubule cells to extend and migrate toward a specific destination. In angiogenesis, the major branching signal is Vascular Endothelial Growth Factor (VEGF); in the respiratory system, Fibroblast Growth Factor (FGF) signaling is critical in both mammalian lung and insect trachea development (Cardoso and Lü, 2006; Hoeben et al., 2004; Metzger and Krasnow, 1999). In each case, branching signals activate a receptor tyrosine kinase, which transduces the Ras-ERK signaling cassette and promotes cell proliferation and cell motility (Koch and Claesson-Welsh, 2012; Metzger and Krasnow, 1999; Morrisey and Hogan, 2010). FGF signaling is also crucial for guiding cell migration during vertebrate and invertebrate gastrulation. FGF4 and FGF8 in the early mouse and chick gastrula direct the migration of epiblast cells out of the primitive streak (Ciruna and Rossant, 2001; Yang et al., 2002).

In angiogenesis, branching signals immediately induce the specification of endothelial tip cells that lead the sprouting and migration of new vessel branches
Phng and Gerhardt, 2009). Tip cells, which are located at the leading edge of extending sprouts, are characterized by extensive filopodia, by their migratory activity, and by the expression of Dll4, the transmembrane ligand for Notch signaling. The activation of cells adjacent to the tip cells activates Notch signaling; these cells adopt the fate of stalk cells (SCs), which have fewer filopodia, are less motile, and follow the tip cells in the sprouting process (Hellström et al., 2007). Through a lateral inhibition mechanism, each sprout is led by a single tip cell that is followed by SCs.

The branching morphogenesis of the Drosophila trachea system is governed by FGF signaling (Ghabrial et al., 2003; Sutherland et al., 1996). Tracheal primordia are specified in each side of the T2 to A8 segment as a cluster of 60 to 80 cells. After invagination, the tracheal primordial cells start expressing the FGF receptor (FGFR) Breathless (Btl) (Klambt et al., 1992). Drosophila FGF, also known as Branchless (Bnl), which is expressed at specific locations of the mesodermal and ectodermal tissues surrounding each tracheal primordium, activates Bnl/FGF signaling in a subset of tracheal cells that form the primary branches (Klambt et al., 1992; Sutherland et al., 1996). Delta and an active phosphorylated form of ERK (dp-ERK) are strongly expressed at the tip of each primary branch (Gabay et al., 1997; Ikeya and Hayashi, 1999). Through lateral inhibition, Delta-positive cells converge into a single cell in each branch, and this cell has numerous filopodia and strong migratory activity (Klambt et al., 1992; Llimargas, 1999).

Two types of cells differentiate from the tip of migrating tracheal branches in later
embryonic stages. Fusion cells (FCs) form anastomoses in the dorsal trunk, lateral trunk, dorsal branch, cephalic branch, and ventral branch by adhering in a pairwise manner and converting into a torus shape to connect the lumen (Caviglia and Luschnig, 2014; Gervais et al., 2012; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). Terminal cells (TCs) differentiate to extend long cytoplasmic extensions (terminal branches) that cover target tissues and exchange air with the intracellular lumen (Guillemin et al., 1996; Samakovlis et al., 1996a). After the primary branches are specified and have navigated toward their specific directions, Bnl/FGF signaling performs a second tracheal function, that of promoting TC differentiation (Gervais and Casanova, 2011; Lee et al., 1996; Reichman-Fried and Shilo, 1995) and navigation (Miao and Hayashi, 2015). Some tracheal branches develop both an FC and a TC and extend the terminal branch from the tube-connection point. While some mechanisms that suppresses emergence of tip cells has been elucidated (Caviglia and Luschnig, 2013; Chen et al., 1998), how the two types of tip cells are selected from the pool of Btl/FGFR-active, migration-competent branch tip cells after Notch-induced lateral inhibition is not understood.

Here we addressed the cell-type diversification of Btl/FGFR-activated branch tip cells. We show that the early FC marker Escargot (Esg) plays a central role in tip-cell diversification by promoting the expression of another FC gene, Dysfusion (Dys), and suppressing the expression of the TC gene Drosophila Serum Response Factor (DSRF). In addition, Esg suppresses Bnl/FGF signaling, partly by downregulating the
FGF-signal transducer Downstream of FGF (Dof). Therefore, the fusion competence of specific tracheal branches is acquired through a suppression of the default TC fate by Esg.

