Cell, Vol. 99, 211–220, October 15, 1999, Copyright ©1999 by Cell Press

Oxygen Regulation of Airway Branching in *Drosophila* Is Mediated by Branchless FGF

Jill Jarecki,† Eric Johnson,† and Mark A. Krasnow*
Howard Hughes Medical Institute
Department of Biochemistry
Stanford University School of Medicine
Stanford, California 94305-5307

Summary

The *Drosophila* tracheal (respiratory) system is a tubular epithelial network that delivers oxygen to internal tissues. Sprouting of the major tracheal branches is stereotyped and controlled by hard-wired developmental cues. Here we show that ramification of the fine terminal branches is variable and regulated by oxygen, and that this process is controlled by a local signal or signals produced by oxygen-starved cells. We provide evidence that the critical signal is Branchless (Bnl) FGF, the same growth factor that patterns the major branches during embryogenesis. During larval life, oxygen deprivation stimulates expression of Bnl, and the secreted growth factor functions as a chemoattractant that guides new terminal branches to the expressing cells. Thus, a single growth factor is reiteratively used to pattern each level of airway branching, and the change in branch patterning results from a switch from developmental to physiological control of its expression.

Introduction

Although there has been substantial progress in elucidating the genetic programs that control early stages of organ development, little is known about how physiology and environmental factors interface with the hard-wired developmental programs to achieve organ structures of optimal design. The role of the environment in shaping the nervous system has long been appreciated (Goodman and Shatz, 1993), but the environment and tissue physiology also play important roles in the development of many other tissues as well. The structures of bones and muscles, for example, are altered by weightbearing and exercise (Smith and Gilligan, 1996), and the structures of the mammalian vascular system and lung are regulated by oxygen physiology (Cunningham et al., 1974; Shweiki et al., 1992). For each organ, a fixed developmental program sets out the general structure of the organ early in development, and the structure is refined in a plastic phase that modifies organ form to match function. Recently, the embryonic program that controls the stereotyped development of the major branches of the *Drosophila melanogaster* tracheal (respiratory) system was elucidated (Sutherland et al., 1996). Here we describe how oxygen physiology patterns the fine terminal branches during the subsequent plastic phase of tracheal development.

*To whom correspondence should be addressed (e-mail: krasnow@cmgm.stanford.edu).
† These authors made equal contributions.

The *Drosophila* tracheal system is an elaborate network of epithelial tubes that ramifies throughout the body (Manning and Krasnow, 1993). Oxygen enters the network through the spiracular openings, and passes through primary, secondary, and terminal branches to reach the tissues. The network develops in the embryo by sequential sprouting of branches from 20 epithelial sacs, each composed of ~80 cells (Samakovlis et al., 1996). The entire branching sequence occurs exclusively by cell migration and changes in cell shape. Initially, six small groups of cells migrate out from each sac and form primary branches. This process is controlled by Branchless (Bnl), a homolog of mammalian fibroblast growth factors (Sutherland et al., 1996; Shilo et al., 1997; Metzger and Krasnow, 1999). The gene is expressed in six clusters of cells arrayed around each sac. The secreted growth factor activates the Breathless (Btl) FGF receptor (FGFR), a receptor tyrosine kinase expressed on nearby tracheal cells, and guides their migrations as they grow out and assemble into primary branches (Klambt et al., 1992; Reichman-Fried et al., 1994; Lee et al., 1996). The Bnl pathway also patterns secondary branch sprouting, but by a different mechanism. High levels of Bnl induce expression of secondary branch genes, such as the *pointed* ETS domain transcription factor in the cells at the tips of the outgrowing primary branches, stimulating these cells to form unicellular tubes called secondary branches (Lee et al., 1996; Sutherland et al., 1996). In this way, the Bnl pathway specifies the stereotyped pattern of major branches in the embryo.

Most secondary branch cells subsequently express terminal branch genes such as *blistered* (*pruned*) and go on to form elaborate networks of fine terminal branches in the larva (Guillemin et al., 1996; Samakovlis et al., 1996). The structure and pattern of terminal branches differs dramatically from the previous generations of branches (Ruhle, 1932; Wigglesworth, 1983; Manning and Krasnow, 1993). Each terminal branch arises as a long, thin (0.1–1 μm diameter) cytoplasmic extension that grows out on the target tissue and forms an intracellular lumen, allowing oxygen to pass through the terminal tracheal cell to reach the tissue. Each terminal cell repeats this process of cytoplasmic extension and lumen formation dozens of times, forming an elaborately branched structure resembling a neuron. In many tissues, terminal branches contact almost every cell, providing each with its own oxygen supply.

Unlike the pattern of the major branches, which is simple and stereotyped, the pattern of terminal branching is exceedingly complex and variable. A half century ago, Wigglesworth (1954) carried out a series of experiments on the large hemipteran insect *Rhodnius prolixus* that demonstrated the plasticity of terminal branching and the key role of oxygen in the process. Blocking oxygen delivery to tissues by surgical removal of tracheal branches or by plugging the spiracular openings led to a compensatory increase in terminal branching. Implanting a metabolically active tissue induced terminal branches from neighboring segments to grow in and

target the implant. One model Wigglesworth proposed to explain these results was that metabolically active tissues produce a signal that attracts tracheal branches. Although the identity of the signal is unknown, it was suggested to be an anaerobic metabolite (Wigglesworth, 1954; Pihan, 1972).

In this paper, we investigate the effect of oxygen tension on terminal branch sprouting in *Drosophila* and show that the process is regulated by a local signal or signals from oxygen-starved tissues in the larva. We provide evidence that the critical signal is Bnl FGF, the same signal that patterns outgrowth of the major branches in the embryo. During larval life, low oxygen stimulates expression of Bnl, and the secreted growth factor guides new terminal branches to the expressing cells to supply them with oxygen. Thus, a single growth factor is reiteratively used to pattern each level of tracheal branching, and the dramatic change in branch patterning during development results from a switch from developmental to physiological control of its expression.

Results

Terminal Branching in *Drosophila* Is Regulated by Local Oxygen Need

We investigated the effect of ambient oxygen levels on the development of terminal branches by counting the number of mature, air-filled branches in larvae reared under different oxygen tensions. We also examined the effect of oxygen tension on the initial step in the formation of terminal branches, the extension of cytoplasmic projections from terminal cells, using a tau-GFP reporter that specifically labels tracheal cells. Both analyses gave similar results. There were 68% more cytoplasmic extensions and mature branches in first instar larvae grown for 20 hr under 5% O_2 compared to siblings grown under normal atmospheric oxygen (21% O_2). Conversely, larvae grown under high oxygen tension (60% O_2) had fewer branches than normoxic controls (Figures 1A–1C; Table 1). Similar effects were observed at each larval stage and at different positions in the animal (Figures 1D–1F; Table 1). The morphology of the branches was also affected by oxygen. Under high oxygen tension the terminal branches were shorter and straight with few side branches, whereas under the low oxygen condition they were long and tortuous with many side branches (Figures 1D–1F). Thus, oxygen is an important regulator of terminal branching, influencing the initial budding and the final number and morphology of the branches.

