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Cell Autonomy of HIF Effects in *Drosophila*: Tracheal Cells Sense Hypoxia and Induce Terminal Branch Sprouting

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SUMMARY

Drosophila **tracheal terminal branches are plastic and have the capacity to sprout out projections toward oxygen-starved areas, in a process analogous to mammalian angiogenesis. This response involves the upregulation of FGF/Branchless in hypoxic tissues, which binds its receptor Breathless on tracheal cells. Here, we show that extra sprouting depends on the Hypoxia-Inducible Factor (HIF)-α homolog Sima and on the HIF-prolyl hydroxylase Fatiga that operates as an oxygen sensor. In mild hypoxia, Sima accumulates in tracheal cells, where it induces *breathless*, and this induction is sufficient to provoke tracheal extra sprouting. In nontracheal cells, Sima contributes to *branchless* induction, whereas overexpression of Sima fails to attract terminal branch outgrowth, suggesting that HIF-independent components are also required for full induction of the ligand. We propose that the autonomous response to hypoxia that occurs in tracheal cells enhances tracheal sensitivity to increasing Branchless levels, and that this mechanism is a cardinal step in hypoxia-dependent tracheal sprouting.**

INTRODUCTION

Animal physiology and development are modulated by a wide variety of external stimuli, such as temperature, light, nutrient availability, and oxygen tension. Adaptation to environmental conditions often involves upregulation or repression of specific sets of genes that contribute to maintain the homeostasis of cells or whole organisms. Animals typically respond to low oxygen tension (hypoxia) by improving oxygen transport from respiratory surfaces to tissues, and by shifting ATP production from oxidative mechanisms to anaerobe glycolysis. In many lower species, enhanced tolerance to hypoxia results from a marked and controlled reduction of metabolism (Hochachka, 1986; Hochachka et al., 1996). In mammals, oxygenation is optimized through various physiological adaptations, including an increase of heart and respiratory frequency, synthesis of more red blood cells, vasodilatation, and angiogenesis. Angiogenesis is a widespread developmental process that is common to many physiological and pathological conditions such as formation of the placenta, endometrial growth, wound healing, ischemic heart disease, stroke, and cancer (Carmeliet, 2003; Folkman and Klagsbrun, 1987). It encompasses the formation of new capillaries from pre-existing blood vessels, and it is triggered when hypoxic cells secrete angiogenic growth factors, mainly the Vascular Endothelial Growth Factor (VEGF), which binds its receptors on the endothelial cells of blood vessels (Ferrara et al., 2003; Levy et al., 1995; Shweiki et al., 1992). Transcriptional induction of VEGF in oxygen-starved cells largely depends on the accumulation of Hypoxia-Inducible Factors (HIFs) (Carmeliet et al., 1998; Pugh and Ratcliffe, 2003), although nontranscriptional, hypoxia-dependent mechanisms, such as VEGF mRNA stabilization and translational induction, also contribute to the angiogenic response (Levy et al., 1996a, 1996b). HIF proteins are heterodimers of basic-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) proteins (Wang et al., 1995), in which the β subunit is constitutive and the α subunit is regulated by oxygen through various concurrent mechanisms, including oxygen-dependent proteasomal degradation (Huang et al., 1998; Pugh et al., 1997), transcriptional coactivator recruitment (Hewitson et al., 2002), and subcellular localization (Kallio et al., 1998; Luo and Shibuya, 2001). HIF-α polyubiquitination, which precedes proteolysis, depends on the hydroxylation of two key prolyl residues, localized in the so-called oxygen-dependent degradation domain (ODDD) (Ivan et al., 2001; Jaakkola et al., 2001). Hydroxylation is mediated by 2-oxoglutarate/iron(II)-dependent prolyl-4-hydroxylases, which use dioxygen as a cosubstrate for catalysis and, hence, are considered to be bona fide oxygen sensors (Bruick and McKnight, 2001; Epstein et al., 2001).

In *Drosophila melanogaster*, the cellular machinery that mediates the transcriptional response to hypoxia is highly conserved with the mammalian HIF system (Gorr et al., 2004, 2006; Lavista-Llanos et al., 2002; Nagao et al., 1996). The fly HIF is also a bHLH-PAS heterodimer in which the oxygen-regulated α subunit is a protein called Similar (Sima) (Bacon et al., 1998; Nambu et al., 1996), whereas the constitutive β subunit is named Tango (Ma and Haddad, 1999; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). Sima is stable in hypoxia but is readily degraded

in normoxia (Gorr et al., 2004); degradation requires oxygen-dependent hydroxylation of a specific prolyl residue (Arquier et al., 2006), which is mediated by a prolyl hydroxylase domain (PHD) protein encoded by the *fatiga* gene (Bruick and McKnight, 2001; Centanin et al., 2005; Lavista-Llanos et al., 2002). Consistent with this, *fatiga* loss-of-function mutants show high levels of Sima protein in normoxia and, consequently, the constitutive induction of Sima target genes.

In insects, the circulatory system is rudimentary and, with few known exceptions of hypoxic specialists (Burmester and Hankeln, 2007), does not participate in oxygen transport. Instead, oxygen reaches organs and tissues of the body directly through the tracheal system, a network of ramified, interconnected, and progressively narrowing air-containing epithelial tubes (Affolter et al., 2003; Ghabrial et al., 2003). By midembryogenesis, the *Drosophila* tracheal system develops from ectodermal primordia, in a process that largely depends on guided cell migration (Samakovlis et al., 1996). Chemoattraction of tracheal cells is exerted by the fibroblast growth factor (FGF) homolog Branchless (Bnl), which is expressed in small cell clusters in nontracheal tissues (Sutherland et al., 1996) and binds to the FGF receptor Breathless (Btl) on the surface of tracheal cells (Klambt et al., 1992). Bnl expression is highly dynamic; once a tracheal leading cell has reached a Bnl-positive cluster, *bnl* expression ceases and the gene is turned on again in a new cluster a few cell diameters further on the track of the forming branch. Thus, *bnl* is turned on and off many times along the path of the growing branches (Sutherland et al., 1996). By the end of embryogenesis, this genetically specified phase of tracheal development is complete and the general branching pattern has been established. At this stage, specialized cells in all tracheal branches—the terminal cells—differentiate and acquire the capacity to sprout out projections called terminal branches (Guillemin et al., 1996). In larval stages, the terminal branches are plastic and have the capacity to ramify and invade oxygen-starved areas in target tissues (Jarecki et al., 1999), in a process analogous to mammalian angiogenesis (Metzger and Krasnow, 1999). It has been shown that Bnl protein accumulates in hypoxia, and that its ectopic expression in nontracheal cells results in the attraction of tracheal terminal branches that sprout out from neighboring terminal cells. Thus, it has been proposed that the angiogenic-like response of the *Drosophila* tracheal system is triggered by hypoxia-inducible expression of Bnl in nontracheal cells, which promotes the formation of new terminal ramifications (Jarecki et al., 1999).

Here, we show that *Drosophila* larval tracheal terminal cells can induce gene expression in hypoxia with high sensitivity, and that they can respond to the hypoxic stimulus by upregulating the receptor Btl. This transcriptional response, which is dependent on the oxygen sensor Fatiga and the transcription factor Sima, contributes to the tracheal morphological changes occurring in larvae exposed to hypoxia. Our results, therefore, expand the previous "Bnl-centered model" (Jarecki et al., 1999) and demonstrate that tracheal cells play an active role in sensing oxygen levels and responding to hypoxia. We propose that upon exposure to hypoxia, tracheal cells execute a cell-autonomous response that turns on a "searching mode" that sensitizes them to available and increasing levels of the ligand. We speculate on the implications of this mechanism in mammalian angiogenesis.

