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# Supporting Online Material for

## Dual Origin of Tissue-Specific Progenitor Cells in *Drosophila* Tracheal Remodeling

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**This PDF file includes:**

**Supporting Online Material**

## Materials and methods

### Fly strains

*btl*$^{724}$ is a previously described null allele (*1*). *trachealess-lacZ* (*1-eve-1*) is a viable P[*lacZ*] insertion in *trachealess* (*2*). The Gal4/UAS system (*3*) was used with *btl*-Gal4 (*4*) to drive UAS transgene expression throughout the tracheal system, and with *esg-Gal4* (*5*) and *P127-Gal4* (*6, 7*) to drive expression in the SB. UAS responders were: *UAS-DsRed*, *UAS-DsRed2nls* ("*UAS-nDsRed")*, *UASi-GFPhp* (*1*), *UAS-DNbtl* (*8*), *UAS-λbtl* (*9*), and *UAS-Apc2-GFP* (*10*). To distinguish tracheal cells with recent onset of *btl-Gal4* expression from those that continuously express the transgene, a "molecular timer" strain (*w*; *btl-Gal4*, *UAS-GFP*; *UAS-DsRed;* (*1*)) was developed in which *btl-Gal4* drives expression of both UAS-GFP and UAS-DsRed. Because DsRed matures into a fluorescent protein more slowly than GFP (*11*), tracheoblasts with new onset of *btl-Gal4* expression fluoresce green for 34-48 hours before DsRed is detected and then appear yellow, whereas larval tracheal cells that continuously express both proteins during development appear yellow throughout this period. All crosses were carried out at 25°C unless noted otherwise.

### Immunostaining and microscopy

Larvae and pupae were dissected by ventrally filleting, then fixed and immunostained (*12*). Primary antibodies were anti-beta-galactosidase (mouse (Sigma), used at 1:10 dilution; rabbit (Cappell), 1:250), mouse anti-Armadillo (Developmental Studies Hybridoma Bank, 1:100), mouse anti-BrdU (Becton-Dickinson, 1:10), mouse anti-Blistered/DSRF (gift from M. Gilman, 1:200) (*13*), rabbit anti-GFP (Molecular Probes, 1:250), and rabbit anti-phospho-histone H3 (Upstate Biochemical, 1:100). Fluorescent secondary antibodies (Jackson Immunoresearch) were used at 1:300. DAPI (100 ng/ml) was used to stain nuclei. Specimens in Figure S1 were counterstained with rhodamine-conjugated chitin-binding probe (New England Biolabs) to label tracheal lumens. Stained specimens were analyzed and digital images captured on confocal (BioRad MRC 1024 or Leica SP2 AOBS) or conventional (Zeiss Axiophot) florescence microscopes.

### Lineage tracing of SB cells

*w; esg-Gal4/AyGal4, UAS-GFP; UAS-flp*$^{122}$*/+* animals were used in which *esg*-Gal4 (*5*) or P127-Gal4 (*6, 7*) drive *UAS-FLP* (Flybase) expression in SB cells. FLP recombinase permanently activates the "Flp-out" AyGal4 transgene (*14*), which in turn drives permanent expression of UAS-GFP (Flybase). Wandering third instar larvae and stage P5 pupae were immunostained for GFP and Arm to identify GFP-expressing tracheal clones. Control experiments showed that all tracheoblasts including SB and DB tracheoblasts robustly express *UAS-GFP* under control of *Act-Gal4*, the product of FLP action on *AyGal4*.

### Clonal analysis and fate mapping of DB cells

For clonal analysis, the MARCM system (*15*) was used in which 2-6 hour old *y w hs-flp*$^{122}$*; FRT40A, FRTG13, btl-Gal4, UAS-GFP/FRTG13 tubulin-Gal80* embryos raised at 25° were placed at 38°C for 45 minutes to transiently induce FLP recombinase and generate sporadic tracheal cells expressing GFP. Animals were returned to 25°C to continue development, and third instar larvae were dissected, fixed, and some samples stained overnight with rhodamine-conjugated chitin binding protein. Clones of GFP-expressing DB cells were analyzed by conventional fluorescence microscopy. Of 219 tracheal metameres scored, 63 contained marked cells in anterior DBs (Fig. S1).