To determine if the effects of oxygen on terminal branching were globally regulated or mediated by local signals produced by oxygen-starved tissues, small regions of the larvae were deprived of their normal oxygen supply. This was accomplished by generating clones of *blistered*$^-$ tracheal cells, which are unable to form terminal branches, leaving the surrounding region without its normal tracheation. We found that the *blistered*$^+$ tracheal cells in neighboring segments grew into the detracheated region, sprouting 40% (n = 25) more terminal branches than normal, whereas *blistered*$^+$ tracheal cells not bordering the clone were unaffected (Figures 1G and 1H and data not shown). To determine if it was the absence of terminal branches or just the absence of an oxygen supply that induced neighboring branches to grow, we tested clones of *synaptobrevin*$^-$ tracheal cells that extend terminal branches but are unable to form a lumen and deliver oxygen to the target (E. J., unpublished data). Even in the presence of the nonfunctional *synaptobrevin*$^-$ terminal branches, neighboring wild-type tracheal cells grew into the oxygen-starved region (Figure 1I). This implies that it is lack of oxygen delivery to the region, not some other function of missing terminal branches, that causes neighboring tracheal cells to respond. We conclude that terminal branching is regulated by local oxygen need, and that oxygen-starved cells produce a signal or signals that can attract tracheal branches from as far as one segment away.

bnl Is Reexpressed during Larval Development in Tracheal Target Tissues

We sought to identify the tracheogenic factor secreted by oxygen-starved tissues. Previously it was shown that during embryonic development *bnl* is expressed dynamically at the specific positions where primary branches and secondary branches form, and expression shuts off as these events are completed near the end of embryogenesis (Sutherland et al., 1996). When *bnl* expression was examined by in situ hybridization at later stages of development, we found that the gene turned back on by the first larval instar and continued to be expressed throughout larval life. However, in contrast to its highly restricted expression pattern in the embryo, the gene was broadly expressed in the larva, including all tissues that become heavily tracheated with terminal branches. In particular, the three most highly tracheated tissues—gut, muscles, and central nervous system (CNS)—all showed generalized expression of *bnl* during the first and second larval instars when terminal branches are sprouting (Figures 2A–2C). Thus, *bnl* is expressed in the appropriate tissues at the right time to regulate terminal branching in the larva. Several tissues that are not tracheated, including the epidermis and salivary gland (Figure 2F), did not express *bnl*, strengthening the correlation between *bnl* expression and terminal branching. The correlation was not absolute though, as several other tissues with few or no branches, including the imaginal discs, heart, and fat body, did express significant levels of *bnl* RNA (Figures 2D and 2E and data not shown). However, in no case did a terminally tracheated tissue not express *bnl*.

Bnl Is a Potent Inducer and Chemoattractant for Terminal Branches

To test whether Bnl regulates terminal branching, we first overexpressed the gene during larval development using the GAL4-UAS system (Brand and Perrimon, 1993). Ubiquitous expression of *bnl* driven by hsGAL4 for just 1 hr during the first larval instar caused a dramatic proliferation of terminal branches. However, unlike normal terminal branches, which distribute evenly over their targets (Figure 3H), these remained clumped together in a large tangled mass near their point of origin (Figure 3J). A dramatic proliferation of terminal branches also resulted when *bnl* was persistently expressed throughout larval development in specific target tissues such

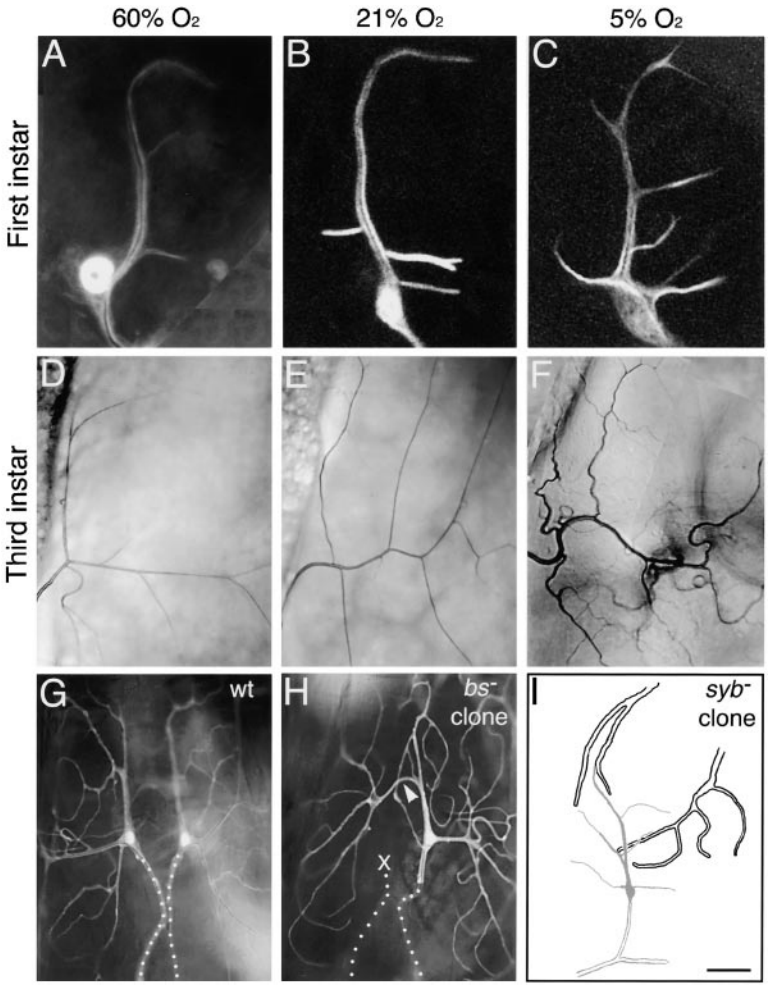

Figure 1. Effect of Global and Local Oxygen Deprivation on Tracheal Terminal Branching

(A–C) Effects of ambient oxygen level on first instar larvae. Confocal fluorescence images of the dorsal branch terminal cell (dorsal view, anterior left) of a Tr3 tracheal metamere in living UAS-*tau*GFP, UAS-nGFP; *btl*-GAL4 first instar larvae reared in room air (21% O_2) and then transferred to 60% (A), 21% (B), or 5% (C) O_2 for 20 hr. The GFP markers are expressed specifically in tracheal cells and allow visualization of the nucleus and cytoplasmic extensions, most of which will develop a lumen and become mature terminal branches. Note the increasing number of cytoplasmic extensions with decreasing O_2 tension.

(D–F) Effect of ambient oxygen level on third instar larvae. Wild-type larvae were reared in room air and then transferred to 60% (D), 21% (E), or 5% O_2 (F) for 40 hr. Bright-field images of mature, air-filled terminal branches (dorsal view, anterior up) emanating from a Tr3 dorsal branch terminal cell are shown. Note the increasing number of branches with decreasing O_2 tension and their increased tortuosity under the low oxygen condition. (F) is a montage.

(G–I) Local regions lacking their normal tracheal supply were created by inducing clones of *blistered*$^-$ or *synaptobrevin*$^-$ tracheal cells. The response of nearby *blistered*$^+$ or *synaptobrevin*$^+$ terminal cells was monitored. (G) Pair of normal Tr3 dorsal branches in a third instar larva, visualized as in (A)–(C) (dorsal view, anterior up). The right and left dorsal branches (highlighted by dotted lines) end in terminal cells that ramify on the right and left dorsolateral muscles, respectively. Terminal cell nuclei show intense expression of the nGFP marker. (H) Similar view of a *blistered*$^-$ terminal cell clone. The left dorsal branch is truncated (X) because of the *blistered*$^-$ clone, and the *blistered*$^+$ terminal cell on the right has compensated by sprouting extra branches (arrowhead) that cross the midline to supply muscles on the left. (I) Composite tracing of multiple focal planes surrounding a *synaptobrevin*$^-$ tracheal clone in the lateral trunk of a third instar larva. Normally, tracheal branches avoid their neighbors but here *synaptobrevin*$^+$ terminal branches (black outline) are invading a region occupied by nonfunctional (lumenless), *synaptobrevin*$^-$ terminal branches (gray fill).

Bar, 7 μm for (A)–(C), 25 μm for (D)–(F), 50 μm for (G)–(I).

as the CNS, gut, and somatic muscle fibers using other GAL4 drivers (see Experimental Procedures). Overexpression of *bnl* in the CNS, for example, transformed the normal ladder-like pattern of tracheal branches (Figure 3C) into a massive tangle of branches that completely covered the CNS (Figure 3E). Thus, *bnl* is a potent inducer of terminal branching. Although similar effects were seen in most other tissues examined, the heart and imaginal discs, which normally express *bnl* but lack tracheal branches, failed to attract branches even when the gene was overexpressed. This suggests that *bnl* expression or activity is regulated posttranscriptionally in these tissues.