Results

Dorsal branch development in *Drosophila*

To investigate the mechanism of divergent cell-fate determination under Bnl/FGF signaling, we focused on the dorsal branch (DB), which migrates dorsally and fuses with another DB from the contralateral side at the dorsal midline (Kato et al., 2004; Samakovlis et al., 1996b). At stage 15, the DB tips have reached the dorsal margin of the dorsal epidermis (DE) and remain fixed to the same location in the DE (Kato et al., 2004). DB tips are brought to the dorsal midline by the dorsal-closure movement (Kato et al., 2004; Kato et al., 2016). At this stage, three cell types are distinguished by specific cell shapes and marker-gene expression (Fig. 1I): the FC (green), which is located in the dorsal-most position and mediates branch fusion; the TC (red), which is located on the anterior side of the DB tip and sprouts a long terminal branch ventrally along the compartment boundary; and the SCs (blue), which are tandemly aligned behind the FC (Fig. 1I). The FC and TC are marked by the expression of the transcriptional factors Esg and *DSRF*, respectively (Fig. 1F,H, J) (Guillemin et al., 1996; Tanaka-Matakatsu et al., 1996). At early stage 14, the DB tip consisted of a pair of cells, of which the anterior-most cell expressed Esg (Fig. 1G, I, J) but did not
express DSRF at a detectable level (Fig. 1E-E”).

To elucidate the role of Bnl/FGF signaling in DB development, we examined the expression of Dof (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998), an intracellular protein that acts downstream of Btl/FGFR and upstream of Ras. Dof is specifically expressed in cells expressing either Drosophila Btl/FGFR or Heartless (Htl), and is needed for the activation of MAPK signaling via FGF signaling (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Dof is first expressed in all tracheal cells during stages 10 to 12, and is then strongly expressed in the tip cells at stage 13 (Supplementary Fig. S1). Dof expression in the trachea is similar to the RNA expression of dof and btl (Ohshiro and Saigo, 1997; Vincent et al., 1998). At stage 14, Dof expression was concentrated in the TC, although it was still expressed in the FC and, though weakly, in SCs (Fig. 1A, A’). After stage 15, when the TC extends long terminal branches, Dof was expressed only in the TC (Fig. 1B, B’), which also activated ERK (Fig. 1C,D), These observations suggested that Dof can be used as a marker to trace Bnl/FGF signaling in tracheal cells.

**Differential roles of Esg and Dys in FC specification**

Our data indicated that the onset of Esg expression coincides with the FC specification. Dys is a basic helix-loop-helix (bHLH)-PAS transcription factor expressed in FCs (Jiang and Crews, 2003; Jiang and Crews, 2006). Although both Esg and Dys are required for DB fusion, whether their roles in the fusion process
overlap has been not addressed. Thus, we used live imaging to compare the phenotypes of mutant esg and dys embryos. In control embryos at late stage 15, FCs from each side extend numerous filopodia and contact each other at the dorsal midline, establish new cell adhesion interfaces, and change into a compact torus shape with a very short lumen to connect the two DBs (Fig. 2A, B-B”, Supplementary Movie S1) (Gervais et al., 2012; Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). In esg-mutant embryos, FCs reached the dorsal midline and contacted each other, but failed to establish new adhesion interfaces; they instead continued to elongate into an extensively winding form with what appeared to be a TC-like internal lumen (Fig. 2C, D-D”, Supplementary Movie S2). This live-imaging analysis confirmed a previous observation, based on fixed preparations, that esg-mutant FCs acquire a TC-like character (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996).

In dys-mutant embryos, the FCs also failed to complete fusion (Jiang and Crews, 2006). High-resolution live imaging revealed differences in the FC phenotypes between the dys and esg mutants. The FCs in dys mutants visualized with btl-RFPmoe formed fewer filopodia than did the controls and esg mutants labeled with the same marker, and migration of FC in dys mutant was retarded: in 54% of the metameres (22 out of 41 fusion point, 11 embryos) the FCs failed to reach the dorsal midline or to contact other FCs (Fig. 2E, F-F”, Supplementary Fig. S2, Supplementary Movie S3, contact failure rate in control: 0.02%, 1/59 fusion point, 11 embryos; esg-: 12.5%, 2/16 fusion point, 3 embryos). No TC-like behavior or appearance of luminal
structure was observed in dys-mutant FCs (Fig. 2F”). These observations indicate that esg and dys play distinct roles in tracheal-branch fusion.

**Epistatic relationship of esg, dys, and DSRF**

To understand the relationship between esg and dys, it was important to clarify their epistatic relationship. We found that the Esg expression in DB FCs was unaltered in the dys-mutant background (Fig. 3A1, A2), consistent with a previous report that an esg-lacZ reporter remains expressed in embryos injected with double-strand dys-transcript RNA (Jiang and Crews, 2003). Jiang and Crews (2003) reported that Dys expression is lost in FCs of the DB, the lateral trunk, and the first three ganglionic branches in esg-mutant embryos, but is maintained in DT. Down-regulation of Dys expression in the FC of DB was confirmed (Fig. 3B3). However, the antibody staining experiment was insufficient to verify if Dys expression was totally diminished in esg mutants. In order to address this point, we used more sensitive method of monitoring FC-specific enhancer activity of dys (Jiang et al., 2010)(Jenett et al., 2012) and found that the dys FC enhancer remained active in DB and DT of esg mutant trachea. (Fig. 3D, E). We concluded that esg-dependent and esg-independent pathways coordinately regulate FC-specific expression of dys.