RESULTS

Fatiga and Similar Mediate Tracheal Terminal Sprouting

To address whether Sima and Fatiga participate in the regulation of tracheal terminal sprouting, we began by analyzing in detail phenotypic alterations of the tracheae of third-instar larvae exposed to hypoxia. For quantitative purposes, we focused on the dorsal branch of the third segment (Figure 1A), whose terminal cell has a characteristic branching pattern, typically comprising of a main cellular process from which straight cellular extensions of about 1 μm diameter (hereafter, "Thick Terminal Branches" [TTBs]) project and thinner extensions ramify thereafter (Figures 1B and 1C). The average number of TTBs at the third dorsal branch of wild-type larvae maintained in normoxia was 5.65 (N = 116) (Figure 1D; Table 1). In hyperoxia (60% O_2), we observed a slight but significant reduction of TTBs (4.82; N = 125) (Figure 1E; Table 1), whereas in hypoxia (5% O_2), the average TTB number increased to 8.76 (N = 109) (Figure 1F; Table 1). Of note, this increase in the number of TTBs was paralleled by a similar increase in the number of thinner terminal projections. Third-instar larvae displayed an average of 17.0 ± 2.7 thin projections at the third dorsal branch in normoxia (N = 35) and an average of 30.7 ± 3.4 (N = 56) thin projections at 5% O_2, implying that, upon hypoxic exposure, the number of thin terminal projections increase to an extent similar to that of the TTBs (Table 1). These results confirm the tight correlation between tracheal terminal branching and oxygen levels that has been known since Wigglesworths' pioneering studies in the 1950s (Jarecki et al., 1999; Wigglesworth, 1983), and they establish the number of TTBs as a good read out to analyze the extent of terminal branching. Next, we tested whether *fatiga* mutant larvae, which are known to accumulate high levels of Sima protein in normoxia (Centanin et al., 2005), have alterations in the number of ramifications. We used the fga^1/fga^9 allelic combination that can develop to the third-larval instar, and we observed that, under normoxic conditions, these larvae displayed an average of 9.49 TTBs (N = 72) (Figure 1G) and 43.6 ± 5.3 thin terminal projections (N = 22) at the third dorsal branch, an overall number of ramifications even higher than that of wild-type larvae exposed to hypoxia (Table 1). To test whether increased levels of Sima can account for the excess of TTBs in *fga* mutant larvae, we analyzed *fga sima* double homozygous individuals, which are viable to adulthood (Centanin et al., 2005). In *fga sima* double mutant larvae, the number of TTBs (5.14; N = 66) and thin terminal projections (18.2 ± 3.3; N = 14) reverted to wild-type levels (Figure 1I; Table 1), which, in turn, was very similar to those of *sima* homozygous mutants (5.17 TTBs; N = 71 and 18.9 ± 2.7 thinner projections; N = 18) (Figure 1H; Table 1) (Centanin et al., 2005). These findings suggest that the extraterminal sprouting phenotype observed in *fga* mutants is due to increased levels of Sima protein.

To gather additional evidence of the participation of the Fatiga (Fga)-Sima system in tracheal hypoxia-dependent plasticity, we focused on a different oxygen-dependent modification that is typical of hypoxic larvae: upon exposure to 5% O_2, most tracheal branches become tortuous and, in particular, ganglionic branches (the branches that reach the central nervous system) (Figure 1J) adopt a ringlet appearance (Figures

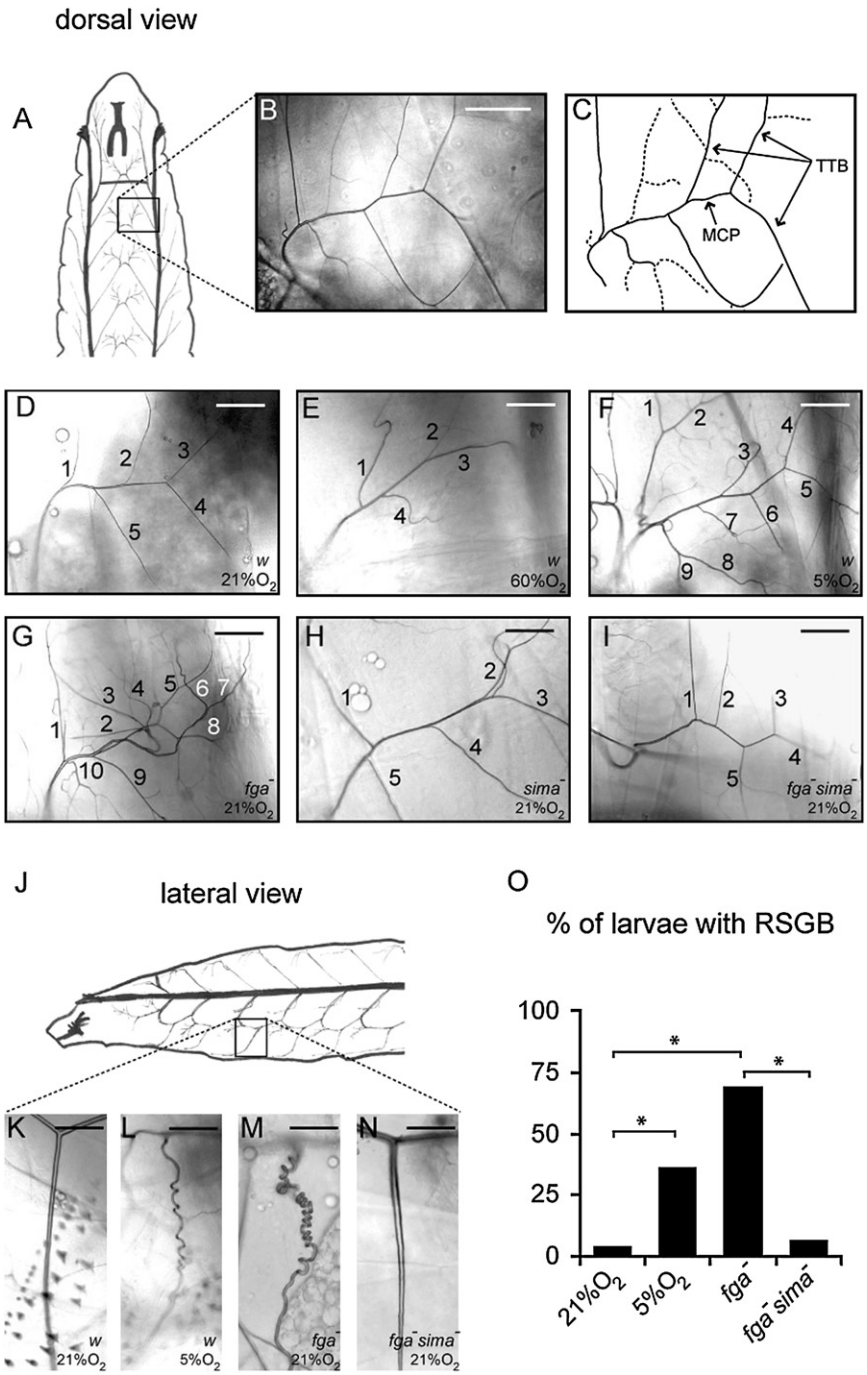

Figure 1. Tracheal Plasticity in Response to Hypoxia Depends on the Prolyl Hydroxylase Fatiga and the HIF-α Homolog Similar

(A) Scheme of a dorsal view of a *Drosophila* third-instar larva showing the position of a third segment dorsal branch terminal cell.

(B and C) (B) Photograph and (C) scheme showing the morphology of a terminal cell of a wild-type larva grown in normoxic conditions. A typical dorsal branch terminal cell comprises one main cellular process (MCP) from which five main cellular extensions of more than 1 μm diameter, the "thick terminal branches" (TTBs), project. Thinner terminal branches ramify from TTBs (dashed lines in [C]).

(D) The five TTBs of a normoxic wild-type larva are shown.

(E and F) (E) At 60% O_2 (hyperoxia), the number of TTBs is reduced; (F) at 5% O_2 (hypoxia), more TTBs project from the terminal cell.

(G) In *fatiga* (*fga*) mutant larvae, extra sprouting of TTBs occur, mimicking the effect of hypoxia (H and I).

(H and I) In (H) *sima* mutants or (I) *fga sima* double mutants, the number of TTBs is similar to that of wild-type normoxic larvae (quantification of these phenotypes is depicted in Table 1).

(J) Scheme of a lateral view of a larva marking the position of a ganglionic branch.

(K) Ganglionic branches of third-instar wild-type larvae grown in normoxia are straight.

(L) A proportion of the larvae exposed to hypoxia exhibit ringlet-shaped ganglionic branches (RSGBs).

(M) *fga* mutant larvae also display RSGBs.

(N) RSGBs do not occur in *fga sima* double mutant larvae.

(O) Proportion of third-instar larvae of different genotypes or grown in hypoxia displaying at least one RSGB.

*Significant differences, $p < 0.0001$, Fisher's test. Scale bars are 50 μm in (B)–(I) and 25 μm in (K)–(N).