Ectopic expression experiments demonstrated that

Table 1. Effects of Ambient Oxygen on Terminal Branching

			Number of Terminal Branches		
Expt	Stage	Branch	60% Oxygen	21% Oxygen	5% Oxygen
I	L1	Tr3 DB	ND	5.6 ± 0.2 (100%)	9.4 ± 0.3 (168%)
II	L1	Tr3 DB	3.6 ± 0.1 (77%)	4.7 ± 0.2 (100%)	ND
III	L2	Tr5 LF	ND	7.8 ± 0.3 (100%)	12.5 ± 0.5 (160%)
IV	L3	Tr3 DB	ND	20.4 ± 0.4 (100%)	33.8 ± 0.6 (166%)
V	L3	Tr3 DB	14.7 ± 0.4 (72%)	20.5 ± 0.6 (100%)	ND

Larvae were placed at the indicated O_2 tensions for 20 hr (experiments I, II, III) or 40 (IV, V), and the number of cytoplasmic extensions (I, II) or mature, air-filled terminal branches (III, IV, V) was counted at the indicated larval stage. Values given are the average (± SE) for 13–28 counts. Normalized values relative to the 21% oxygen condition are given in parentheses. ND, not determined.

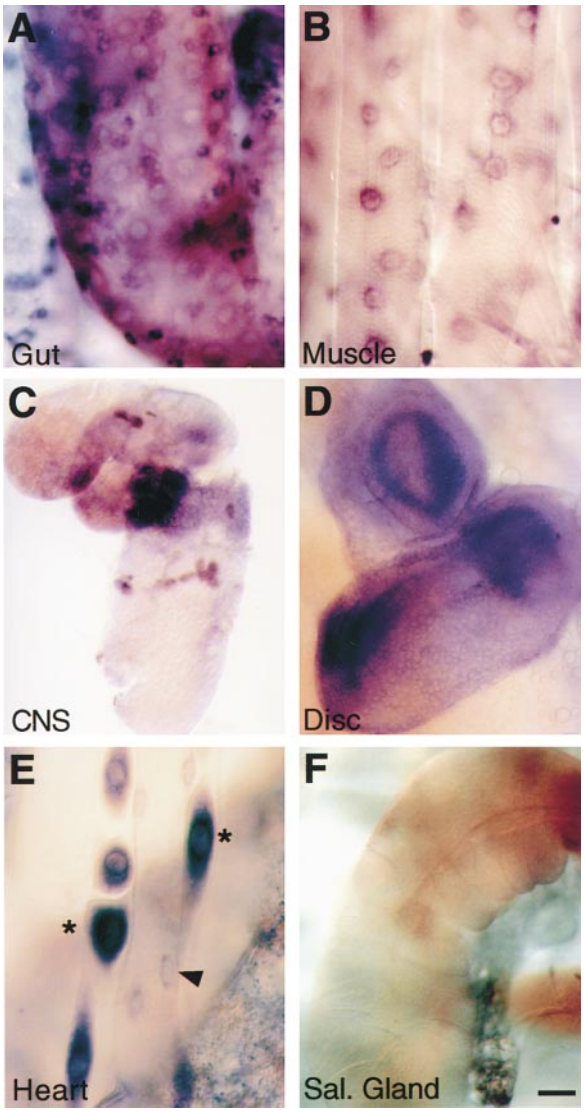

Figure 2. Expression of *bnl* in Larval Tissues

In situ hybridization of larval tissues with a *bnl* mRNA probe. Widespread expression (purple/blue stain) is seen throughout most tissues with transcript concentrated around each nucleus.

(A) Gut, third instar.

(B) Somatic muscles, third instar.

(C) CNS, second instar. Expression is seen throughout the CNS except in the neuropile of the ventral nerve cord. The dark regions are areas of tissue overlap.

(D) Eye-antennal disc, third instar.

(E) Heart, third instar. High levels of expression are seen in the pericardial cells (*) and lower levels in cardial cells (arrowhead).

(F) Salivary gland. No expression is detected.

A control probe for *bnl* antisense strand showed no significant staining in any tissues (not shown). Bar, 30 μm for (A), (B), (E), and (F); 10 μm for (C), and 20 μm for (D).

Bnl functions as a chemoattractant that can guide terminal branches to new tissues and even to individual cells within a tissue. For example, when *bnl* was expressed in the developing salivary glands, which normally do not express *bnl* (Figure 2F) or receive tracheal branches (Figure 4A; n = 32), 82% of expressing glands (n = 55) now received terminal branches from tracheal cells that supply nearby tissues (Figure 4B). Similar results were obtained when *bnl* was overexpressed in a single somatic muscle fiber within a field of muscle fibers that normally all express *bnl* at similar levels: outgrowth of LG terminal branches was redirected to muscle fiber 12 (Figure 4D) and away from their normal targets at the ventral midline (Figure 4C). This implies that terminal branches can sense different levels of Bnl and grow toward the area of highest concentration.

The ability of terminal branches to target a source of Bnl was most dramatically shown in larvae engineered to express elevated levels of Bnl in individual cells. An hsFLP transgene was used to activate expression of *actin*-GAL4 in random cells, which in turn drove expression of UAS-*bnl* (and UAS-GFP to fluorescently mark the ectopic Bnl-expressing cells). Expression of Bnl in single cells caused a local proliferation of terminal branches that grew out to the expressing cells, arborizing on the cells and almost completely engulfing them (Figure 4E). We also found a case in which a terminal branch had bifurcated to target two Bnl-expressing cells separated by several cell diameters (Figure 4F). This implies that a tracheal cell can simultaneously sense and respond to two nearby sources of Bnl. Targeting of terminal branches to individual Bnl-expressing cells occurred over distances of up to 10 cell diameters, indicating that the secreted growth factor can diffuse substantial distances across a tissue. Thus, Bnl is a terminal branch attractant that can provide long-range and extremely precise targeting information—down to the level of individual cells.

Bnl Is a Dosage-Sensitive Regulator of Terminal Branching

Because the complete absence of *bnl* causes embryonic lethality, we investigated the requirement for *bnl* during larval terminal branching by examining the effects of more subtle alterations in *bnl* activity and of a conditional mutation in the Bnl pathway. There was a striking dependence of terminal branching on *bnl* gene dosage (Figures 3A–3J). The number of terminal branches was reduced 30% to 50% in heterozygous (bnl^{P1}/+) and hemizygous (*Df(3R)DlBX12*/+) mutants, whereas larvae with an extra copy of *bnl* (*Dp(3;3)C123.3*/*Df(3R)P47*) had 25% more branches than normal (Figure 3K). Terminal branches in larvae carrying an extra copy of *bnl* exhibited a tortuous morphology (Figure 3I) similar to that observed in animals reared under low oxygen (Figure 1F). When Bnl pathway activity was severely compromised by expressing a dominant-negative form of the Breathless FGF receptor (Reichman-Fried and Shilo, 1995) in the terminal tracheal cells of the larva, there was nearly complete (90%) inhibition of terminal branching in subsets of terminal cells (Figures 3A, 3F, and 3K).