For fate mapping of identified DB cells (Figs. 2, 3; Tables 1, S1), similar experiments were done using a modified MARCM system (*1*) in which tracheal clones express *UAS-GFP* and all tracheal cells express *UAS-nDsRed*. Second instar larvae were anesthetized with ether (*12*) to screen for ones containing GFP-expressing DB tracheal cells, and the positions and identities of the GFP-expressing cells were recorded. Larvae were then placed in individual vials and allowed to continue development. After pupation, animals were dissected, fixed, and stained as above to assess the fate of marked DB cells.

To generate $btl^{724}$ (*1*) mutant clones (Fig. S3, Table S2), the MARCM system (*15*) was used as in the clonal analysis above except the embryo genotype was *y w hs-flp*$^{122}$*; btl-Gal4, UAS-GFP;* $btl^{724}$ *FRT2A/ tubulin-Gal80 FRT2A* to generate sporadic *btl* mutant tracheal cells expressing GFP, and animals were dissected 24 hours after pupariation. Filleted pupae were fixed, immunostained for Blistered/DSRF, and visualized by confocal microscopy. *y w hs-flp*$^{122}$*; btl-Gal4, UAS-GFP; FRT2A/ tubulin-Gal80 FRT2A* animals treated and stained identically served as wild-type control. Clone size was determined by cell counts through confocal stacks, and CT fate assigned based on morphological criteria (multiple lumens, nucleus separate from main tube) and Blistered/DSRF immunostaining.

**BrdU incorporation assay for endoreplication of nuclear DNA**

Eggs from *trachealess-lacZ* animals were collected for 4 hours on food containing BrdU at 0.1 mg/ml (*16*). After hatching, larvae eat the food and proliferating cells and endoreplicating larval cells incorporate the label. Newly molted third instar larvae were dissected, treated for 90 minutes with DNAse I (Fermentas) at 25 u/ml, and immunostained for beta-galactosidase to identify tracheal cells and for BrdU to detect cells with nuclear incorporation of BrdU.

**Tracheal expression of Breathless FGFR variants**

*btl-Gal4* driver (*4*) and a ubiquitously expressed, temperature sensitive repressor (*tubulin Gal80TS;* (*17*)) were used to express constitutively active (*UAS-λbtl*; (*9*)) or dominant negative Btl (*UAS-DNbtl*; (*8*)) transgenes in the developing pupal tracheal system. *btl-Gal4*, *UAS-GFP*/ *tubulin Gal80TS* ; *btl-Gal4*, *UAS-GFP*/ *UAS-DNbtl* (or *UAS-λbtl*) animals were grown at 18°C until late second instar and then shifted to 30°C to inactivate Gal80TS and allow Gal4 induction of UAS transgenes. After development to P5 pupal stage, animals were dissected, fixed and stained with anti-Arm and DAPI. UAS-GFP was used to monitor induction specificity and level. Animals of identical genotype maintained at 18°C served as controls.

**Supporting text**

**Dorsal Branch clonal analysis**

Random tracheal cells were permanently marked with GFP by transient induction of a *hsFLP* transgene in 2-6 hr old embryos, and marked clones of DB cells in Tr1-5 were identified and characterized late in larval life (Fig. S1). Thirty-seven of 63 DB clones analyzed contained exclusively DB tracheoblasts; the rest contained persisting larval DB cells or a combination of larval cells and tracheoblasts. Tracheoblast clones contained from 2-47 cells (mean=13.5$\pm$9.2 S.D.) and were largely coherent except for limited mixing with unmarked cells at clone boundaries. Four DBs contained two well separated tracheoblast clusters that we presume are rare double clones in which two progenitors were initially labeled. Tracheoblast clones occupied various positions along the DB but never extended beyond the DB: dorsal clones extended to the end of the DB stalk and ventral clones terminated at its base. The positions and number of tracheoblast clones (40 clones) relative to the number of clones of larval DB1 and DB2 cells (18 clones), cells that are not replaced at metamorphosis (see Fig. S2), suggest that DB tracheoblasts arise from a pool of ~4 or 5 progenitors that are distributed along the DB stalk or become distributed along it as the progenitors spread.

We obtained 18 clones of larval DB1 and DB2 cells (which are not replaced at metamorphosis) but only 6 clones of larval SB3-7cells (stalk cells). indicating that most stalk cell clones die or transform into another cell type, such as DB tracheoblasts.