1K and 1L). We quantified the proportion of larvae exhibiting at least one ringlet-shaped ganglionic branch (RSGB) and found that, in normoxic larvae, all ganglionic branches were straight (Figure 1K), whereas upon exposure to hypoxia (5% O_2), 36.4% of the larvae (N = 22) displayed at least one RSGB (Figures 1L and 1O). Among fga^1/fga^9 larvae, the proportion of individuals exhibiting at least one RSGB (Figure 1M) dramatically increased (Figure 1O), with 69.2% of them being RSGB positive (N = 13). Strikingly, in *fga sima* double mutants, the incidence of RSGBs was reduced again to almost wild-type levels (N = 30) (Figures 1N and 1O), suggesting that RSGBs in *fga* mutant larvae are also provoked by increased levels of Sima. These results paralleled those regarding the regulation of terminal sprouting, and they support the notion that oxygen-dependent tracheal plasticity of *Drosophila* larvae is controlled by the oxygen-sensing prolyl-4-hydroxylase Fga, through the regulation of Sima protein abundance.

Tracheal Terminal Cells Respond to Low Oxygen Levels with High Sensitivity

To examine if accumulation of Sima in extratracheal tissues can account for extra sprouting, we overexpressed Sima protein in random clones outside the tracheae. No effect on tracheal sprouting was observed whatsoever, but, instead, long cellular processes protruded from Sima-expressing cells (see

Table 1. Number of Thick Terminal Branches at the Third Dorsal Branch of Third-Instar Larvae of Various Genotypes at Different Oxygen Concentrations

Genotype, [O_2]	Thick Terminal Branches	N	Variation
w, 21% O_2	5.65 ± 0.09	116	100%
w, 60% O_2	4.82 ± 0.11[a]	125	85%
w, 5% O_2	8.76 ± 0.17[a]	109	155%
fga$^-$, 21% O_2	9.49 ± 0.23[a]	72	168%
sima$^-$, 21% O_2	5.17 ± 0.14	71	91%
sima$^-$ *fga*$^-$, 21% O_2	5.14 ± 0.13	66	91%
btl$^{-/+}$, 21% O_2	5.48 ± 0.68	56	97%
fga$^-$/*fga*$^-$ *btl*$^-$, 21% O_2	7.45 ± 0.16[b]	84	125%
bs$^{-/+}$, 21% O_2	5.37 ± 0.66	38	95%
fga$^-$/*fga*$^-$ *bs*$^-$, 21% O_2	7.79 ± 1.12[b]	42	138%
btl-Gal4/+	5.96 ± 0.14	74	100%
btl-Gal4/*UAS*-Sima	7.74 ± 0.17[c]	105	130%
btl-Gal4/*UAS*-Btl	7.89 ± 0.19[c]	85	132%

TTBs of the third dorsal branch were counted under a bright-field microscope. Values are means ± SE. "Variation": normalized values of TTBs in comparison to the TTB number in the control condition.
[a] Significant differences compared to wild-type control larvae (*w*).
[b] Significant differences compared to *fatiga*$^-$ homozygous larvae (Fga$^-$).
[c] Significant differences compared to *btl*-Gal4/+ control larvae ($p < 0.001$; one-way ANOVA and Bonferroni's postcomparison test).

the Supplemental Data and Figures S1 and S2, in the Supplemental Data available with this article online). Given Sima's ability to induce long cellular processes, we hypothesized that perhaps accumulation of Sima inside the tracheal cells contributes to tracheal terminal sprouting in hypoxia. Therefore, we sought to analyze hypoxia-dependent transcription in the tracheal cells. In previous studies, we had analyzed hypoxia-dependent transcription by using a Sima-dependent reporter and found that it is induced in tracheal cells with higher sensitivity than in any other tissue (Figure 2A) (Centanin et al., 2005; Lavista-Llanos et al., 2002). To characterize this phenomenon in more detail, we analyzed the expression of the same reporter upon exposure to more mild hypoxic conditions. At 15% O_2, the expression of the *ldh*-Gal4 *UAS*-LacZ.GFP$_n$ reporter was clearly observed in terminal cells, whereas expression in the remainder of the tracheal system was weak (Figure 2B). Remarkably, in normoxia (21% O_2), the expression of the reporter was still evident in terminal cells, whereas no expression at all could be detected in other tracheal cells (Figures 2C, 2D, and 2G). To test whether this expression in terminal cells responds to oxygen levels, we exposed the larvae to 60% O_2. No expression at all could be detected in these conditions (Figures 2E and 2G), indicating that gene expression in tracheal terminal cells is exquisitely sensitive to changes in oxygen levels. Moreover, the observed reporter expression was entirely dependent on Sima, as it was absent in *sima* homozygous mutant larvae at 21% O_2 (Figure 2F). Altogether, these results suggest that Sima-dependent transcription in tracheal terminal cells might contribute to tracheal extra sprouting.

Accumulation of the HIFα Protein Similar in Tracheal Cells Is Necessary for Extra Sprouting in Hypoxia

To address if Sima is required specifically in tracheal terminal cells for extra sprouting in hypoxia, we used the MARCM technique (Lee and Luo, 1999) to generate *sima* homozygous mutant cells positively labeled with EGFP, which were analyzed in third-instar larvae exposed to hypoxia (5% O_2). In larvae bearing EGFP-positive wild-type MARCM control cells, EGFP-expressing cells ramified to an extent very similar to that of their contralateral EGFP-negative cells (9.3 ± 1.6 versus 9.2 ± 1.9 TTBs; N = 28) (Figures 3A and 3C; Figure S3A). In contrast, *sima* homozygous mutant (EGFP-labeled) dorsal terminal cells exhibited a number of TTBs that was remarkably lower (6.4 ± 1.85; N = 40) than that of their EGFP-negative contralateral terminal cells, which continued to display an extra sprouting phenotype (Figures 3B and 3C; Figure S3A) (9.2 ± 2.9 TTBs; N = 40). Of note, in 87.5% of the segments analyzed in these mosaic larvae, *sima* mutant terminal cells displayed less TTBs than their contralateral EGFP-negative control cells (Figure 3C), strongly suggesting that accumulation of Sima in terminal cells is required for tracheal extra sprouting in hypoxia. Interestingly, we observed that in 43% of the segments displaying a Sima mutant dorsal terminal cell (N = 65), an ectopic wild-type terminal cell occurred (data not shown), probably compensating for reduced oxygenation in the area. For better comparison between *sima* mutant and wild-type terminal cells, the segments bearing an ectopic terminal cell were excluded from the quantitative TTB analysis (Figures 3A–3C).

Given the requirement of Sima in terminal cells for extra sprouting, we next investigated whether the accumulation of Sima in tracheal cells is sufficient for inducing terminal ramifications. Since *btl*-Gal4-driven tracheal overexpression of Sima provoked first-instar larvae lethality, we restricted induction of Sima with a thermosensitive variant of the Gal80 transcriptional repressor (Gal80^{ts}) (McGuire et al., 2004). It was reported that Gal80^{ts} is fully active at 18°C, but that it is inactive above 30°C; intermediate temperatures such as 25°C or 28°C result in low but detectable expression of the UAS transgene (McGuire et al., 2004). Thus, we explored different temperature conditions to induce a mild, nonlethal overexpression of Sima in the tracheae. When individuals were grown at 18°C until the second larval instar and transgene expression was then induced by shifting the temperature up to 30°C for 48 hr, we observed a clear increase of terminal branch sprouting (Figures 3D and 3E), with an average of 7.74 TTBs (N = 105), a number of ramifications significantly higher than that observed in the control larvae (5.96 TTBs; N = 74 [Table 1]). Remarkably, in some of the larvae overexpressing Sima, a dramatic extra sprouting phenotype was observed: terminal branches were long, extremely ramified, and bundled together in the same direction (Figures 3F and 3G). A similar phenotype was frequently observed in *fga* mutant larvae (Figure 3H). These results show that elevated Sima levels in tracheal cells are sufficient to induce tracheal terminal branching.