We next studied the expression of the TC marker DSRF. In esg mutants, DSRF-positive cells were duplicated in 94% of the DBs (66 out of 70 DBs, 10 embryos, Fig. 3C3, Fig. 4B), consistent with a model in which Esg negatively
regulates DSRF and suppresses the TC phenotype in FCs (Samakovlis et al., 1996b). On the other hand, the number of DSRF-positive cells was unchanged in the dys mutants (Fig. 3C2, Fig. 4B). The Esg and Dys expressions were unaltered in DSRF mutants (Fig. 3A4, B4), suggesting that Esg regulates dys and DSRF independently.

To further characterize the changes in tip-cell specification in mutant embryos, we studied LacZ expression under the control of the esg enhancer (esg-lacZ). The long persistence of the LacZ product permits monitoring of the current and past transcriptional states of esg in mutant cells. In control embryos, the DB tip contained pairs of DSRF+ (TC type) and esg-lacZ+ single-positive cells (FC type) (Fig. 4A class 1, B, C1). In 74% of the esg-mutant DBs, TC-type cells were paired with DSRF, esg-lacZ double-positive cells (mixed-identity type, 52 out of 70 DBs, 10 embryos, Fig. 4A, B, C2); we called this combination class 2A. This finding implied that one of the tip cells, once activated the esg enhancer, expressed DSRF. In 20% of the cases, both of the two tip cells were of the mixed type (14 out of 70 DBs, 10 embryos, class 2B, Fig. 4B, C3). In dys embryos, the majority of the DB tip cells (93%) were class 1, with a minor fraction of other classes (42 out of 45 DBs, 7 embryos, Fig. 4B).

To further verify this epistasis model, we generated esg;dys double mutants. The double-mutant trachea showed a variety of defects, including the appearance of intracellular lumen in the leading cells (similar to the esg mutant) and delayed DB migration (similar to the dys mutant) (Fig. 2G, H", Supplementary Movie S4). Class 2 tip-cell phenotypes were seen in 36% of the double-mutant DBs (Fig. 4CF, 17 out of
47 DBs, 5 embryos). In addition, we observed a novel phenotype in which the FCs were lost (class 3A, 3B, single tip cells of TC or mixed type, Fig. 4C4, C5). The remaining TCs sometimes bifurcated and extended dorsal and ventral protrusions (Fig. 2G, I-I", Supplementary Movie S5). This single tip-cell phenotype was characterized by single tip cells with DSRF expression, and was seen in 62% of the double-mutant DBs (class 3A and 3B, 29 out of 47 DBs, 5 embryos, Fig. 4B, C4, C5).

In some case of the double mutant DB, *esg-lacZ* positive cells were found in DB stalk (Fig. 4D, E, E', 3 out of 29 single tip cell DB), indicating that misplacement of prospective FC in stalk accounts for some case of the single tip-cell phenotype.

Based on these observations, we concluded that *esg* and *dys* have distinct roles in FC specification: *esg* specifies fusion competence by promoting E-cadherin expression (Tanaka-Matakatsu et al., 1996) and suppressing TC differentiation (Samakovlis et al., 1996b), and *dys* specifies migratory competence by promoting filopodia formation (Jiang and Crews, 2006). The simultaneous loss of *esg* and *dys* caused FC mis-specification, resulting in transformation of the cells into TCs (class 2, 36%) or a loss of mutant FCs (class 3, 62%). In either case, the DB dorsal extension was delayed.

**Esg suppresses Bnl/FGF signaling in tracheal tip cells**

As shown above, Esg but not Dys suppresses the DSRF expression in FCs (Fig. 3C2, C3). Since DSRF expression depends on Bnl/FGF signaling (Sutherland et al., 1996),
we examined whether Esg regulates Bnl/FGF signaling. We first characterized the regulation of Bnl/FGF signaling in FCs by altering the expression of Btl/FGFR. We reduced the Btl/FGFR expression by expressing RNAi constructs driven by btl-Gal4, and observed that the Bnl/FGF-signaling markers Dof, dpERK, and DSRF were strongly inhibited (Fig. 5B, E, compare to Fig. 5A, D). Remarkably, Btl/FGFR overexpression by an FC-specific dys-Gal4 increased the Dof expression in FCs. This treatment, however, did not induce ectopic ERK activity or DSRF expression (Fig. 5C, F). The failure of ectopic Btl/FGFR to increase the Bnl/FGF signaling may be due to the limited source of Bnl/FGF.

Next, we studied the effect of Esg on Dof, dpERK, and DSRF. In esg mutants, Dof and dpERK were ectopically elevated in FCs (Fig. 5G, J). This elevation of Dof and dpERK was due to overactivation of FGF receptor, since ectopic Dof and dpERK was partially reduced by simultaneous down-regulation of btl (Fig. 5I, L). In contrast, Esg overexpression strongly reduced the Dof, dpERK, and DSRF in TCs (Fig. 5H, K). These results demonstrated that Esg suppresses Bnl/FGF signaling at the level of FGF receptor activation in tracheal tip cells.