Even at artificially high levels of Bnl, terminal branching remained sensitive to different Bnl levels. Because GAL4 activity in the GAL4-UAS system increases at higher temperatures (Brand et al., 1994), we were able to modulate the levels of *bnl* overexpression by varying temperature. In wild-type third instar larvae, there were an average of 6.3 terminal branches on muscle fiber 12 (Figure 3L; n = 32). In 5053-GAL4>UAS-*bnl* larvae reared at 25°C, there were more than twice the normal

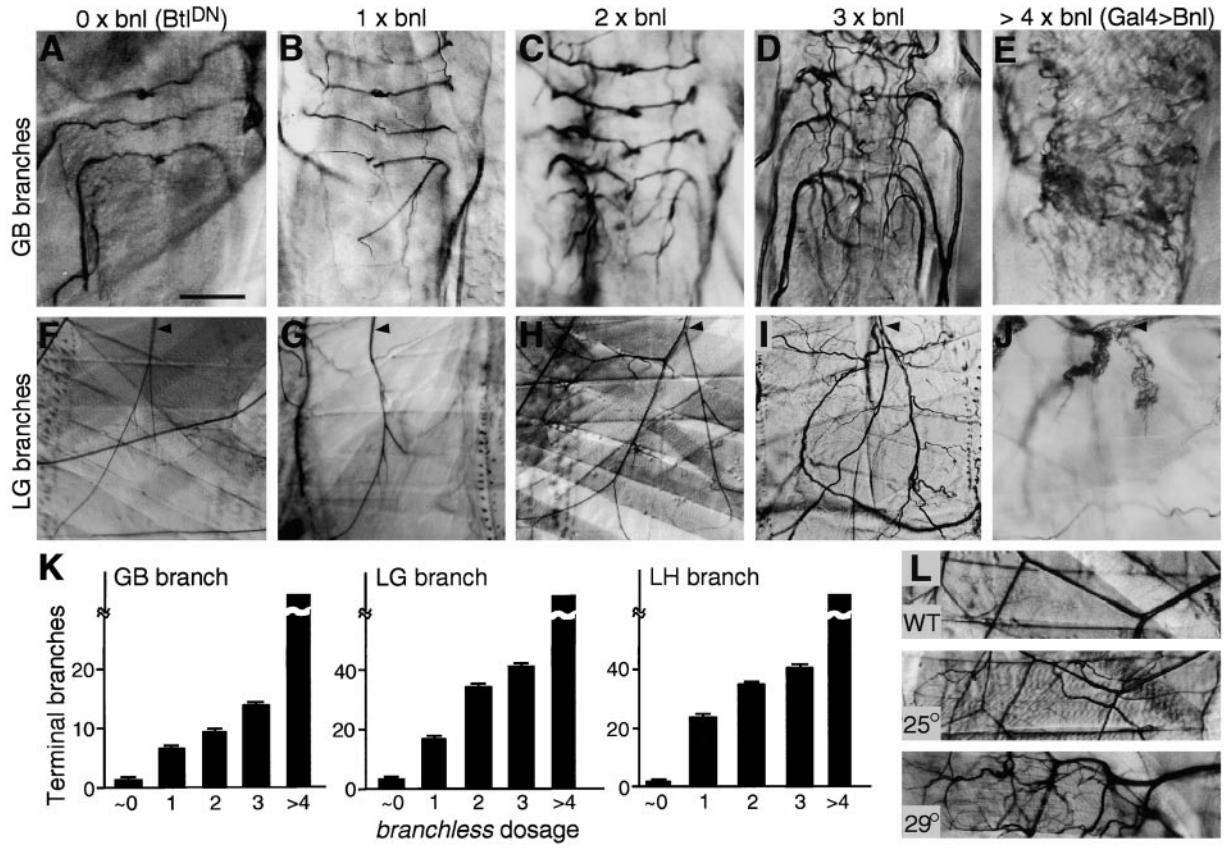

Figure 3. Dependence of Terminal Branching on *bnl* Dosage

Close-up views of several ganglionic branches (GBs) (A–E; ventral view, anterior up) and a lateral trunk LG branch (F–J; dorsal up, anterior left) in third instar larvae under conditions of increasing *bnl* pathway activity as indicated. In wild type (C and H), GBs enter the CNS from both sides forming a ladder-like pattern (C), and the LG grows toward the ventral midline and ramifies on ventral muscles (H). (A and F) Low Bnl pathway activity due to tracheal expression of UAS-*breathless*^{DN268}, a dominant-negative receptor, driven by A9-GAL4 (A) or *blistered*23.26-GAL4 (F). (B and G) *bnl*^{P1}/+ heterozygous larvae. Similar effects were seen with a hemizygote (*Df(3R)D1BX12*/+). (C and H) Wild type. (D and I) Triploid *bnl* larvae (*Dp(3;3)C123.3/Df(3R)P47*). The ventral nerve cord is elongated in this genetic background (D). (E and J) High *bnl* activity due to overexpression of a UAS-*bnl* transgene driven by A9-GAL4 (E) or hsGAL4 (J). (K) Quantitation of GB, LG, and LH terminal branches (average ± SE) in third instar larvae under the different conditions of Bnl pathway activity. (L) Increasing activity of a UAS-*bnl* transgene increases terminal branches on muscle fiber 12. (Top) Wild-type third instar larva. (Middle) 5053A-GAL4>UAS-*bnl* larva reared at 25°C. There are twice the normal number of terminal branches. Extras all arise from rerouting of LG terminal branches. (Bottom) 5053A-GAL4>UAS-*bnl* larva reared at 29°C to increase GAL4-dependent expression of Bnl. There are about four times the normal number of terminal branches. The extras arise from LG as well as LH and LF, the two branches that normally tracheate this muscle. Bar, 50 μm for (A)–(E); 150 μm for (F)–(J); and 100 μm for (L).

number (15.6, n = 34), and the number nearly doubled again to 25.9 (n = 26) when the rearing temperature was increased to 29°C to further increase Bnl expression (Figure 3L). There was little effect of temperature on terminal branch number in larvae lacking the UAS-*bnl* transgene (data not shown). We conclude that *bnl* is a critical, dosage-sensitive regulator of terminal branching, and that it functions over a wide range of concentrations.

Oxygen Regulates Bnl Expression

The above results show that both ambient oxygen level and Bnl are important regulators of terminal branching. This suggested that oxygen might exert its effect by altering the expression or activity of Bnl. We assayed Bnl protein levels by immunoblot analysis of larvae subjected to different oxygen environments. Larvae reared under 5% oxygen for 36 hr expressed 2-fold more Bnl than normoxic (21% oxygen) controls, whereas larvae placed under 60% oxygen expressed low or undetectable levels of Bnl (Figure 5A). Although the magnitude of the effects was not large, it is sufficient to account for the observed effects of hypoxia on branching because of the extreme sensitivity of the process to *bnl* dosage described above. This implies that Bnl is the key mediator of this hypoxic response.

We also examined Bnl levels in regions of local hypoxia created by clones of *blistered*$^{-}$ tracheal cells. We focused the analysis on the few larvae in which the neighboring wild-type branches had not yet grown into the region so it presumably remained hypoxic. In each case examined, Bnl expression was increased in the syncitial muscle fiber or fibers normally supplied by the tracheal clone, whereas the neighboring muscles as well as the contralateral muscles all showed normal Bnl levels (Figure 5B). We conclude that Bnl expression is locally increased in hypoxic cells.