During mammalian angiogenesis, HIF is required in endothelial cells for inducing the expression of VEGF, which, in turn, exerts an autocrine effect that is required for angiogenesis (Tang et al., 2004). We therefore sought to examine if *branchless* (*bnl*) expression is required in tracheal terminal cells for hypoxia-driven extra sprouting. We used the MARCM technique to generate EGFP-labeled *bnl*^{P1} homozygous dorsal terminal cells, and we studied

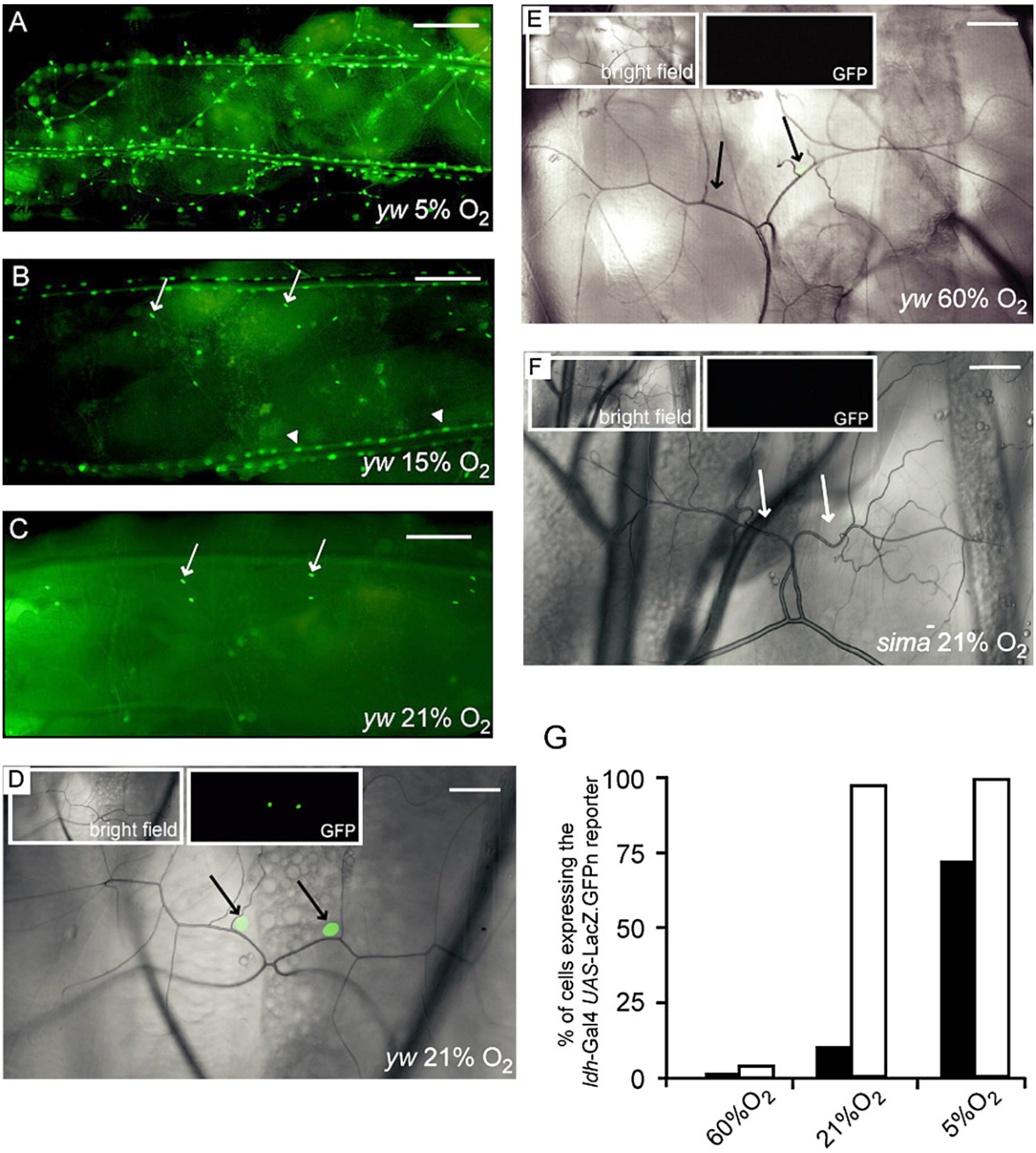

Figure 2. Tracheal Terminal Cells Can Induce Hypoxia-Dependent Transcription with High Sensitivity

(A) Expression of an *Idh*-Gal4 *UAS*-LacZ.GFP$_n$ reporter can be visualized exclusively in the nuclei of tracheal cells of third-instar larvae exposed to 5% O_2 for 4 hr.

(B) At 15% O_2, the expression of the reporter in the tracheal system is remarkably weaker. Tracheal terminal cells are marked with arrows; cells of the dorsal trunk are marked with arrowheads.

(C) In normoxia, expression of the reporter can be detected only in the terminal cells (arrows).

(D) Dorsal branch terminal cells of third-instar normoxic larvae; expression of the reporter can be seen in the nuclei of these cells (arrows).

(E and F) In (E) wild-type larvae exposed to hyperoxia (60% O_2) or in (F) normoxic *sima* homozygous mutants, the hypoxia-inducible reporter cannot be detected; the arrows indicate the approximate position of the terminal cell nuclei.

(G) Quantification of the proportion of third-instar larvae expressing the reporter in the terminal cells of the dorsal branch (white columns) or also expressing the reporter in other cells of the dorsal branch (black columns), at different oxygen concentrations.

Scale bars are 250 μm in (A)–(C) and 50 μm in (D)–(F).

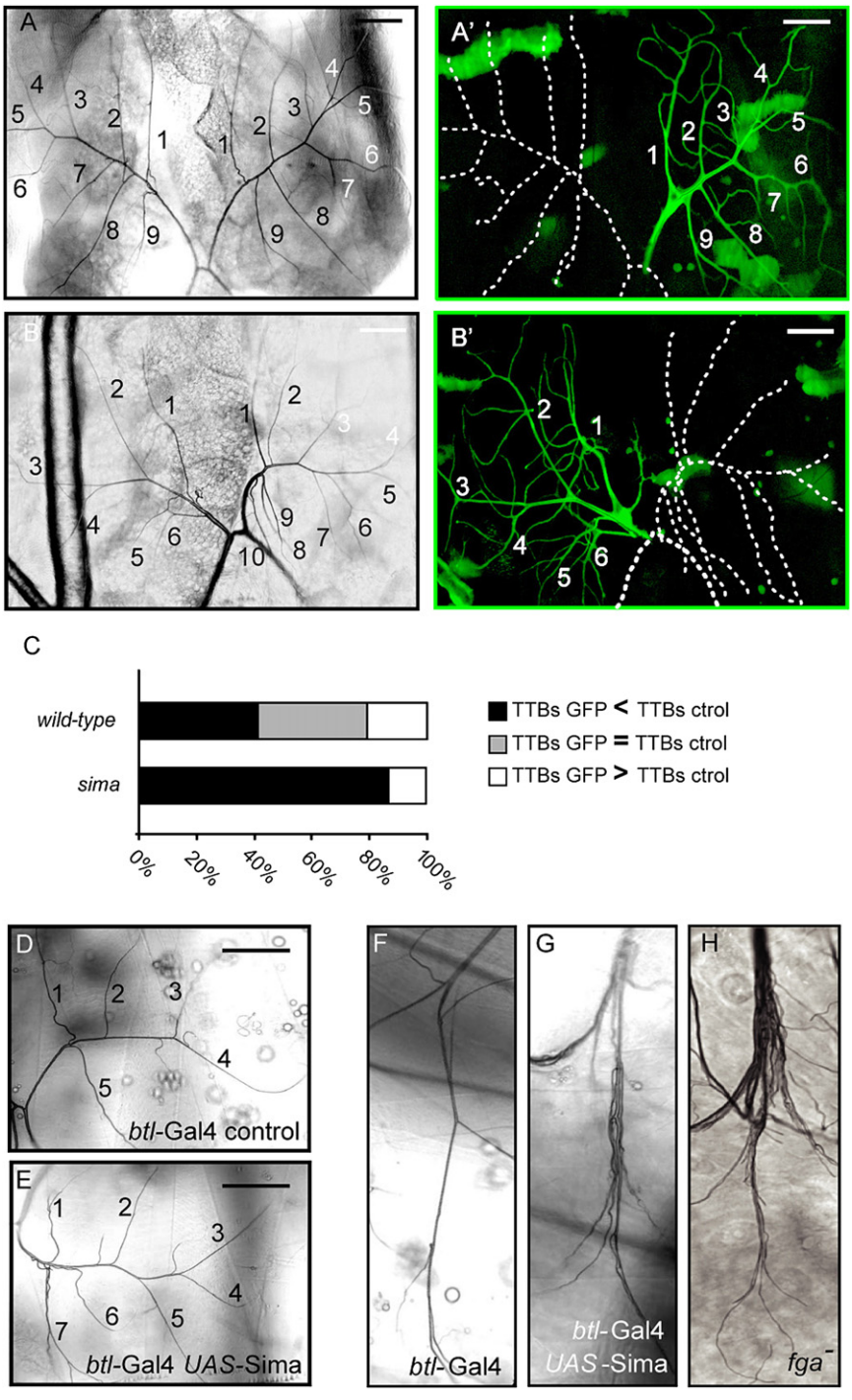

Figure 3. Expression of Similar in Terminal Cells Is Required for Tracheal Branching, and Its Overexpression in Tracheal Cells Mimics Extra Sprouting in Hypoxia

Sima homozygous mutant terminal cells were generated and assayed for their capacity to undergo extra sprouting in hypoxia.