The TC posterior-to-anterior position shift depends on esg

We noted that in control stage-14 DBs, when Esg expression was first detected, cells with high ERK activation and Dof expression were positioned posterior to the Esg+ tip cells (Fig. 1C, G, G’, I). Later, at stage 15, dpERK- and Dof-positive cells began
expressing DSRF and were found anterior to Esg+ cells (Fig. 1D, H, H', I). To clarify the reason for this positional shift, we observed the FC location relative to other tracheal cells. At the onset of expression of the esg enhancer Gal4 at early stage 14, the FCs were found in direct contact with the epidermis, and prospective TCs were positioned on top of the FCs (Supplementary Fig. S3A, A", Supplementary Movie S6). At late stage 14, prospective TCs moved anteriorly and made direct contact with the epidermis (Supplementary Fig. S3B-B”). Based on these observations, we concluded that this shift in position involves the migration of prospective TCs with high Bnl/FGF-signaling activity toward the anterior.

We next examined the role of esg in the TC anterior migration. We counted cells expressing esg-LacZ and DSRF, and detected this TC positional shift in 85% of the control embryo DBs (24 out of 28) at stage 15. The TC position shifts occurred at a similarly high frequency in dys mutants (90%, 38 out of 42). We also counted the frequency of TC positional shifts in type 2A branches of esg mutants. We found that esg-lacZ-, DSRF+ cells (authentic TCs) were positioned anterior to esg-lacZ+, DSRF+ cells (prospective FCs transformed into TCs) in 56% of the cases (29 out of 52). Therefore, the frequency of anterior positional shifts of prospective TCs was reduced to a near-random level. This result suggested that the proper anterior-posterior (AP) positioning of TCs and FCs is established by TC migration that depends on esg.
**Esg modulates Bnl/FGF-induced cell migration**

The TCs and FCs had contrasting migratory behaviors at stage 15. While TCs migrated from the posterior to the anterior of the DB tip and extended the terminal branch ventrally, FCs stayed near the leading edge of the DE, became polarized dorsally, and extended filopodia to contact FCs coming from the contralateral side. The direction of terminal branch migration is controlled by a combination of Hedgehog and Dpp signaling (Kato et al., 2004). In addition, localized Bnl/FGF is an attractive cue for terminal branch migration (Miao and Hayashi, 2015). Since paired TCs and FCs are exposed to very similar levels of signaling ligands, their distinct migratory behaviors must reflect their cell-type-specific interpretation of the signaling environment. To test this hypothesis, we compared the FC and TC responses to ectopic Bnl/FGF. We previously showed that TCs respond positively, extending the terminal branch toward cells that ectopically express Bnl/FGF from heat-shock (HS)-Bnl/FGF induced by laser (Miao and Hayashi, 2015).

We used IR-LEGO (Kamei et al., 2009) to induce Bnl/FGF ectopically in a prospective fusion position of early stage 14 embryos, and tracked the FC responses by live imaging (Fig. 6A-D, A’-D’, Supplementary Movie S7). At stage 14, all of the FCs migrated normally (Fig. 6B, B’). At late stage 15, when the FCs in control branches migrated to the dorsal midline and fused, the migration of the FC adjacent to the ectopic Bnl/FGF was arrested (7 out of 10 cases, Fig. 6C, C’, C’’, D, D’, D’’), or made a detour around the Bnl expressing cells (Supplementary Fig. S5). In contrast,
HS-eGFP expression only in prospective fusion position did not affect fusion cell migration and fusion (8 out of 8 cases, Supplementary Fig. S4 and Supplementary Movie S9). This finding suggested that Bnl/FGF signaling inhibits FC migration, in contrast to its role as an attractant for TCs.

To investigate whether $esg$ contributes to the inhibitory migratory response to Bnl/FGF, we repeated the experiment in $esg$-mutant embryos. The DB migration was indistinguishable in laser-treated and control segments (Fig. 6I-L, I’-L’, Supplementary Movie S8). The prospective FC extended a long terminal branch across the site of ectopic Bnl/FGF expression and reached the dorsal midline (5 out of 5 cases, Fig. 5K, K’, K”, L, L’, L”). These findings indicated that Esg regulates the inhibitory migratory response of FCs to Bnl/FGF.
Discussion