**Supporting figures**

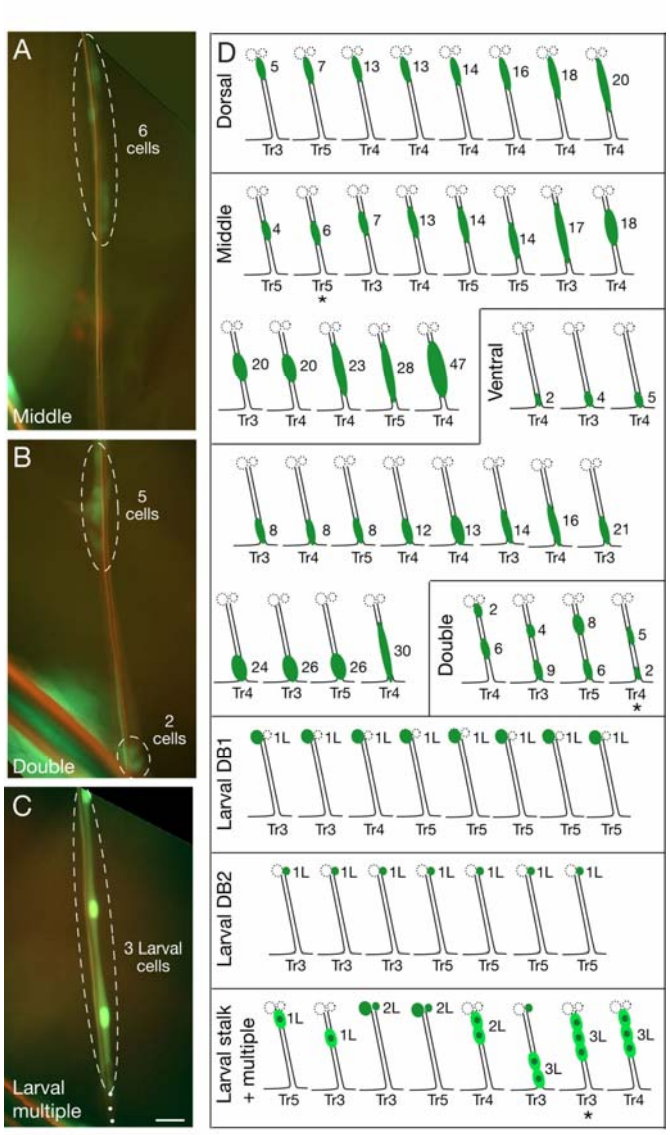


**Figure S1. Clonal analysis of DB tracheoblasts.** Clones were generated by transient induction of FLP recombinase at 2-6 hrs of development in *hs-FLP$^{122}$; FRT40A, FRTG13, btl-Gal4, UAS-GFP/FRTG13 tubulin-Gal80* embryos. GFP-expressing DB clones were scored in third larval instar. **(A-C)** Representative DB clones. Tracheal lumen is labeled with rhodamine-conjugated chitin binding protein (red). Bar, 20 μm (A-C) **(D)** Schematics of 63 DB clones (green) showing clone size, position, approximate number of cells (L, larval cells), and tracheal metamere. *, clones shown in A-C. Large dashed circle, larval DB1 cell. Small dashed circle, larval DB2 cell. Larval stalk cells (green with dark green center) were distinguished from tracheoblasts (solid green) by their distinctive morphology, nuclear size, and GFP intensity