(A) A bright-field image showing two control dorsal terminal cells of a third-instar larva exposed to hypoxia (5% O_2 for 60 hr).

(A′) The cell on the right is labeled with EGFP, whereas the cell on the left is not marked. The EGFP-labeled cell and its contralateral (nonlabeled) cell both display identical branching capacity (A).

(B and B′) Whereas the control cell on the right undergoes extra sprouting in hypoxia (ten TTBs), the EGFP-labeled cell on the left, which is homozygous mutant for *sima*, has a reduced number of branches (six TTBs).

(C) Comparison of the number of TTBs in EGFP-labeled dorsal terminal cells with their contralateral unlabeled cells in larvae exposed to hypoxia. EGFP-labeled and nonlabeled cells display a similar number of TTBs in wild-type control larvae, whereas the number of TTBs in EGFP-labeled cells that are homozygous for a *sima* mutation is lower than that of their contralateral nonlabeled cells, in 87.5% of the segments analyzed (n = 40; Student's t test; $p < 10^{-5}$).

(D) In *btl*-Gal4 control larvae, ramifications of dorsal branch terminal cells are normal.

(E) In larvae overexpressing *sima* in the tracheal system, extraterminal ramifications are observed.

(F) Morphology of the anterior lateral trunk of a wild-type larva.

(G) An anterior lateral trunk of a larva with tracheal overexpression of *sima* showing numerous terminal projections that bundle together in the same direction.

(H) A similar phenotype is observed in *fatiga* mutant larvae.

Scale bars are 50 μm in (A) and (B) and 25 μm in (D) and (E).

their ability to ramify upon exposure to hypoxia as described above. Because Bnl is required for terminal cell differentiation at late embryogenesis (Sutherland et al., 1996), and given that the bnl^{P1} allele is haploinsufficient, most of the analyzed larval segments lacked at least one dorsal terminal cell. Therefore, we initially studied bnl^{P1} (EGFP-labeled) homozygous mutant cells in those rare segments that retained their terminal cells in both dorsal branches. bnl^{P1} homozygous (EGFP-labeled) cells in these segments ramified to a similar extent as their contralateral (EGFP-negative) terminal cells (Figures S3B and S3C). Mutant cells displayed an average of 7.3 ± 1.1 TTBs (N = 12), whereas their contralateral control cells exhibited 6.4 ± 1.4 TTBs. Note that the number of TTBs in these control cells was reduced compared to that of hypoxic wild-type larvae (Table 1) due to the haploinsufficiency of bnl^{P1}/+ individuals (Jarecki et al., 1999). These results show that *bnl* expression is not required in terminal cells for hypoxia-dependent extra sprouting.

We noticed that in most segments lacking one dorsal terminal cell (17/24), the contralateral terminal cell compensated for the lack of oxygen by generating extra branches that crossed the dorsal midline and invaded the hypoxic area (Jarecki et al., 1999) (Figures S4A and S4B). Remarkably, this compensatory behavior was also executed by bnl^{P1} homozygous terminal cells

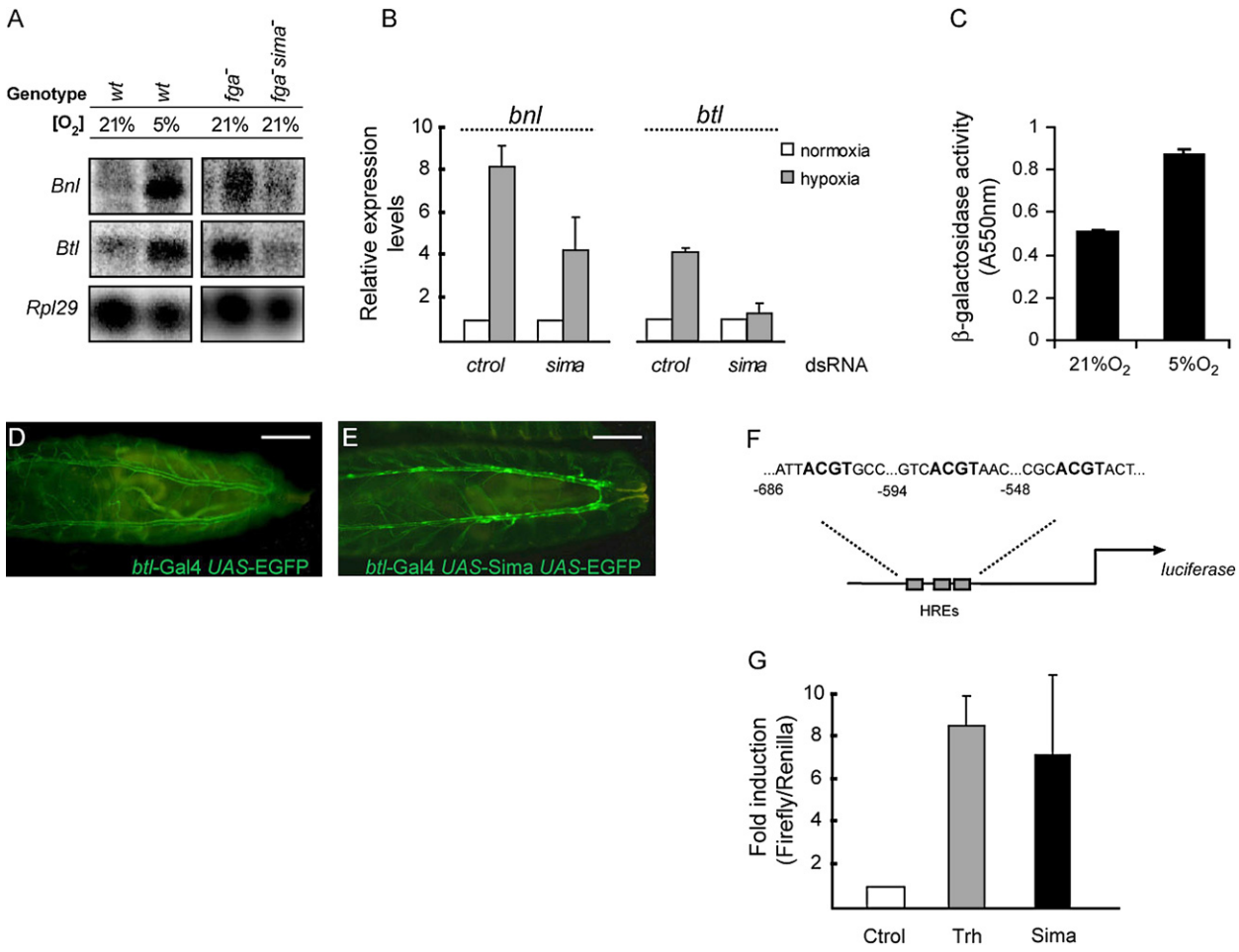

Figure 4. The *breathless* Transcript Is Induced in Hypoxia or after Overexpression of Similar

(A) Northern blot of total RNAs extracted from larvae grown in normoxia or exposed to hypoxia (5% O_2). Both *branchless* (*bnl*) and *breathless* (*btl*) transcripts are induced in hypoxia or in *fatiga* (*fga*) homozygous mutant larvae. Upregulation of the two transcripts is abrogated in *fga sima* double mutants.

(B) Induction of *bnl* and *btl* expression in S2 cells in hypoxia (1% O_2 for 24 hr); upregulation of *bnl* is partially inhibited by the addition of *sima* double-stranded RNA, whereas *btl* induction was completely inhibited by this treatment.

(C) β-gal enzymatic activity in extracts prepared from larvae of the H82Δ3 *btl* enhancer-trap line; expression of LacZ is induced in hypoxia.

(D) *btl*-Gal4-driven expression of a membrane-targeted *UAS*-EGFP element decorates the tracheal system.

(E) When *sima* is coexpressed under control of the same Gal4 driver, expression of EGFP is strongly upregulated, suggesting that Sima induces *btl* transcription. The scale bars in (D) and (E) are 500 μm.

(F) *btl*-luciferase reporter for S2 cell transfection; the nucleotide sequence of the *btl* enhancer used in the reporter includes three HIF-responsive elements (HREs) that are highlighted in bold font.

