Supplemental Data

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

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OVER-EXPRESSION OF SIMA IN NON-TRACHEAL CELLS

Having established that tracheal remodeling in hypoxia depends on Sima, we sought to examine whether accumulation of Sima in non-tracheal tissues can promote terminal sprouting. It was previously reported that over-expression of Bnl in non-tracheal random clones in 3rd instar larvae promotes the formation of new terminal branches, which are guided towards the Bnl-expressing cells (Jarecki, Johnson et al. 1999) (Figure S1A). To test if Sima can provoke an effect similar to Bnl, we induced over-expression of Sima in random clones by using the flip-out technique (Nellen, Burke et al. 1996). The cells over-expressing Sima did not attract the outgrowth of tracheal terminal branches (Figure S1B), but instead, executed a cell-autonomous response characterized by the projection of long filopodia that extended from Sima-expressing cells in random directions (Figures S1C and S1D). Yet, it is possible that rapid Sima degradation accounts for the lack of chemotactic response of tracheal terminal branches upon overexpression of Sima in the clones. In order to investigate this possibility, we performed flip-out expression of a Sima protein variant (SimaP850A) in which the prolyl residue that is subjected to hydroxylation has been replaced by an alanine and thus, is stable even in normoxic conditions (Arquier, Vigne et al. 2006; Wappner et al. unpublished results). Overexpression of SimaP850A in random clones failed to attract tracheal terminal branches (Figure S2), mimicking the results obtained upon overexpression of wild type Sima (Figure S1B). This suggests that rapid Sima degradation in the clones does not account for the lack of tracheal chemo-attraction and extra-sprouting. Altogether, these observations suggest that it is unlikely that accumulation of Sima in non-tracheal tissues is sufficient for inducing extra-sprouting in hypoxic larvae.
Figure S1. Effect of the Over-Expression of *branchless* or *sim* in Extra-Tracheal Cells

(A) Flip-out over-expression of *branchless* in random clones outside the tracheae attracts the outgrowth of numerous tracheal terminal branches; the ectodermal cell over-expressing *branchless* is marked with EGFP (A’). (B) Flip-out over-expression of *sim* does not provoke the same effect on tracheal outgrowth, as terminal branch sprouting is not induced. (C) In control flip-out clones expressing only GFP the morphology of ectodermal cells is normal; (D) cells that over-express *sim*, project long subcellular processes in random directions.
Figure S2. Effect of the Over-Expression of a Non-Degradable Variant of Sima (SimaP850A) in Extra-Tracheal Cells

(A) Bright field microscopy showing 3rd instar dorsal tracheal branches; (B) fluorescent image showing an EGFP-labeled cell expressing SimaP850A; (C) the merge image shows that tracheal branches are not attracted towards the EGFP-labeled cells.
Figure S3. Sima Is Essential for Terminal Branch Extra-Sprouting in Hypoxia, while Branchless Is Dispensable

(A) Sima mutant cells fail to generate extra-branches in hypoxic larvae. Frequency distribution of the differences in the number of TTBs between EGFP-positive dorsal terminal cells and their EGFP-
negative contralateral cells in MARCM experiments examining sima function in these cells. White columns: MARCM control experiment (both cells are wild type) (N=29); black columns: MARCM sima experiment (EGFP-positive cells are homozygous mutant for sima) (N=40). Note that sima mutant cells usually have less ramifications than their contralateral terminal cells, whereas in the control experiment the two terminal dorsal cells have a similar number of ramifications. (B-C) branchless mutant terminal cells can undergo extra-sprouting in hypoxia. bnlP1 EGFP-labeled homozygous mutant terminal cells were generated in heterozygous larvae, and the number of TTBs in these cells was compared with that of their contralateral (non-labeled) control cells upon exposure to hypoxia. (B) Bright field microscopy showing that the two dorsal terminal cells have a similar number of TTBs; (C) fluorescent image showing that the cell on the left expresses EGFP and therefore, is mutant for bnl.
Figure S4. *branchless* Mutant Terminal Cells Retain the Capacity to Invade the Hypoxic Contralateral Hemisegment that Lacks Its Own Terminal Cell

EGFP-labeled *bnt*¹ homzygous mutant cells have been generated by using the MARCM technique. Most segments of *bnt*¹ heterozygous larvae lack one dorsal terminal cell; the right terminal cell is missing in panels A and B. In these segments lacking one terminal cell, the remaining cell projects ramifications across the midline (thick dashed line), compensating for poor oxygen supply at the contralateral hemisegment. (A) Bright field microscopy; (B) fluorescent image, showing that the
terminal cell depicted in (A) is not labeled with EGFP and therefore, is not mutant for \textit{bnl}. Solid lines mark the regular branches projected by the single terminal cell towards the ipsilateral (left) hemisegment; dashed lines mark extra-branches projected by the same cell into the contralateral (right) hemisegment. (C,D) A \textit{bnl} homozygous mutant cell (labeled with EGFP) has identical capacity to invade the contralateral hemisegment (left) that lacks its own terminal cell.