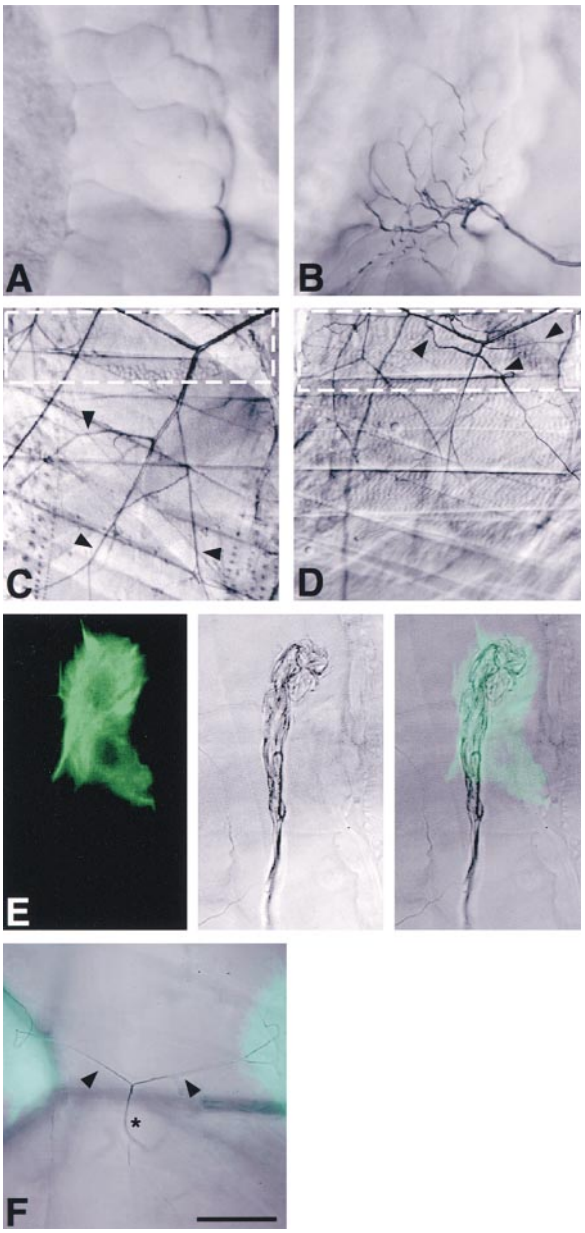

Figure 4. Bnl Functions as a Chemoattractant for Terminal Branches

(A) Normal salivary gland with no tracheal branches.

(B) Salivary gland in a 71B-GAL4>UAS-*bnl* larva. Bnl expression in the salivary gland has attracted terminal branches from a nearby tissue. The temperature dependence of GAL4 activity was used to demonstrate that the critical period of *bnl* expression for attracting branches to the salivary gland occurs during larval development (data not shown).

(C) LG lateral trunk branches in wild type larva (dorsal up, anterior left). The LG terminal branches (arrowheads) extend toward the ventral midline and target the ventral muscle fibers. Muscle fiber 12 (boxed) is tracheated by LG in only 6% of segments (n = 67).

(D) Similar view of a 5053A-GAL4>UAS-*bnl* larva that overexpresses Bnl in muscle fiber 12 during larval development. LG terminal branches (arrowheads) are directed to this muscle fiber in 87% of segments (n = 61).

(E) Fluoresence (left), bright-field (center), and merged images (right) of a 2-cell clone of Bnl- and GFP-expressing cells in the epidermis induced by FLP-mediated recombination. A rope-like tangle of terminal branches has grown out and ramified on the expressing cells.

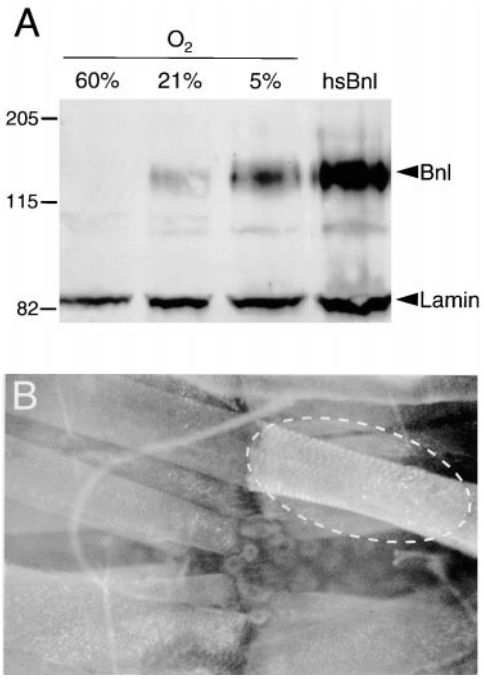

Figure 5. Hypoxia Induces Bnl Expression

(A) Bnl immunoblot of extracts of third instar larvae reared for 36 hr at the oxygen tensions indicated. Larvae expressing a heat-inducible *bnl* transgene are shown in lane 4 as reference. The blot was reprobed with an antiserum against nuclear lamin Dm0 to confirm equal loading. Scanning densitometry showed there was a 2-fold increase of Bnl levels (normalized to lamin Dm0) in the hypoxic (5% O_2) relative to the normoxic (21% O_2) condition, and no detectable Bnl in the hyperoxic (60% O_2) condition. Similar results were obtained in five independent experiments, except that in some there was low but detectable Bnl in the hyperoxic condition. Molecular weights of markers (in kDa) are shown at left.

(B) Bnl immunofluorescence in a region around a *blistered*$^-$ clone. A bright-field view of the same region (not shown) demonstrated that the syncitial muscle fiber indicated (dashed oval) is missing its normal tracheal supply. This muscle fiber, but none of the neighboring muscle fibers, shows elevated Bnl immunofluorescence. Similar results were obtained in the two other clones examined.

Discussion

We have investigated how oxygen controls terminal branching of the *Drosophila* airways, and there are two major conclusions from the results. First, terminal branching is regulated by a local signal or signals produced by oxygen-starved cells. Second, the critical signal is Bnl FGF. The identification of the key pathway by which cellular oxygen physiology controls airway branching leads to a simple model of how the pattern of terminal branches is specified. Although the pattern is complex and variable, it is not at all random. Rather, we propose that branching is very precisely controlled by the FGF pathway to match the oxygen needs of larval tissues.

(F) Merged fluorescence and bright-field images of the edges of two separated Bnl- and GFP-expressing cells induced as above. A nearby tracheal branch (asterisk) has split and each sprout (arrowheads) directly targets one expressing cell.

Bar, 90 μm for (A)–(D), 25 μm for (E) and (F).

A Local Signal from Oxygen-Starved Tissues Regulates Terminal Branching

Two types of experiments support the conclusion that oxygen-starved tissues in *Drosophila* produce a signal that controls tracheal branching. First, rearing larvae under low oxygen caused increased sprouting of terminal branches throughout the animal, whereas high oxygen tension had the opposite effect. Second, depriving local regions of oxygen in genetic mosaics caused increased sprouting of tracheal cells bordering the hypoxic areas. A similar tracheal response is observed near rapidly growing tumors in *Drosophila* (E. J., unpublished data). Both global and local hypoxia triggered the outgrowth of new cytoplasmic extensions from terminal tracheal cells, which targeted the hypoxic area and developed into complex arrays of fine branches that supplied the region with oxygen. The results imply that oxygen-starved tissues produce a local signal that induces tracheal sprouting and guides branches to the signaling source. The signal is apparently diffusible as it can influence tracheal cells in neighboring segments. Classical embryological experiments have provided evidence for a hypoxia-induced tracheal chemoattractant with similar characteristics in other insects, suggesting that such a signal is quite general (Wigglesworth, 1954; Locke, 1958; Pihan, 1971).

Bnl as the Hypoxic Signal

Our results provide evidence that the critical hypoxic signal is Bnl FGF. First, *bnl* RNA is expressed broadly in the larva and in all tissues that become heavily tracheated during larval life. Second, Bnl is a potent inducer of terminal branching. Increasing Bnl expression in various tissues with *bnl* transgenes caused a striking proliferation of terminal branches that formed dense tangles that in some cases completely covered the tissue. Third, there was a strong correspondence between *bnl* gene dosage and terminal branch number that held over a wide range of Bnl levels. Furthermore, blocking the response of tracheal terminal cells to Bnl with a dominant-negative form of its receptor can prevent almost all terminal branching. These results show that the Bnl pathway is necessary and sufficient for terminal branching in vivo, and the dosage dependence implies that it is the critical, although not necessarily the only, determinant (see below). Finally, we observed that oxygen-deprivation stimulated expression of Bnl whereas increased oxygen suppressed expression, and the magnitude of these effects can account for the observed effects of oxygen on terminal branch number and morphology. Thus, we propose that Bnl is the hypoxic signal postulated by Wigglesworth a half century ago.