(G) The *btl*-luciferase reporter transfected in S2 cells is strongly induced upon transfection of plasmids encoding Trachealess (Trh) or Similar (Sima).

at a high frequency (13/14) (Figures S4C and S4D), supporting the notion that *bnl* expression in tracheal terminal cells is dispensable for compensatory extra sprouting toward oxygen-starved areas.

In those rare segments of larvae bearing *sima* homozygous mutant terminal cells (Figure 3B) in which the contralateral control cell was missing (N = 3), we noticed that mutant cells failed to execute the compensatory extra sprouting response, and, instead, terminal cells from neighboring segments projected extra ramifications to the hypoxic region (Figure S5). Once again, these observations are consistent with a model in which expression of Sima in terminal cells is necessary for terminal branch sprouting in hypoxia.

Similar Induces *breathless* Transcription

It was previously reported that Bnl protein accumulates in larvae exposed to hypoxia (Jarecki et al., 1999), but the mechanism involved has not been reported. Northern blot analyses of RNAs from third-instar larvae demonstrated a robust induction of both *bnl* and *btl* transcripts after 4h exposure to 5% O_2, in comparison with normoxic controls (Figure 4A). Consistent with this, *fga* homozygous mutant larvae exhibited a clear induction of the two genes, and this induction was abrogated in *fga sima* double mutants (Figure 4A). These results suggest that the Fga-Sima system participates in both *bnl* and *btl* induction in hypoxia. In order to gain insights on this regulation, we sought to test directly whether hypoxic induction of *bnl* and *btl* is

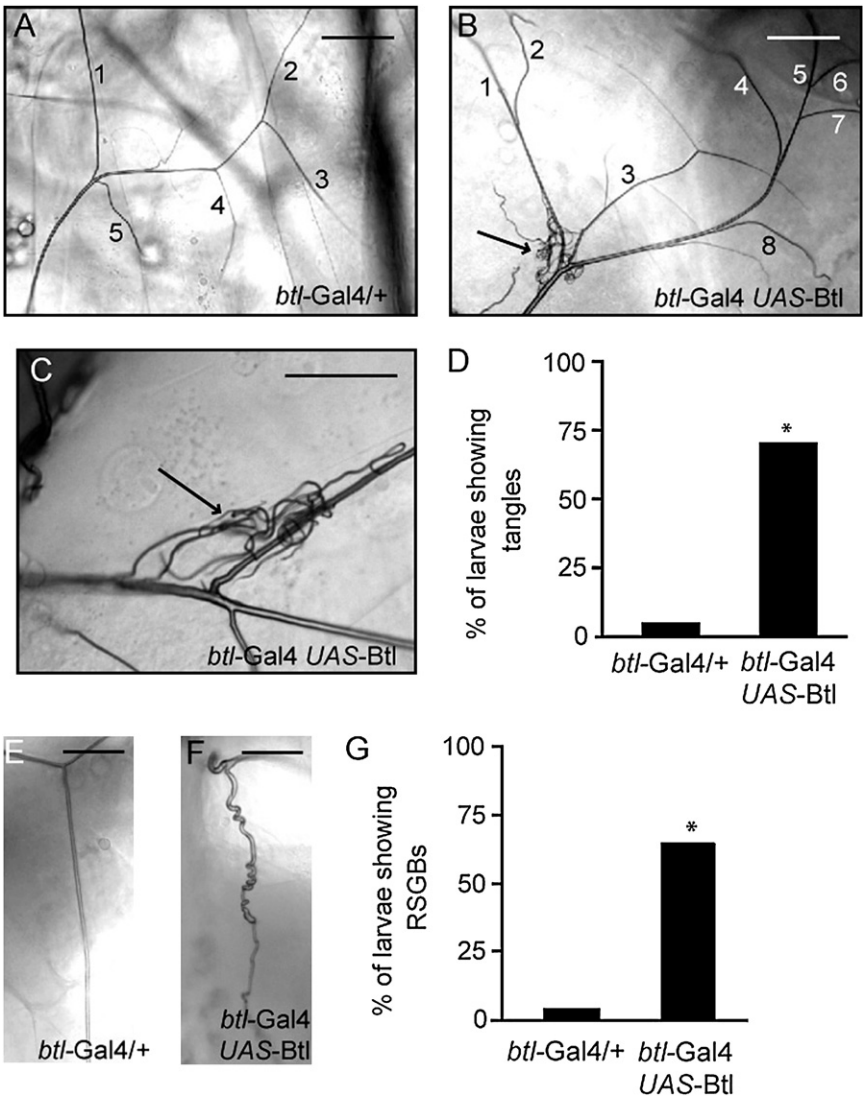

Figure 5. Overexpression of *breathless* in the Tracheal System Induces Extra Sprouting and Other Phenotypes Typically Observed in Hypoxic Wild-Type Larvae

(A) *btl*-Gal4 control larvae display a wild-type branching phenotype.

(B) Upon tracheal-specific overexpression of Btl, extra sprouting of terminal cells was induced, giving rise to tracheal tangles (arrow).

(C) Detail of a tracheal tangle (arrow) induced in *btl*-Gal4 *UAS*-Btl third-instar larvae.

(D) Quantification of the proportion of larvae exhibiting tracheal tangles upon tracheal overexpression of Btl.

(E) Ganglionic branches of *btl*-Gal4/+ controls are straight.

(F and G) (F) In larvae overexpressing Btl in the tracheal system, ringlet-shaped ganglionic branches (RSGBs) are frequently observed (quantification of this phenotype is shown in [G]).

*Significant differences, $p < 0.0001$, Fisher's test. Scale bars are 50 μm in (A) and (B) and 25 μm in (C), (E), and (F).

mediated by Sima. Since *sima* mutant larvae fail to survive in hypoxia (Centanin et al., 2005), these studies were carried out in cell culture. Real-time PCR analyses of *bnl* and *btl* expression in hypoxic or normoxic S2 cells revealed that the two transcripts are strongly induced in hypoxia in this experimental setting (Figure 4B). Remarkably, addition of *sima* double stranded RNA abrogated *btl* induction completely, whereas hypoxic induction of *bnl* was blocked only partially (Figure 4B). This suggests that *btl* hypoxic induction is Sima-dependent, whereas upregulation of *bnl* is partially mediated by Sima. To have another estimation of the extent of *btl* induction in hypoxia, we determined β-galactosidase activity in extracts prepared from the H82Δ3 *btl-lacZ* enhancer-trap line. The activity in extracts of individuals exposed to 5% O_2 was 86% higher than that of normoxic extracts (Figure 4C), confirming that *btl* is induced in hypoxic larvae.

Next, we focused on tracheal-specific induction of the *btl* transcript. In tracheal cells, the expression of *btl* normally depends on a bHLH-PAS protein named Trachealess (Trh) (Isaac and Andrew, 1996; Wilk et al., 1996). In addition, the *btl* gene can be induced upon Bnl binding and subsequent activation of its own signaling pathway (Ohshiro et al., 2002). Therefore, it is expected that *btl* induction in hypoxia depends, at least in part, of Bnl protein that activates the Btl pathway. Yet, in light of the observation that tracheal cells, and especially terminal cells, have the capacity to induce Sima-dependent transcription with high sensitivity, it is conceivable that *btl* is a direct Sima target gene in the tracheal system. To examine this possibility, we overexpressed Sima specifically in tracheal cells under control of a *btl*-Gal4 driver, in 1st instar larvae that also contained a *UAS*-EGFP reporter element. Expression of the EGFP reporter in *btl*-Gal4; *UAS*-Sima *UAS*-EGFP larvae was remarkably higher than in *btl*-Gal4; *UAS*-EGFP controls (Figures 4D and 4E), suggesting that Sima can induce *btl* transcription in tracheal cells.

Next, we examined if this regulation occurs also in embryos and found that indeed, Sima can mediate *btl* induction during embryogenesis as well (see Supplemental Data and Figure S6). Since Sima can induce *btl* transcription in both embryonic and larval stages, we next sought to gather direct evidence of *btl* transcriptional induction by Sima in an S2 cell culture system by examining Sima's ability to promote the expression of an HRE-containing *btl*-luciferase reporter (Figure 4F), which was previously shown to be activated by Trachealess (Jin et al., 2001; Ohshiro and Saigo, 1997). As depicted in Figure 4G, transfection of a Sima expression plasmid can induce the *btl* HRE-luciferase reporter to a similar extent as transfection of a Trachealess-expressing plasmid. These results strengthen the notion that Sima is able to activate *btl* transcription.