Esg coordinates the sequential specification of FCs and TCs

In the *Drosophila* tracheal system, the combined actions of Bnl/FGF and Wingless specify tip-cell fate by stimulating ERK signaling and the expression of *esg* and the Notch ligand *Delta* (Chihara and Hayashi, 2000; Ikeya and Hayashi, 1999; Llimargas, 1999; Llimargas, 2000). Delta is broadly expressed in all tracheal cells and is upregulated in the tip region. Through lateral inhibition, the tip-cell fate is restricted to single cells that begin expressing *esg* at stage 13 (Ikeya and Hayashi, 1999; Llimargas, 2000; Steneberg et al., 1999). The rest of the tracheal cells activate Notch signaling and acquire the SC fate by suppressing FC-marker genes and ERK signaling (Ikeya and Hayashi, 1999; Llimargas, 1999). After FC specification, the second mode of Bnl/FGF signaling begins. Btl/FGFR activation after this stage results in the specification of a TC expressing DSRF (Lee et al., 1996; Sutherland et al., 1996). Our findings show that the Esg-dependent suppression of Bnl/FGF signaling makes the FC insensitive to Bnl/FGF, after which the adjacent tip cell increases its Bnl/FGF signaling to the highest level found among DB cells and begins specification of the TC fate (Fig. 7A). At late stage 14, prospective TCs shifted position to the anterior side and begin expression of the TC marker DSRF (Fig. 7B). Therefore, FCs and TCs are sequentially specified by Bnl/FGF, and Esg plays a central role in changing the target of Bnl/FGF signaling from FCs to TCs.
Distinct roles of Esg and Dys

Our genetic epistasis analysis indicated that \textit{esg; dys} double mutants displayed phenotypes more severe than respective single mutants, indicating \textit{esg} and \textit{dys} have unique functions. High Dys expression in DB FC depends on \textit{esg} (Jiang and Crews, 2003), but \textit{esg}-independent pathway through the \textit{dys} FC enhancer stimulates low level of \textit{dys} transcription in \textit{esg} mutant background. Those pathways, together with \textit{dys}-independent \textit{esg} function collectively specify the FC character. Since SRF expression was unaltered in \textit{dys} mutants, the suppression of Bnl/FGF signaling was mainly ascribed to the \textit{dys}-independent, \textit{esg}-dependent pathway. \textit{dys} plays a role in branch migration through stimulating the filopodia formation (Jiang and Crews, 2006). Since \textit{esg} lies upstream of \textit{dys} expression, \textit{esg} is placed as a primary determinant of the FC fate. It should be noted that \textit{esg} and \textit{dys} functions are dispensable for the fusion of DT, where guidance of FGF producing cells plays a major role (Wolf et al., 2002).

Differential control of tip-cell migration

We found that the differential migratory behavior of DB tracheal tip cells occurs in two steps. The first is an antero-posterior positional shift in TCs and FCs occurring at late stage 14 (Fig. 7). The second is a polarized cell protrusion extending along the dorso-ventral axis at stage 15, when the FC sends filopodia toward the dorsal midline, and the TC sends the terminal branch ventrally. These differential migratory
behaviors can be explained by the sequential action of Bnl/FGF signaling as follows: at stage 14, bnl mRNA is expressed in stripes in each epidermal segment, and the DB tips are associated with the basal surface of bnl-positive epidermal cells (Kato et al., 2004). FC differentiation begins just after Esg is expressed in the anterior cell of the tip-cell pair (Fig. 7A). Bnl/FGF signaling in FCs declines as Esg expression increases, and the posterior of the paired tip cells, which is the prospective TC, elevates its Bnl/FGF signaling to the highest level (Fig. 7A), and, attracted to the source of the Bnl/FGF, migrates over the FC to complete the AP positional change (Fig. 7B).

The second phase of migration starts at stage 15. DSRF expression starts in the TC, which at this time has started to send a terminal branch along the AP compartment boundary. The ventral orientation of the terminal branch is determined by the positioning of the TC body relative to the epidermal region with the highest Bnl/FGF expression, the restriction of terminal-branch elongation to the Hedgehog-expressing P compartment, and the repulsive effect of Dpp expressed in the dorsal midline (Kato et al., 2004). FCs, on the other hand, have low ERK activity and are not positionally restricted by the Bnl/FGF source. In this regard, it is surprising that FCs were repulsed by ectopic Bnl/FGF expressed by a local laser heat shock. In esg mutants, the TC-like tip cells transformed from FCs were not repulsed by Bnl/FGF. We speculate that in the presence of Esg, Btl/FGFR’s ERK-signaling branch is suppressed in FCs, while other branches such as PI3K-AKT may still be
active and able to instruct a repulsive response to ectopic Bnl/FGF. In this regard, Akt phosphorylates Trachealless and regulates its nuclear localization (Jin et al., 2001). However, Akt’s role in tracheal-branch migration is not known.

**Esg regulates Dof and Bnl/FGF signaling**

We showed that the expression of Dof protein is elevated in the tip region of tracheal branches. This pattern is similar to that of *dof* and *btl* mRNA and requires Btl/FGFR (Fig. 1), indicating that Dof expression can be used as a read-out of Btl/FGFR signaling in tracheal cells. Interestingly, Btl/FGFR overexpression elevated the Dof in FCs. Since ERK signaling was not elevated in this condition (probably because the amount of Bnl/FGF available for receptor activation or other molecules in the signaling cascade was limited), it is likely that the increased level of Dof observed in the cytoplasm was due to protein stabilization by its interaction with Btl/FGFR, as occurs in yeast cells (Battersby et al., 2003). We have shown that Esg inhibits a high accumulation of Dof in FCs (Fig. 5). Since *esg* and *btl* double knockout eliminated ectopic Dof and ERK activation in FC, hyper activation of FGF receptor is likely the cause of *esg* mutant phenotype. Future work should be directed to elucidate the molecular mechanism of Esg-dependent suppression of Dof and FGF signaling.