\textbf{Figure S5. sima Mutant Terminal Cells Loose the Capacity to Invade the Hypoxic Contralateral Hemisegment that Lacks Its Own Terminal Cell}

(A) An EGFP-labeled dorsal terminal cell homozygous for a \textit{sim}a mutation (arrow) fails to invade the contralateral (right) hemisegment lacking its own terminal branch. Instead, a terminal cell from a neighboring segment projected ramifications into the hypoxic territory. (B) Scheme of the micrograph
depicted in panel A; the sima homozygous terminal cell is marked in green, whereas compensatory branches projecting from a terminal cell of a neighboring segment are shown in black.

**breathless regulation by Sima in embryos**

The btl transcript is first detected at embryogenesis stage 8, in ten tracheal placodes at each side of the embryo, that soon afterwards invaginate to give rise to tracheal pits (Glazer and Shilo 1991) (Figure S6A). btl expression in tracheal pits depends on the overlapping expression of Trh and Drifter, the latter, a POU domain transcription factor that is expressed in the embryo in 13 pits (10 pits overlapping Trh plus 3 additional pits) (Anderson, Certel et al. 1996). Thus, when Trh is ectopically expressed under control of a heat shock promoter, 1 to 3 additional btl-expressing pits are induced (Wilk, Weizman et al. 1996; Zelzer, Wappner et al. 1997) (Figure S6B). In order to answer whether Sima can similarly induce btl gene expression, we over-expressed Sima under control of a hs-Gal4 driver, and observed 1 to 3 btl-positive extra pits in the position where Drifter is known to be expressed (Figure S6C). These observations support the notion that Sima can promote btl transcription.

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**Figure S6. breathless Induction by Overexpression of Sima in Embryos**

(A) breathless mRNA in situ hybridization reveals expression in ten tracheal placodes on each side of stage 10 wild type embryos (t1-t10); (B) upon hs-Gal4 driven over-expression of Trachealess, extra-tracheal placodes expressing breathless are induced (arrows). (C) When Sima was over-expressed instead of Trachealess, breathless ectopic expression in extra-tracheal placodes was detected even more strongly (arrows).
EFFECT OF *Breathless* or *Blistered* Gene Dosage on Terminal Branch Sprouting

We have shown above that *fga* mutant larvae display extra-terminal ramifications (Figure 1G); in *fga* mutants that were heterozygous for a *btl* mutation, the average number of TTBs at the 3rd dorsal branch was severely reduced in comparison to that of *fga* mutants (Figures S7A and S7B; Table 1). Since *btl* heterozygous larvae in normoxia display a normal number of TTBs, the above results suggest that Btl dosage is critical for extra-sprouting upon accumulation of Sima.

The *pruned/blistered* (*bs*) gene encodes the *Drosophila* Serum Responsive Factor (DSRF), a transcription factor that mediates Btl pathway activation (Guillemin, Groppe et al. 1996). Thus, we explored if reduction of *bs* gene dosage impairs the branching potential of the terminal cells in a *fga* mutant. *fga* homozygous mutant larvae that were heterozygous for *bs* displayed less TTBs than *fga* homozygous control larvae (Figures S7A and S7C; Table 1). Since the number of TTBs in *bs/+* larvae was not significantly different from that of wild type individuals (Table 1), we conclude that DSRF is likely involved in tracheal extra-sprouting upon activation of the Btl pathway in hypoxia.

**Figure S7.** The Branching Capacity of *fatiga* Mutants Is Impaired upon Reduction of *breathless* or *blistered* Gene Dosage

(A) Tracheal extra-sprouting phenotype of *fatiga* homozygous mutant larvae, revealed by the high number of TTBs; the number of TTBs is reduced in *fatiga* homozygous larvae that are heterozygous for *breathless* (B) or *blistered* (C).