Overexpression of Breathless in Tracheal Cells Mimics Hypoxia-Dependent Tracheal Remodeling

The above results raised the possibility that in hypoxia, accumulation of Sima in tracheal cells may provoke upregulation of *btl* transcription, which in turn, induces the morphological changes that occur in hypoxia. We therefore examined whether reducing the gene dosage of *btl* or its downstream effector *blistered* (*bs*) will impinge on tracheal terminal sprouting. We found that indeed, the sprouting capacity of tracheal terminal cells of *fga* mutant larvae that were heterozygous for *btl* or *bs* was reduced in comparison with *fga* homozygous controls (see Supplemental Data and Figure S7; Table 1).

We next sought to examine if upregulation of Btl in the tracheae of wild-type individuals is sufficient to provoke extra sprouting. We overexpressed Btl in larval tracheal cells through a *btl*-Gal4 driver, and observed a clear increase of terminal branching in these larvae (Figures 5A and 5B), with an average of 7.89 TTBs at the third dorsal branch (N = 85) (Table 1). Moreover, overexpression of *btl* mirrored the results obtained upon tracheal overexpression of Sima, since in *btl*-Gal4; *UAS*-Btl third-instar larvae, a dramatic extra sprouting phenotype was frequently observed, where terminal branches formed "tangles" that tended to wrap onto larger tracheal branches (Figures 5B–5D). Finally, RSGBs also occurred at high frequency in larvae overexpressing Btl in the tracheae (Figures 5E–5G), phenocopying *fga* mutants (Figure 1M). These observations indicate that, under conditions of constant Bnl levels in target tissues, upregulation of Btl in tracheal cells is sufficient to provoke morphological changes identical to those occurring in hypoxia.

DISCUSSION

Oxygen Sensing and Tracheal Development

We have analyzed the role of the *Drosophila* HIF-α homolog Sima and the oxygen-sensing prolyl-4-hydroxylase Fga in tracheal terminal branching. It is assumed that during embryonic stages, tracheal development depends on hard-wired developmental cues, and, later, in larval stages, tracheal terminal branching is driven by local hypoxia in the target tissues (Ghabrial et al., 2003). The observations carried out in this study indicate that the tracheal system of *sima* mutant third-instar larvae is indistinguishable from that of wild-type individuals, including the pattern of terminal branches. Thus, our results imply that if terminal branching during normal development was mediated by tissue hypoxia, the mechanism involved in such a local response should be Sima independent. This is a remarkable difference between *Drosophila* tracheogenesis and the development of the mammalian vascular system, in which HIF proteins are critically required for both vasculogenesis and developmental angiogenesis.

We have also shown that Sima does play a cardinal role in hypoxia-dependent tracheal terminal branch sprouting, as well as in the formation of terminal branches that compensate for poor oxygenation in exceptional situations in which a neighboring branch is missing. Sima-dependent extra sprouting is negatively regulated by the oxygen-sensing prolyl-4-hydroxylase Fga, since *fga* mutants displayed an extra sprouting phenotype that was even stronger than that observed in wild-type individuals exposed to hypoxia. This extra sprouting phenotype is, to our knowledge, the first demonstration that loss of function of a HIF-prolyl hydroxylase can provoke an angiogenic-like phenotype. Thus, it seems reasonable to expect that conditional knockdown of mammalian PHDs in an appropriate cell type will promote angiogenesis.

Tracheal Terminal Cells Are Specialized Oxygen Sensors

The long-standing paradigm for mammalian angiogenesis is that low oxygen levels trigger HIF accumulation in target tissues, which, in turn, mediates VEGF induction that, upon binding to VEGF receptors on endothelial cells, attracts the outgrowth of newly formed blood capillaries. Nevertheless, this apparently passive role of endothelial cells has recently been challenged. It has been demonstrated that in endothelial cell-specific HIF-α knockout mice the angiogenic response is impaired (Tang et al., 2004), highlighting a central role of the oxygen-sensing machinery in endothelial cells.

Here, we have shown that the specialized *Drosophila* tracheal cells that respond to hypoxia by projecting angiogenic-like subcellular processes— i.e., the terminal branches—are apparently more sensitive to hypoxia than any other cell type in the larva. The sensory threshold to induce Sima-driven gene activation in these cells is shifted to near-normoxic oxygen tension. An alternative interpretation of our data is that tracheal terminal cells are similarly sensitive but more hypoxic than other cells, thereby inducing hypoxia-dependent transcription with higher sensitivity. In either case, the results suggest that Sima-dependent transcription within the tracheal terminal cells is part of the mechanism of oxygen sensing and tracheal extra sprouting.

To test this hypothesis directly, we generated EGFP-labeled *sima* homozygous mutant terminal cells, and we found that the ability of these cells to ramify upon a hypoxic stimulus is largely impaired. Furthermore, we examined whether overexpression of Sima in the tracheae can provoke the angiogenic-like response, and we found that, indeed, expression of Sima restricted to the tracheal system is sufficient to induce extra sprouting. In contrast, overexpression of Sima—or of a nondegradable variant of Sima—in flip-out random clones outside the tracheae failed to provoke a similar phenotype, suggesting that accumulation of Sima in these cells is not sufficient for extra sprouting. Interestingly, in these Sima flip-out clones, a cell-autonomous response was observed, in which long subcellular processes projected from the cells that overexpressed Sima. Thus, although it is clear that *bnl* is induced in hypoxia and attracts the extension of terminal branches (Jarecki et al., 1999), our data support the notion that Sima is necessary, but not sufficient, for *bnl* induction in hypoxia (Figure 6).

Induction of the FGF Receptor Breathless in Tracheal Cells Promotes Extra Sprouting

We have investigated which Sima target genes might be responsible for tracheal extra sprouting in *fga* mutants or upon exposure of wild-type larvae to hypoxia. Northern blot analyses indicated that *bnl* and *btl* are both upregulated in mildly hypoxic larvae or *fga* mutants. However, *bnl* homozygous EGFP-labeled terminal cells of larvae exposed to hypoxia retained their branching capacity, suggesting that extra sprouting in hypoxia is not mediated by an autocrine effect of Bnl, upon Sima-dependent

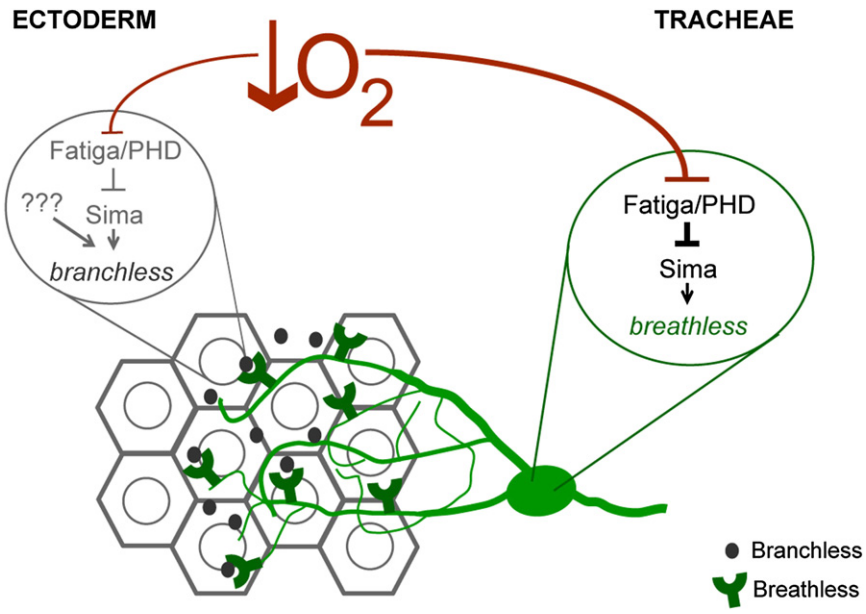

Figure 6. Model for Tracheal Sprouting in Response to Hypoxia

Tracheal terminal cells can sense hypoxia through the oxygen sensor Fatiga, resulting in accumulation of the HIF-α subunit Sima in these cells. Sima accumulation provokes induction of *btl*, and augmented levels of the receptor Btl sensitize the cells to available levels of the ligand Branchless, resulting in terminal branch outgrowth. In nontracheal tissues, such as the ectoderm, *branchless* (*bnl*) is coordinately induced in hypoxia; Sima is necessary, but not sufficient, for *bnl* induction.

induction in tracheal cells. On the other hand, *btl* is directly induced by Sima in tracheal cells, and, consistent with this, overexpression of Btl in tracheal cells was sufficient to mimic the phenotypes of larvae exposed to hypoxia. Thus, our data suggest that Sima-dependent transcriptional induction of *btl* in tracheal terminal cells is a critical step of the angiogenic-like response of the tracheal system in hypoxic larvae.