Taken together, Esg acts as a central coordinator for tip-cell specification in the fusion branch, first by modulating Bnl/FGF signaling and second by controlling the FC-specific cell-shape conversion (Kato et al., 2016). The robust maintenance of *esg*
expression throughout fusion-branch migration and fusion is central to the stereotyped branching pattern of the *Drosophila* trachea. An Esg-like tip-cell regulator has not been identified in vertebrate blood vessels, which may explain the instability of tip-cell fate in blood vessels. The lack of a robust tip-maintenance program may allow the frequent conversion of tip cells and SCs that is essential for the flexibility seen in vessel remodeling.

**Materials and Methods**

**Fly stocks**

The following fly stocks were used in this study: *btl-Gal4* (Shiga et al., 1996), *UAS-GFP-moe* (Chihara et al., 2003), *HS-eGFP, HS-bnl* (Miao and Hayashi, 2015), *UAS-GFP-N-lacZ* (Shiga et al., 1996), *esg^{G66B}/CyO* (Whiteley et al., 1992), *dys^2*, *dys^3* (Jiang and Crews, 2006), and *bs^{PZ}* (Montagne et al., 1996). *UAS-esg* (Fuse et al., 1994), *UAS-btl wt* (Lee et al., 1996), *UAS-btl RNAi (y[1] sc^{*} v[1]; P[y^{+t7.7} v^{+t1.8}=TriP.HMS02038]attP2 ), UAS-dof/CyO* (Vincent et al., 1998), *btl-RFP-moe* (a gift from Markus Affolter), and *GRM13C07-Gal4 (dys-Gal4)* (Jenett et al., 2012) were described previously and were obtained from the authors or from BDSC. *esg_FC-Gal4* driver was constructed by inserting the FC enhancer of the *esg* genomic region (Kato et al., 2016). These stocks were cultured at 25°C.
IR-LEGO

The IR-LEGO system (IR-LEGO-1000, Sigma-Koki Co., Ltd., Saitama Japan) was combined with a confocal microscope (FV1000, Olympus) equipped with GaAsP detectors. The IR laser was introduced through the lateral camera port of an inverted microscope (IX81, Olympus). The setting was as previously described (Miao and Hayashi, 2015).

Live imaging

Confocal images were acquired using a laser-scanning confocal microscope (FV1000, Olympus) equipped with a PlanApo 60x NA1.40 oil IR lens; 512x512 pixel images of 1-µm-thick sections were captured every 1, 2, 3, or 5 minutes for 2 or 3 hours with a 1x, 2x, or 3x zoom with GaAsP detectors. The images were de-noised and projected with the in-house software Malma (Kagayaki Kato, unpublished).

Immunofluorescence and antibodies

The primary antibodies were: mouse anti-dpERK (1:1000, Sigma-Aldrich M9692 clone MAPK-YT); mouse anti-DSRF (1:1000); rat anti-Esg (1:100) (Fuse et al., 1994); rabbit anti-Dys (1:800, a gift from Lan Jiang); rabbit anti-Dof (1:200, a gift from Maria Leptin); and rabbit anti-β-gal (1:1000, Cappel). The chitin-binding probe (CBP) (1:50) was prepared from a bacterial-expression construct according to the protocol by
Yinhua Zhang (New England Biolabs). The secondly antibodies were: anti-mouse DIG biotin-sp conjugated (1:500, used to enhance dpERK staining, Jackson Laboratory), TSA-direct Cy3 (1:50 in amplification diluent), anti-mouse IgG Alexa 546 (1:500, Molecular Probes), anti-mouse IgG Alexa 633 (1:500, Molecular Probes), anti-rat IgG Cy3 (1:500, Jackson Laboratory), anti-rabbit IgG Alexa 555 (1:500, Molecular Probes), and anti-rabbit IgG Alexa 633 (1:500, Molecular Probes).
Author Contributions

G.M. and S.H. conceived the project, G.M. obtained the experimental data, and G.M. and S. H. wrote the manuscript.