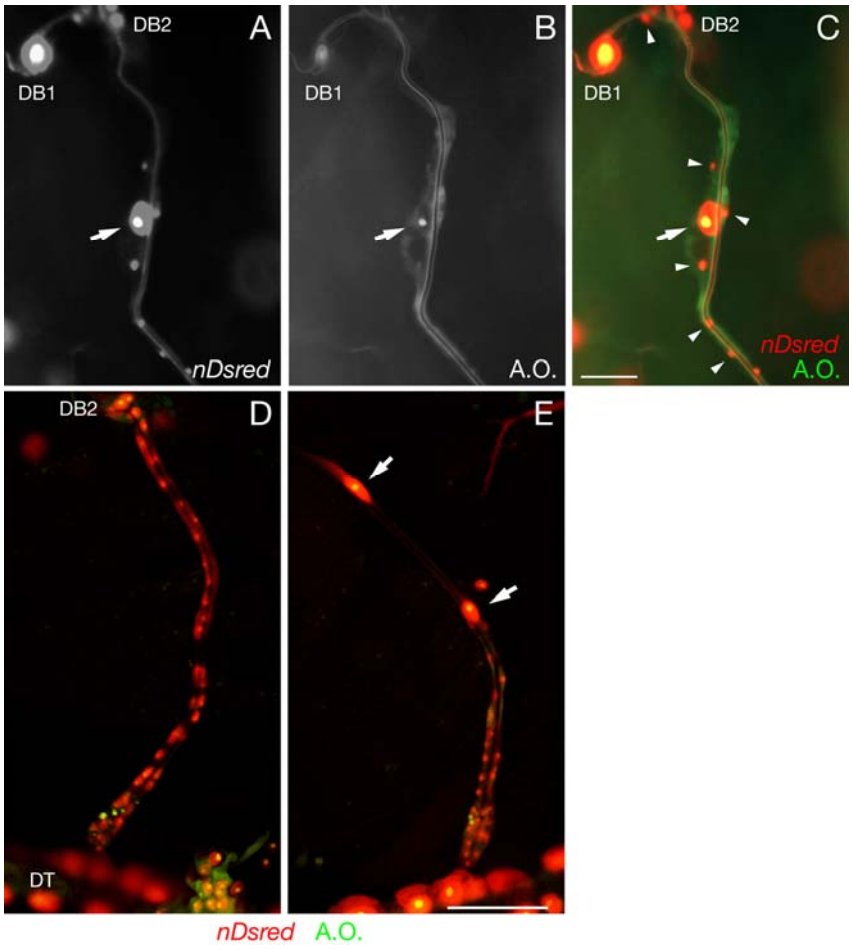


**Figure S2. Some larval DB cells undergo cell death at metamorphosis**. Standard (A-C) and confocal fluorescence micrographs (D,E) of anterior DBs of *y w hsFLP*$^{122}$*; btl-Gal4, UAS-GFP, UAS-nDsRed; FRT82B UASi-GFPhp* white prepupa stained with acridine orange (AO). Tracheal nuclei express nDsRed and dying cells have an AO$^{+}$ nuclear dot. (A-C) Larval terminal cell (DB1) branches and lumens have degenerated and cell is dying, its standard fate at metamorphosis. Larval DB fusion cells also die at metamorphosis but the one shown (DB2) is not yet AO$^{+}$. Sproradic larval stalk cells also die, like the one indicated (arrow) with an AO$^{+}$ nuclear dot and vacuolated cytoplasm. It is surrounded by five DB tracheoblasts with small, DsRed$^{+}$ nuclei (arrowheads). Bar in C, 50 μm. (D, E) Pair of contralateral DBs in process of remodeling. In (D), tracheoblasts fill the entire DB stalk from fusion cell (DB2) to dorsal trunk (DT). Fusion cells are dying and are AO$^{+}$, as are several tracheoblasts at base of DB. In contralateral DB (E), two larval DB stalk cells (arrows) are dying so ventral tracheoblasts of the DB will have to proliferate and spread dorsally to replace them. Bar in E (D, E), 75 μm.

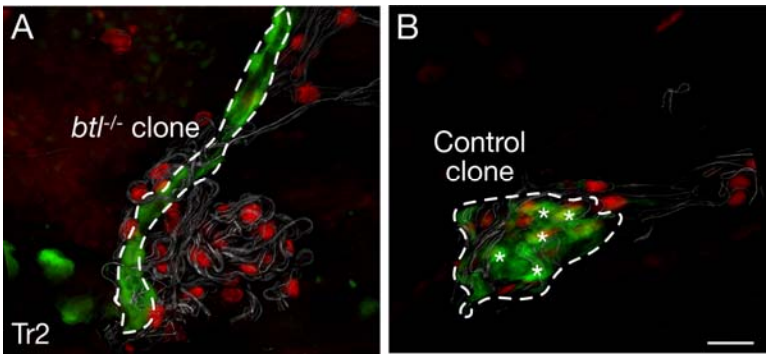


**Figure S3. Clonal analysis of *breathless* FGFR in pupal tracheoblasts.** *btl*$^{724/724}$ (A; clone b5, Table S2) and wild-type control (B; clone w4) MARCM clones in Tr2. Clones (outlined) were marked with GFP (green) and double-stained with a CT nuclear marker (α-Blistered/DSRF, red). Clones shown are same size (14 cells) and position in Tr2. Note *btl*$^{724/724}$ clone has no CT cells whereas wild-type clone has five (asterisks), although for two, the α-Blistered staining is not visible in micrograph. Bar, 25 μm.