In summary, we propose that tracheal cells respond to hypoxia in an autonomous manner, by promoting the accumulation of Sima, which induces expression of the receptor Btl, thereby increasing sensitivity of these cells to the ligand Bnl. Concomitantly, Bnl is induced in hypoxic target tissues through a mechanism that also involves the participation of Sima, and serves to cue the outgrowth of terminal branches toward O_2-starved areas (Figure 6).

During angiogenesis, vertebrate VEGF receptors are upregulated in endothelial cells of blood vessels that invade hypoxic tissues, and, particularly, Flt-1 induction is HIF dependent (Gerber et al., 1997; Waltenberger et al., 1996). Endothelial-specific overexpression of VEGF receptors might reveal to what extent this induction is a cardinal step in the angiogenic response to hypoxia.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies used in this study were *yw*,*UAS*-Sima and *ldh*-Gal4 *UAS*-LacZ.GFP$_n$ (Lavista-Llanos et al., 2002); *btl*-Gal4 (Shiga et al., 1996); *UAS*-Btl (Dossenbach et al., 2001); *UAS*-Trh (Wilk et al., 1996); *UAS*-Bnl (Sutherland et al., 1996); *fga*^{1}, *fga*^{9}, and *sima*^{07607} (Centanin et al., 2005); *btl*^{H82} (Klambt et al., 1992); *btl*$^{H83\Delta3}$ (Reichman-Fried and Shilo, 1995); and *tubulin*-Gal80^{ts} (McGuire et al., 2004).

Quantification of Tracheal Phenotypes

yw, *fga*, *sima*, and *fga sima* larvae were grown at 25°C, 21% O_2 at a density of 20 larvae per vial. Second-instar *yw* larvae were placed at 5% or 60% O_2 by using a Forma Scientific 3131 incubator. Third-instar larvae were anesthetized with ether or in ice-cold PBS (Phophate Buffer Saline), and ramifications of the third dorsal branch were counted under a bright-field microscope (Olympus BX-60). For studying *btl*-Gal4 *UAS*-Btl larvae, embryos were placed at 18°C until the second larval Instar, and they were then transferred to 28°C; the resulting tracheal phenotype was analyzed at the third larval instar. For Sima overexpression experiments, *tubulin*-Gal80^{ts} *btl*-Gal4 *UAS*-Sima *UAS*-CD8GFP or *tubulin*-Gal80^{ts} *btl*-Gal4 *UAS*-EGFP (control) larvae were kept at 18°C until the second larval instar and were then transferred to 30°C to allow for the expression of the transgene. GFP-expressing larvae were sorted out and analyzed as described above.

Generation of Mutant Tracheal Terminal Cells

MARCM clones were generated as previously described (Ghabrial and Krasnow, 2006). Briefly, flies carrying hs-FLP; actin-Gal4 UAS-GFP; FRT82B Tubulin-Gal80 were crossed with FRT82B bnl^{P1}, FRT82B sima^{07607}, or FRT82B flies alone as a control. The progeny of the different crosses was heat shocked at 38°C for 40 min, 1–4 hr postfertilization. Eggs were maintained in vials until the first larval instar and were then transferred to 5% O_2 until they have reached the third larval instar. Larvae were checked for GFP expression in terminal cells under a fluorescent dissection microscope, sacrificed, and tracheal phenotypes were analyzed by using a fluorescence microscope (Olympus BX-60).

breathless and *branchless* Expression Studies

For *btl* mRNA in situ hybridization experiments *hs*-Gal4 *UAS*-Trh or *hs*-Gal4 *UAS*-Sima embryos were collected for 1.5 hr, developed at 25°C for 1.5 hr, heat shocked for 30 min at 37°C, developed for 2 hr at 25°C, and fixed with formaldehyde. An antisense digoxigenin-labeled RNA probe for *btl* was synthesized with T7 RNA polymerase (Roche Diagnostics, Manheim) by using as a template a PCR product bearing a T7-binding site on the 3′ end of a *btl*-specific primer. Northern blots and β-gal activity assays were carried out by following standard procedures. Briefly, total RNA was isolated from normoxic or hypoxic (5% O_2) wild-type (yw) third-instar larvae by using the Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. Aliquots of RNA (15–20 μg) were run on denaturing 6% formaldehyde/1% agarose gels, on which the 18 S rRNA and the processed half molecules of the 28 S rRNA (i.e., 28 Sa and 28 Sb) migrated as one major band that was between 1.7 and 2 kb in size. RNA was blotted in 20× SSC buffer onto positively charged nylon membranes (Biodyne®B, 0.45 μm; Pall Corporation, USA) and hybridized for 16 hr at 42°C with 20–30 ng of the following RT-PCR-isolated, sequenced, and ^{32}P-labeled cDNA probes: (1) *branchless* (*bnl*), a 938 bp fragment of exon 3 (amino acids 373–684; forward primer (fp): 5′-TACAC GAACGCCATCACGG-3′; reverse primer (rp): 5′-ACCCTATCGCTGGTTT CGCT-3′); (2) *btl*, a 521 bp fragment of exon 2 (amino acids 150–322; fp: 5′-AAAACCGTACAACGGACCGCA-3′; rp: 5′-TCCTCCATCAGGACATTTCCAA G-3′); (3) ribosomal protein L29: 256 bp of the entire open reading frame (fp: 5′-CCGGAGTAGTTTAAGATGGCCAAGTC-3′; rp: 5′-GGGGCAATCA TCTAC AGAGTAACAGG-3′). The next day, membranes were washed three times with 0.1× SSC and 0.1% SDS solution at 55°C and were exposed to phosphoimager screens. After stripping of the membranes, constitutive expression of the *rpL29* gene was assessed.

To measure β-gal activity, $btl^{H82\Delta3}$ third-instar larvae were grown at 5% or 21% O_2, homogenized in lysis buffer (50 mM Tris-HCl [pH 7.8], 2 mM EDTA, 10% glycerol, 2 mM dithiothreitol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), and centrifuged at 2,500 × g for 3 min at 4°C. Supernatant was recovered, and protein concentration was determined by using the Bicinchoninic acid method (Sigma). Enzymatic reactions were carried out by incubating 20–50 μg protein extract with 180 μl reaction buffer, containing 80 mM Na3PO4 (pH 7.3), 102 mM β-mercaptoethanol, 9 mM $MgCl_2$, and 4 mM Chlorophenol Red β-d-galactopyranoside (Roche Diagnostics, Mannheim, Germany) at 37°C, and absorbance at 574 nm was recorded at 10, 30, 60, 120, and 180 min time points. The rate of color development was linear throughout this time period, and values at 2 hr were used. Endogenous background was substracted by using a heat-inactivated sample.

Overexpression of Branchless or Similar in Random Cell Clones

Flip-out expression in random clones was performed as previously described (Nellen et al., 1996). Briefly, larvae with the genotype *hs*-FLP; *Act* > CD2 > Gal4; *UAS*-CD8GFP *UAS*-Sima or *hs*-FLP; *Act* > CD2 > Gal4; *UAS*-CD8GFP *UAS*-Bnl were heat shocked at 37°C during 10 min at the first larval instar and were allowed to develop at 25°C. Resulting third-instar larvae were checked for clones expressing GFP and were analyzed under a fluorescence or confocal microscope (BX-60 Olympus or Zeiss LSM5 Pascal).

Statistical Analysis

Comparisons of TTB numbers between genotypes or oxygen tensions (Figure 1; Table 1) were performed through the one-way ANOVA and Bonferroni's postcomparison test. In MARCM experiments, the Student's t test was used to compare TTBs in GFP-positive and GFP-negative cells (Figure 3). The Fischer's test was utilized to compare frequencies of tangles or RSGBs (Figure 5).

SUPPLEMENTAL DATA

Supplemental Data include experiments describing the effect of the overexpression of Bnl or different Sima variants in nontracheal cells (Figures S1 and S2); the effect of *sima* or *bnl* loss of function in one single terminal cell in larvae exposed to hypoxia (Figure S3); the ability of wild-type, *bnl*, or *sima* mutant single terminal cells to project branches toward a contralateral hemisegment that lacks its own terminal cell (Figures S4 and S5); the ectopic expression of *btl* upon overexpression of Trh or Sima in embryos (Figure S6); and, finally, the effect on the extra sprouting phenotype of *fga* mutants that we observed upon *btl* or *bz* gene dose reduction (Figure S7). These data are available at http://www.developmentalcell.com/cgi/content/full/14/4/547/DC1/.

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