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Competing interests

The authors declare no competing or financial interests.
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Figure 1. Normal Dorsal Branch (DB) development. Anti-Dof staining (A, A’, B, B’), anti-Dof and dpERK co-staining (C, D), anti-Dof and DSRF co-staining (E-E’’, F-F’’),
and anti-Dof and Escargot (Esg) co-staining (G, G', H, H') of btl-Gal4-driven UAS-GFP-moe embryos. (A, C, E, G) Early stage 14. (B, D, F, H) Late stage 15. (A') and (B') are magnified views of (A) and (B), respectively. (E'-E'') and (F'-F'') are single channel images of (E) and (F), respectively. (G') and (H') are x-z views of the area between the yellow lines in (G) and (H), respectively. Scale bar: 10 µm. (I) Schematic drawings of the DB at stages 14 and 15, showing Esg-positive cells (green), dpERK-positive cells (red), and SCs (blue). (J) A summary of gene-expression patterns in DB tip cells.
Figure 2. Differential roles of Escargot (Esg) and Dysfusion (Dys) in fusion cell (FC) specification. Overall views of a btl>GFPMoe embryo (A) and an esg mutant (C), dys mutant (E), and esg, dys double mutant (G). High-magnification snapshots of btl>GFPMoe (B-B”), esg mutant (D-D”), dys mutant (F-F”), and esg,dys double-mutant (H-H”) (I-I”) embryos. Note that btl-RFP-moe was used as a marker for
*dys* mutant, while others are marked with *btl>GFP-moe*. White square brackets: the gap between the FC and its partner cell. Yellow arrows: prospective fusion points. Red arrowheads: ectopic terminal cell (TC)-like cells. Scale bar: 10 µm.
Figure 3. Epistatic relationship of *esg*, *dys*, and *DSRF*. Immunostaining with anti-Escargot (*Esg*) (A1-A4), anti-Dysfusion (*Dys*) (B1-B4), and anti-DSRF (C1-C4) in control (A1, B1, C1), *dys* mutant (A2, B2, C2), *esg* mutant (A3, B3, C3), and *DSRF* mutant (A4, B4, C4) embryos. The expression pattern of *dys* enhancer-gal4 construct detected with UAS-GFPmoesin reporter in *esg* mutant heterozygotes (D, D”) and homozygotes (E, E”). (D, E) Low magnification view. (D’, E’) high magnification view of DB. Yellow arrowheads: *Esg*, *Dys*, *DSRF*, or GFP. Note that anti-Dys antibody gave non-specific labeling of terminal branch that persisted in *dys* mutant embryos. White brackets: the absence of *Esg*, *Dys*, or *DSRF* expression. Scale bar: 10 µm.
Figure 4. Synergistic interaction of *esg* and *dys* mutants.

(A) Five classes of tip cells and their gene expression in different mutants. (B) The frequency of different tip-cell phenotypes in mutants. (C1-C5) Anti-*esg*-lacZ and...
-DSRF staining for the five classes. (D, E, E') *esg-lacZ* expressed in stalk cells in *esg; dys* double mutant embryos. (D) Stage 16. (E) Stage 14. (E') Single section was used to show the localization of lacZ positive cell. Yellow arrowheads: tip cells(C1-C5) or LacZ positive stalk cells(D, E, E'). Scale bar: 10 μm.
Figure 5. Esg suppresses Branchless/FGF signaling in tracheal tip cells. (A-C, G-I) Immunostaining with anti- Dof and anti-DSRF. (D-F, J-L) Immunostaining with anti-Dof and anti-dpERK. Embryos: (A, D) btl>GFP-moe, (B, E) btl>GFP-moe, UAS-btl-RNAi, and (C, F) dys>btl wt. Inset of F shows a single section of a DB tip marked with dotted rectangle. (G, J) btl>GFP-moe, esg-/esg-, (H, K) btl>GFP-moe, UAS-esg, and (I, L) btl>GFP-moe, UAS-btl-RNAi, esg-/esg- embryos. Yellow arrowheads: Dof, DSRF or dpERK. White brackets show the absence of Dof, DSRF, or dpERK expression. Scale bar: 10 µm.
**Figure 6.** Escargot (Esg) modulates Branchless/FGF-induced cell migration. (A-D, A’-D’) Snapshots of HS-eGFP, HS-Bnl, dys>GFP-N, and btl-RFP-moe embryos showing that the induction of ectopic Branchless (Bnl)/FGF inhibited the dorsal branch (DB)-fusion process. (I-L, I’-L’) Ectopic Bnl/FGF was induced in the fusion position in an esg-mutant embryo. (A, A’) (I, I’) Early stage-14 embryo. Asterisk: the heat-shock position. The strong green fluorescence is auto-fluorescence in yolk cells. (B, B’) (J, J’) Late stage-14 embryo. (C, C’)(K, K’) Stage-15 embryo. (D, D’) (L, L’) Stage-16 embryo. (C”, D”, K”, L”) magnified view of boxed region in C, D, K, L. Yellow arrowheads: HS-eGFP. Yellow arrows: prospective fusion points. (E-H) Schematic drawings of the DB-fusion process showing fusion cells (FCs; green), terminal cells (TCs; red), and stalk cells (SCs; blue). Asterisk: the heat-shock position. The blue line shows the leading edge. Scale bar: 10 µm.
Figure 7. Esg coordinates the sequential specification of FC and TC. Schematic drawings the regulatory network under Bnl/FGF signaling and Esg in the DB at late stages 13 to early stage 14 (A) and late stage 14 to later stages (B), showing Esg-positive cells (green), dpERK-positive cells (red), and SCs (blue).
Supplementary Information