Patterning Terminal Branching

The arborization pattern of terminal branches is remarkably complex. Each terminal tracheal cell sprouts dozens of branches that spread out and contact nearly every cell in the target. At first glance, the complex and variable nature of the branch pattern suggests that the process is highly random, with the constraint only that branches fill the available space (Meinhardt, 1976). Our results, however, support a model in which the final pattern, although variable, is not at all random. Instead, branching is precisely controlled during development to meet the oxygen needs of the target cells.

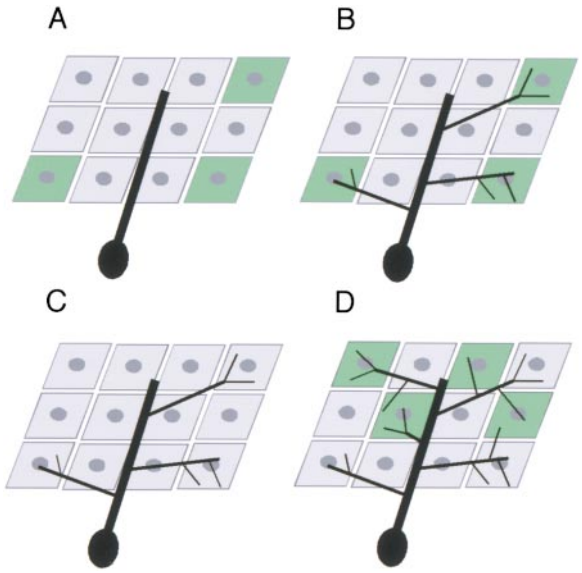

Figure 6. Model for Patterning of Terminal Branching by Bnl

(A) Cells in a target tissue (gray) experience different degrees of hypoxia due to differences in metabolic activity and distance from an existing tracheal branch (black). Bnl expression is increased in the hypoxic cells (green).

(B) The tracheal cell responds by sprouting new terminal branches that grow out toward each Bnl signaling center. When the branch approaches the source, it begins to arborize.

(C) When the new branches mature and supply oxygen to the target after the next molt, hypoxia is relieved and Bnl expression turns off.

(D) Other cells become hypoxic and the process repeats, generating dense tracheal coverage that precisely matches tissue need.

We propose a model in which each cell experiencing an oxygen debt senses the impending crisis and responds by upregulating expression of Bnl (Figure 6A). Bnl FGF diffuses to nearby tracheal cells and stimulates new tracheal branches to form and grow toward each signaling source (Figure 6B). This supplies oxygen to the hypoxic cells and shuts off the signal (Figure 6C). The process is dynamic and repeats itself many times due to the constantly changing balance in cell oxygen need and supply (Figure 6D). Over the course of development, most cells would become hypoxic, serve as an FGF signaling center, and receive an appropriate tracheal supply. Thus, the ultimate pattern of tracheal branches would reflect the complex history of the oxygen needs and Bnl expression pattern of the tissues.

Are all cells equipped with their own oxygen-sensing pathway, and does the pathway have the precision required by the model? The hypoxia inducible factor-1 (HIF-1) transcription factor is a key regulator of the mammalian hypoxic response (Semenza, 1998), and a related activity is also present in *Drosophila* cells (Nagao et al., 1996). Recent studies show that this pathway can be activated by hypoxia in virtually all larval cells (E. J., unpublished data). Under mild hypoxia, the HIF-1 pathway is activated in isolated cells in a tissue, implying that cells trigger the pathway independently. Likewise, we found that Bnl is induced in a syncitial muscle cell

deprived of its oxygen supply, indicating that Bnl expression is regulated independently in individual cells. Furthermore, engineered expression of Bnl in individual cells induced budding of nearby tracheal branches that grew out and targeted the expressing cells, from up to 10 cell diameters ($>$ 100 μm) away. Bnl therefore is a tracheal attractant with the precision required by the model. Remarkably, a terminal tracheal branch is able to simultaneously sense more than one Bnl signal (Figure 4F). This is important during development because terminal cells probably receive Bnl signals from many directions and must discriminate among and respond appropriately to each signal gradient.

Bnl has an additional property relevant to branch patterning—the ability to trigger terminal branch arborization at high concentrations. Far from the source of Bnl, where Bnl concentration is presumably low, Bnl induces branches to grow toward the source. But as branches approach the source, they ramify into many fine branches that cover the expressing cell (Figure 4E). Likewise, gradually increasing the expression of Bnl in a specific muscle fiber first attracts branches from nearby muscle fibers and at higher levels causes many additional branches to sprout (Figure 3L). Thus, low levels of Bnl stimulate guided outgrowth and high levels promote arborization. This contributes to branch pattern by ensuring that the hypoxic target receives the majority of the newly formed branches.

Although our model provides a framework to explain many aspects of terminal branch patterning, the data suggest that factors other than Bnl are also involved. For example, the first terminal branches to sprout are stereotyped and grow toward specific muscle fibers at the end of embryogenesis when Bnl expression is not detected, and later during larval life when all muscles express Bnl some branches show a preference for particular muscles (J. J., unpublished data). The model also does not explain why alterations in *bnl* dosage affect branching, because if the proposed circuitry controlling *bnl* expression were perfectly responsive it should completely compensate for differences in gene dosage. There must be other factors that function in parallel or modulate the Bnl pathway to influence terminal branching. Although overexpression of Bnl or extreme hypoxia can generally overcome these other influences, two tissues, the imaginal discs and the heart, are refractory to Bnl. It is not clear how these tissues survive without a tracheal supply or how they prevent tracheal cells from responding to the Bnl they make.

Reiterative Use of an FGF Pathway to Pattern Tracheal Branching

Our results, together with previous studies of the Bnl FGF pathway, lead to the surprising conclusion that a single growth factor is the major determinant of the complex tracheal branching pattern. In the embryo, Bnl specifies where primary branches bud and the direction they grow, and it also specifies the positions of secondary branch sprouting (Sutherland et al., 1996). Bnl is used again in the larva to pattern terminal branches. At each stage, Bnl acts in conjunction with the same FGF receptor, Breathless. Thus, the FGF pathway is reiteratively used during development to pattern each stage of tracheal branching.

Although the same FGF and FGF receptor are used repeatedly, the pattern and structure of branches change dramatically at each stage. How do the same ligand and receptor generate branches of different structure and pattern? We propose that two molecular switches occur in the Bnl pathway at the end of embryogenesis that explain the different branching outcomes. First, there is a switch in regulation of *bnl* expression, from control by hard-wired developmental regulators such as Dpp (Vincent et al., 1997) to control by oxygen-dependent regulators. The change in regulation of ligand expression explains why the primary and secondary branching pattern is fixed whereas terminal branch sprouting is variable and regulated by oxygen need. The second switch occurs in the signaling pathway downstream of the receptor. During primary branching, Bnl acts as a chemoattractant that guides the migration of groups of tracheal cells as they grow out and assemble into primary branches. Bnl also induces expression of new components of the signal transduction pathway that alter the tracheal response in subsequent branching events. One of the induced genes is *blistered (pruned)*, a key regulator of terminal branching (Guillemin et al., 1996; Sutherland et al., 1996). *blistered* encodes the *Drosophila* homolog of mammalian serum response factor (SRF) and is proposed to function as part of a growth factor–activated transcription complex that drives expression of tracheal genes required for cytoplasmic extension (Affolter et al., 1994; Guillemin et al., 1996). Blistered SRF is not made in time to participate in the initial Bnl signaling events that guide primary branching. However, when the next Bnl signaling events occur during larval life, the presence of this new downstream component (and perhaps others) alters the tracheal response, promoting cytoplasmic outgrowth and formation of fine terminal branches. In this way, modulation of upstream and downstream components of the FGF pathway drives the transition between the disparate phases of tracheal branching.