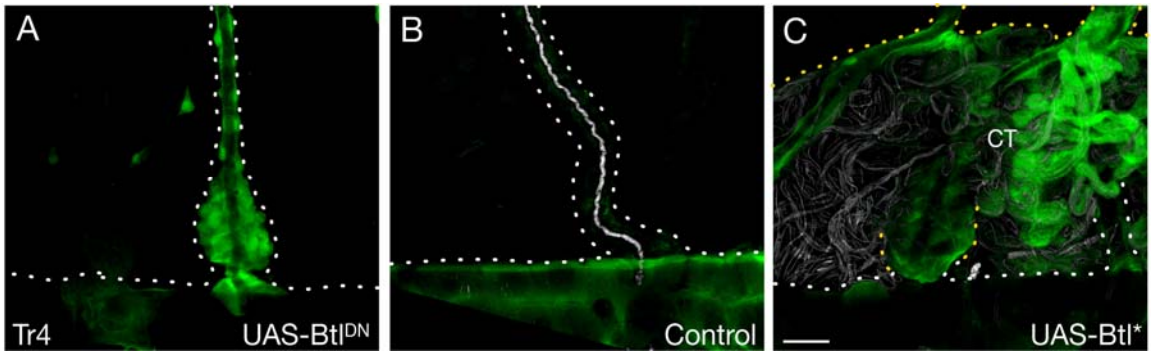


**Figure S4. Effect of dominant negative (Btl$^{DN}$) and constitutively active Breathless (Btl*) in tracheal metamere Tr4.** Experiments were done as in Fig. 3H-J. Note that Btl* induces exuberant coiled tracheoles (CT) even in DBs like Tr4 where this cell type normally never forms. Bar, 25 μm.

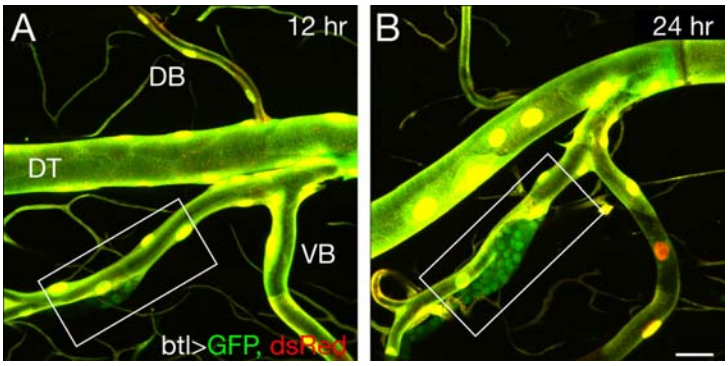


**Figure S5. Low magnification views of Tr4 metameres shown in Figs. 1D, E.** Compare to Fig. 1F'. Tracheoblasts do not appear in DB until about ~30 hr. DB, dorsal branch; DT, dorsal trunk; VB, visceral branch. Bar, 25 μm.