Supplementary Figures

Fig. S1. Dof expression patterns: Anti-Dof staining of embryos carrying btl>GFP-moe. (A) At stage 11, Dof is expressed in all cells in the tracheal primordia that have completed invagination. (B) At stage 12, Dof is expressed in nearly all tracheal cells undergoing Branchless/FGF-induced primary branching. (C) At stage 13, Dof is downregulated in the middle of the trachea, which is populated by stalk cells of the dorsal trunk and dorsal branch (DB). (D) At stage 14, strong Dof expression is maintained in the DB tip and the ventral side (ganglionic branch and lateral trunk). Scale bar: 10 µm.
Fig. S2. *dys* mutant phenotype. DB movement was monitored by *btl-RFP-moe* marker. The number of filopodia in FC was reduced in *dys* mutant (B, identical to Fig. 2F, F’, F’’), compared with control trachea that showed abundant filopodia (A). This figure is presented to show control image of *btl-RFP-moe*. Scale bar: 10µm.
Fig. S3. Antero-posterior positional change of terminal cells at stage 14.

Snapshots from live images of the DB8 tip in an embryo carrying the fusion-cell (FC) marker *esg.FC>GFP-moe* and the pan-tracheal marker *btl-RFP-moe*, which labels terminal cells (TC) and stalk cells (SCs). The x-z and y-z views show sections of the regions marked by yellow lines. (A) When GFP was first detected in the FC at early stage 14, the FC was closely associated with the epidermis (white dotted line) and the RFP single-positive TC was positioned below the FC. (B) Still at stage 14, 30 minutes later: the TC had completed anterior migration and the tip cells were positioned side-by-side next to the epidermis. Orientation of anterior-posterior and dorsal-ventral axis is indicated below A”. Scale bar: 10 µm.
Fig. S4. HS-eGFP did not affect dorsal branch fusion. Snapshots from HS-eGFP, 
dys>GFP-N, btl-RFP-moe embryo show induction of eGFP did not inhibit the 
fusion process of dorsal branch. Asterisk: the heat-shock position. The strong 
green fluorescence is auto-fluorescence in yolk cells and vitelline membrane. 
Yellow arrowheads: HS-eGFP. Yellow arrows: prospective fusion points. Scale 
bar: 10µm.
Fig. S5. Detour of FC around Bnl expressing cells. Snapshots from HS-eGFP, HS-Bnl, btl-RFP-moe embryo. Cells at the heat-shocked position (asterisk) were split into two clusters expressing HS-eGFP (arrowhead in C). Protrusions of the pair of FCs in DB7 navigated through the channel between the two GFP positive cells and contacted with each other. Arrowheads: HS-eGFP positive cells. Scale bar: 10µm.
Movies

Movie S1.

Dorsal branch migration and fusion in a control btl>GFPMoe embryo; 10 confocal sections (1-µm intervals) were taken every 59 sec for 1 hour. Scale bar: 10 µm. Imaging conditions are the same for all movies unless otherwise stated.
Movie S2.

An $esg^{G668}$ homozygote embryo.
Movie S3.

A dys²/dys³ embryo carrying btl-RFP-moe.
Movie S4.

An *esg^{G66B} / esg^{G66B}· dys^{2} / dys^{3}* embryo carrying *btl>GFP-moe*, showing the duplication of the terminal-cell (TC) phenotype (left-side pair, class 2) and single TC phenotype (central pair, class 3). Interval: 172 sec.
Movie S5.

A second example of an \textit{esg}^{G66B} / \textit{esg}^{G66B}; \textit{dys}^2 / \textit{dys}^3 embryo, showing the bifurcation of a single terminal cell.
Movie S6.

The anterior migration of terminal cells (TCs) at stage 14. The TC (magenta) is labeled with \textit{btl-RFP-moe}. Fusion cells were labeled with \textit{esg.FC>GFP-moe}. Anterior, left; dorsal, up. Images were taken every 124 sec.
Movie S7.

Ectopic Branchless (Bnl)/FGF expression arrests fusion-cell (FC) migration. Local IR illumination was directed immediately dorsal to the dorsal-branch tip (asterisk at 0:00:00) in HS-GFP/dys-Gal4; UAS-GFPN-lacZ / HS-Bnl, and btl-RFP-moe embryos. Images were taken every 295 sec. Arrowhead: GFP (and Bnl/FGF). Arrow: the affected FC. Scale bar: 10 μm.
Movie S8.

Branchless (Bnl)/FGF’s inhibition of fusion-cell (FC) migration requires esg. The laser heat-shock experiment was repeated on esg\textsuperscript{G66B} / esg\textsuperscript{G66B}; HS-eGFP / HS-Bnl and btl-RFP-moe embryos. FCs that had transformed into terminal cells were not arrested by ectopic Bnl/FGF. Interval: 176 sec.
Control experiment without Ectopic Bnl. HS-GFP/dys-Gal4; UAS-GFPN-lacZ / btl-RFP-moe embryo received local IR illumination at the position immediately dorsal to the DB tip (asterisk in time 0:00:00) and time lapse images were taken every 313 sec. FC migration and fusion was not affected by HS-eGFP expression.