Implications for Angiogenesis

The mammalian vascular system is a tubular network whose finest branches, the capillaries, generate dense tissue coverage without obvious pattern, like terminal tracheal branches (Risau, 1997). Hypoxia is a potent inducer of angiogenic factors such as VEGFs (Shweiki et al., 1992) and FGFs (Walgenbach et al., 1995), implying that capillary branching is induced at least in part by tissue oxygen need. It is unclear if hypoxia locally controls the growth of each new vessel or if it acts as a more general stimulant to capillary formation. Our results in the *Drosophila* tracheal system raise the possibility that the patterning of blood capillaries is also precisely controlled by local oxygen need. Interestingly, the angiogenic factors regulated by hypoxia and their receptors also play important roles earlier in development during formation of the major blood vessels (Gale and Yancopoulos, 1999). Thus, the reiterative use of a growth factor or growth factor family to pattern both the hard-wired and physiologically regulated stages of branching may be a general strategy to match organ structure to physiological function.

Experimental Procedures

Strains

Canton-S and w^{1118} were the control strains and bnl^{P1} (Sutherland et al., 1996), $blistered^{pruned1}$ (Guillemin et al., 1996), and $synaptobrevin^{25-77}$ (from Tom Schwartz) are null or strong loss-of-function alleles. *Df(3R)DlBX12* removes cytological region 91F–92D, which contains *bnl* (92B), and *Dp(3;3)C123.3/Df(3R)P4* contains duplications of cytogenetic regions 92A–92D and 88B–89D (Perrimon et al., 1991; Lindsley and Zimm, 1992). Strains carrying the following GAL4 and UAS transgenes were used: *btl*-GAL4 and UAS-nGFP (Shiga et al., 1996), UAS-GAPGFP (A. Chiba, personal communication), A9-GAL4 (Lin and Goodman, 1994), 71B-GAL4 (Brand and Perrimon, 1993), 5053A-GAL4 and hsGAL4 (Bloomington Stock Center), *blistered*23.26-GAL4 (K. Guillemin and M. A. K., unpublished data), UAS-*bnl* (Sutherland et al., 1996), UAS-$breathless^{DN268}$ (Reichman-Fried and Shilo, 1995), and *actin*>CD2>GAL4 (Pignoni and Zipursky, 1997). The hsFLP and FRT chromosomes have been described (Xu and Rubin, 1993).

The UAS-*tau*GFP transgene was constructed by ligation of DNAs encoding bovine microtubule-binding protein tau from pKStaulacZ (Callahan and Thomas, 1994) and FITC-shifted GFP from pKEN-GFP2 (Cormack et al., 1996) into the pUAST vector (Brand and Perrimon, 1993). Transgenic strains carrying stable insertions on chromosome 2 were generated by P element–mediated transformation.

Genetic Mosaics

Two- to four-hour-old embryos of genotype *hsFLP; P[w$^+$] P[neoR, FRT 42B]/blistered^{pruned1} P[neoR, FRT 42B]* were heat-shocked for 2 hr at 37°C to induce *blistered*$^-$ clones and then returned to 25°C. Tracheal clones in third instar larvae were identified as dorsal regions of segments A1 or A6 lacking their normal tracheal supply from the Tr3 and Tr8 dorsal branch (DB) terminal cells; the number of air-filled terminal branches emanating from the contralateral, *blistered*$^+$ DB terminal cell were scored under bright-field optics. *blistered*$^+$ DB terminal cells that did not neighbor the region (Tr3 DB for Tr8 DB clones and vice versa) were also scored. As an additional control, *blistered*$^+$ terminal branches were scored in siblings of genotype *P[w$^+$] P[neoR, FRT 42B]/blistered^{pruned1} P[neoR, FRT 42B]* treated in the same manner. We also identified rare *blistered*$^-$ clones in which the affected regions had not yet been tracheated by neighboring *blistered*$^+$ terminal cells. Larvae were dissected open and stained as described (Patel, 1994) with a rabbit anti-Bnl antiserum (D. Sutherland and M. A. K., unpublished data) and a fluorescent secondary antibody. The specificity of the Bnl antiserum was confirmed by enhanced staining of larval clones overexpressing Bnl, generated as described below.

Clones of *synaptobrevin*$^-$ cells were created by exposing 2- to 3-hour-old embryos of genotype *synaptobrevin$^{25-77}$/+; btl*-GAL4/UAS-GAPGFP to 1000 rads of gamma radiation. Tracheal clones were identified in third instar larvae by the absence of oxygen-filled lumens in tracheal branches visualized by GFP fluorescence.

bnl Expression Analysis

Larvae were dissected open at the dorsal midline, fixed in 3.7% formaldehyde, and in situ hybridization was carried out as described (Kopczynski et al., 1996) using digoxigenin-labeled RNA probes made from *bnl* Z3-2 cDNA (Sutherland et al., 1996) and alkaline phosphatase immunohistochemistry (O'Neill and Bier, 1994).

For Bnl immunoblot analysis, second instar larvae were reared in 5%, 21%, or 60% O_2 for 36 hr as described below. The resultant third instar larvae were frozen in liquid nitrogen, dounce homogenized in Laemmli sample buffer, and boiled for 10 min. Extracts were clarified by centrifugation, separated by SDS-PAGE and transferred to nitrocellulose. Bnl protein was detected with protein A–purified rabbit anti-Bnl antiserum preabsorbed against 0- to 2-hour embryos, and HRP/chemiluminescent immunohistochemistry (ECL; Amersham). Blots were reprobed with ADL 84.12 antibody to lamin Dm0 (Smith et al., 1987) and scanned; the ratio of Bnl to Dm0 in each lane was quantitated using IPLab software (Signal Analytics).

Determination of Oxygen Effects on Terminal Branching

UAS-*tau*GFP; *btl*-GAL4 embryos were collected for 6 hr and reared in room air. After hatching, larvae were transferred to agar plates containing a thin layer of yeast paste and placed in a sealed chamber equilibrated with 5% O_2/95% N_2, room air (21% O_2/79% N_2), or 60% O_2/40% N_2. After 20 hr, larvae were either removed from the chamber, etherized, mounted in halocarbon oil, and examined with a Leica fluorescence stereomicroscope, or allowed to develop until the second larval instar (L2) and air-filled branches counted under bright-field optics. Larvae exposed to more extreme conditions (4% or 75% O_2) showed significant mortality after 20 hr. Effects of oxygen on later larval stages were determined in a similar manner except that larvae were reared until late in L1 in room air in vials containing standard cornmeal–molasses fly medium, and the vials were then placed in sealed chambers at different oxygen tensions for 40 hr. Air-filled tracheal branches in L3 larvae were visualized under bright-field optics.

Ectopic Expression of Bnl

Flies carrying the B4-2 UAS-*bnl* transgene (Sutherland et al., 1996) were crossed to flies carrying the hsGAL4 (heat-inducible), 71B-GAL4 (salivary gland beginning at stage 14/15), A9-GAL4 (CNS and tracheal terminal cells beginning at the end of embryogenesis), or 5053A-GAL4 (muscle fiber 12 beginning at stage 14/15) drivers. Wandering third instar larvae were dissected, fixed, and analyzed for tracheal branching defects under D.I.C. optics.

To persistently overexpress Bnl in isolated clones of larval cells, first instar larvae of genotype *actin*>CD2>GAL4/hsFLP; UAS-*bnl*/UAS-*tau*GFP were heat-shocked at 35°C for 45 min and then returned to 25°C. This mild heat shock turns on FLP expression sporadically in isolated cells; FLP-mediated recombination removes the poly(A) site adjacent to CD2 and turns on GAL4, which induces the expression of UAS-*bnl* and UAS-*tau*GFP. Expressing cells were identified in etherized third instar larvae by *tau*GFP fluorescence, and terminal branches were visualized under bright-field optics.

Effects of *bnl* Gene Dosage on Terminal Branching

Individuals carrying one (bnl^{P1}/+ or *Df(3R)DlBX12*/+), two (+/+), or three wild-type copies (*Dp(3;3)C123.3/Df(3R)P47*) of *bnl* were reared in uncrowded bottles at 25°C. Terminal branches emanating from the Tr7-9 GB branches and the Tr3-10 LH and LG branches in third instar larvae were visualized under D.I.C. optics. Expression of UAS-$breathless^{DN268}$, a dominant-negative form of Breathless lacking the kinase domain (Reichman-Fried and Shilo, 1995), was driven in tracheal terminal cells by *blistered*23.26-GAL4 (terminal cells and muscle fibers starting at embryonic stage 15; K. Guillemin and M. A. K., unpublished data) or A9-GAL4 and dramatically inhibited terminal branching from the lateral trunk and GB terminal cells, respectively. Expression driven by hsGAL4 for several hours either at the end of embryogenesis as described (Reichman-Fried and Shilo, 1995) or during the first or second larval instars had no affect on terminal branching, nor did expression in tracheal target tissues driven by 71B-GAL4.

Acknowledgments

We are grateful to D. Sutherland for the Bnl antiserum, and S. K. Chiu, D. Sutherland, and other members of the lab for stimulating discussions. We thank B. Shilo, L. Zipursky, J. Thomas, P. Fisher, T. Schwarz, B. Cormack, A. Chiba, and the Bloomington Stock Center for strains and reagents. This work was supported by an NIH grant (to M. A. K.) and by NRSA postdoctoral fellowships (to J. J. and E. J.). M. A. K. is an investigator of the Howard Hughes Medical Institute.

Received August 13, 1999; revised September 16, 1999.

References

Affolter, M., Montagne, J., Walldorf, U., Groppe, J., Kloter, U., LaRosa, M., and Gehring, W.J. (1994). The Drosophila SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development. Development *120*, 743–753.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as

a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Brand, A.H., Manoukian, A.S., and Perrimon, N. (1994). Ectopic expression in Drosophila. Methods Cell. Biol. *44*, 635–654.

Callahan, C.A., and Thomas, J.B. (1994). Tau-beta-galactosidase, an axon-targeted fusion protein. Proc. Natl. Acad. Sci. USA *91*, 5972–5976.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene *173*, 33–38.

Cunningham, E.L., Brody, J.S., and Jain, B.P. (1974). Lung growth induced by hypoxia. J. Appl. Physiol. *37*, 362–366.

Gale, N.W., and Yancopoulos, G.D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. *13*, 1055–1066.

Goodman, C.S., and Shatz, C.J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell *72* (Suppl.), 77–98.

Guillemin, K., Groppe, J., Ducker, K., Treisman, R., Hafen, E., Affolter, M., and Krasnow, M.A. (1996). The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. Development *122*, 1353–1362.

Klambt, C., Glazer, L., and Shilo, B.Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes Dev. *6*, 1668–1678.

Kopczynski, C.C., Davis, G.W., and Goodman, C.S. (1996). A neural tetraspanin, encoded by late bloomer, that facilitates synapse formation. Science *271*, 1867–1870.

Lee, T., Hacohen, N., Krasnow, M., and Montell, D.J. (1996). Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the Drosophila tracheal system. Genes Dev. *10*, 2912–2921.

Lin, D.M., and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron *13*, 507–523.

Lindsley, D.L., and Zimm, G.G. (1992). The genome of Drosophila melanogaster (San Diego, CA: Academic Press).

Locke, M. (1958). The co-ordination of growth in the tracheal system of insects. Quart. J. Micr. Sci. *99*, 373–391.

Manning, G., and Krasnow, M.A. (1993). Development of the *Drosophila* tracheal system. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 609–686.

Meinhardt, H. (1976). Morphogenesis of lines and nets. Differentiation *6*, 117–123.

Metzger, R.J., and Krasnow, M.A. (1999). Genetic control of branching morphogenesis. Science *284*, 1635–1639.

Nagao, M., Ebert, B.L., Ratcliffe, P.J., and Pugh, C.W. (1996). Drosophila melanogaster SL2 cells contain a hypoxically inducible DNA binding complex which recognises mammalian HIF-binding sites. FEBS Lett. *387*, 161–166.

O'Neill, J.W., and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. Biotechniques *17*, 874–875.

Patel, N. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In Drosophila melanogaster: Practical Uses in Cell and Molecular Biology, L. S. B. Goldstein and E. A. Fyrberg, eds. (San Diego, CA: Academic Press), pp. 445–487.

Perrimon, N., Noll, E., McCall, K., and Brand, A. (1991). Generating lineage-specific markers to study Drosophila development. Dev. Genet. *12*, 238–252.

Pignoni, F., and Zipursky, S.L. (1997). Induction of Drosophila eye development by decapentaplegic. Development *124*, 271–278.

Pihan, J.C. (1971). [Demonstration of a tissue factor acting during morphogenesis of the tracheal system in dipteran insects]. J. Embryol. Exp. Morphol. *26*, 497–521.

Pihan, J.C. (1972). Facteurs intervenant au cours de la morphogenese du systeme tracheen chez les insectes dipteres. Bull. Soc. Zool. Fr. *97*, 351–361.

Reichman-Fried, M., and Shilo, B.Z. (1995). Breathless, a Drosophila FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. Mech. Dev. *52*, 265–273.

Reichman-Fried, M., Dickson, B., Hafen, E., and Shilo, B.Z. (1994). Elucidation of the role of breathless, a Drosophila FGF receptor homolog, in tracheal cell migration. Genes Dev. *8*, 428–439.

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671–674.

Ruhle, H. (1932). Das larvale tracheensystem von Drosophila melanogaster meigen und seine variabilitat. Z. Wiss. Zool. *141*, 159–245.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D.C., Guillemin, K., and Krasnow, M.A. (1996). Development of the Drosophila tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. Development *122*, 1395–1407.

Semenza, G.L. (1998). Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. Curr. Opin. Genet. Dev. *8*, 588–594.

Shiga, Y., Tanaka-Matakatsu, M., and Hayashi, S. (1996). A nuclear GFP/beta-galactosidase fusion protein as a marker for morphogenesis in living Drosophila. Dev. Growth Differ. *38*, 99–106.

Shilo, B.Z., Gabay, L., Glazer, L., Reichman-Fried, M., Wappner, P., Wilk, R., and Zelzer, E. (1997). Branching morphogenesis in the Drosophila tracheal system. Cold Spring Harb. Symp. Quant. Biol. *62*, 241–247.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843–845.

Smith, E.L., and Gilligan, C. (1996). Dose–response relationship between physical loading and mechanical competence of bone. Bone *18*, 455–505.

Smith, D.E., Gruenbaum, Y., Berrios, M., and Fisher, P.A. (1987). Biosynthesis and interconversion of Drosophila nuclear lamin isoforms during normal growth and in response to heat shock. J. Cell Biol. *105*, 771–790.

Sutherland, D., Samakovlis, C., and Krasnow, M.A. (1996). *branchless* encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell *87*, 1091–1101.

Vincent, S., Ruberte, E., Grieder, N.C., Chen, C.K., Haerry, T., Schuh, R., and Affolter, M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the Drosophila embryo. Development *124*, 2741–2750.

Walgenbach, K.J., Gratas, C., Shestak, K.C., and Becker, D. (1995). Ischaemia-induced expression of bFGF in normal skeletal muscle: a potential paracrine mechanism for mediating angiogenesis in ischaemic skeletal muscle. Nat. Med. *1*, 453–459.

Wigglesworth, V.B. (1954). Growth and regeneration in the tracheal system of an insect, *Rhodnius prolixus* (Hemiptera). Quart. J. Micr. Sci. *95*, 115–137.

Wigglesworth, V.B. (1983). The physiology of insect tracheoles. Adv. Insect Physiol. *17*, 86–148.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223–1237.