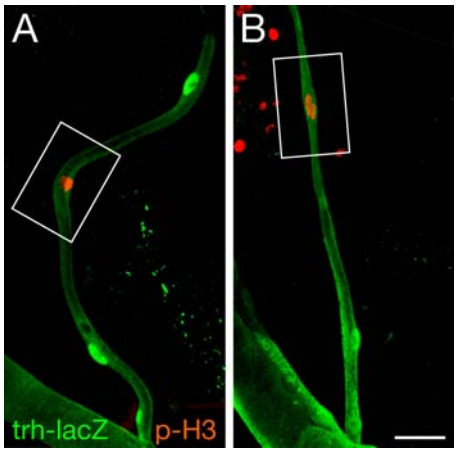


**Figure S6. Low magnification views of dividing DB4 stalk cells shown in Fig. 2D, E.** Compare to Fig. 2F'. Bar, 25 μm

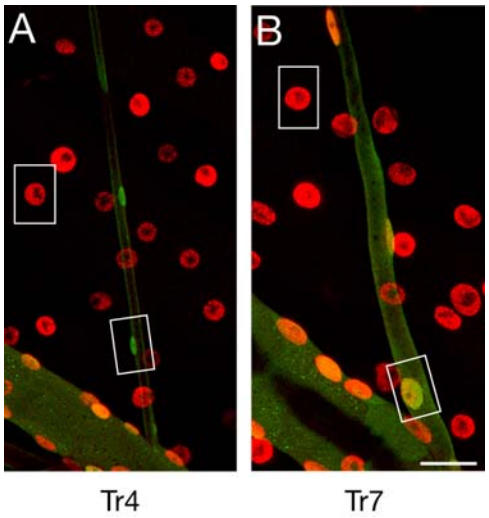


**Figure S7. Low magnification views of the DB stalk cells and nearby epidermal cells in Fig. 2G-J.** Bar, 10 μm.

Table S1. Fate mapping of larval Dorsal Branch cells

| Clone | Marked cell | | No. cells in clone | Clone cell fates | | | | | Class+ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | US^ | MS^ | CT^ | Dead | Lost | |
| A | DB1 | Tr5 | 1† | 0 | † | † | 1 | 0 | 0 |
| B1 | DB1 | Tr3* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| C1 | DB1 | Tr3** | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| D1 | DB1 | Tr3* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| E | DB1 | Tr2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| C2 | DB2 | Tr3** | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| F1 | DB2 | Tr2** | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| G | DB2 | Tr2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| H | DB3 | Tr3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| I | DB3 | Tr2 | 2 | 2 | 0 | 0 | 0 | 0 | I |
| J | DB3 | Tr2 | 5 | 5 | 0 | 0 | 0 | 0 | I |
| C3 | DB3 | Tr3** | 18 | 18 | 0 | 0 | 0 | 0 | I |
| K | DB3 | Tr2 | 8 | 3 | 1 | 4 | 0 | 0 | II |
| L | DB3 | Tr2 | 12 | 7 | 1 | 4 | 0 | 0 | II |
| F2 | DB3 | Tr2** | 12 | 8 | 0 | 4 | 0 | 0 | II |
| M | DB3 | Tr2 | 21 | 18 | 2 | 1 | 0 | 0 | II |
| N | DB4 | Tr3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| D2 | DB4 | Tr3* | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| B2 | DB4 | Tr3* | 4 | 4 | 0 | 0 | 0 | 0 | I |
| O | DB4 | Tr2 | 7 | 7 | 0 | 0 | 0 | 0 | I |
| P | DB5 | Tr2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Q | DB5 | Tr3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| R | DB5 | Tr3 | 14† | 14 | † | † | 0 | 0 | I |
| S | DB5 | Tr2 | 14 | 6 | 2 | 6 | 0 | 0 | II |
| T | DB6 | Tr5 | 1† | 0 | † | † | 1 | 0 | 0 |
| U | DB6 | Tr3 | >6 | >6 | 0 | 0 | 0 | 0 | I |
| V | DB6 | Tr2 | 10 | 4 | 0 | 6 | 0 | 0 | II |
| W | DB7 | Tr3 | 14 | 14 | 0 | 0 | 0 | 0 | I |
| B3 | DB7 | Tr3* | 22 | 22 | 0 | 0 | 0 | 0 | I |
| X | DB5 DB6 | Tr2** | 26 | 8 | 4 | 14 | 0 | 0 | II |
| Y | DB5 DB6 | Tr2** | >30 | Yes | Yes | Yes | 0 | 0 | II |
| Z | DB6 DB7 | Tr2** | >30 | Yes | Yes | Yes | 0 | 0 | II |

^ US, unicellular stalk; MS, multicellular stalk; CT, coiled tracheolar cell

+ Class 0, no proliferation, followed by necrosis or death; Class I, proliferation generating cells of the same fate, Class II, proliferation generating cells of multiple fates

† Clone analyzed at W3L, before cell differentiation.

* Data from DB in which two or three non-adjacent DB cells were labeled

** Data from DB in which two or three adjacent DB cells were labeled

Note: Data from DBs in which only single DB cells were labeled (no asterisks) are also shown in Table 1 and are included here for comparision.

Table S2. Distribution of cell fate in wild type and *btl*$^{724}$ mutant clones

| Clone | Genotype | Segment | No. cells in clone | Cell fates | | |
|---|---|---|---|---|---|---|
| | | | | ST | CT | |
| w10 | wt | Tr2 | 43 | 19 | 24 | |
| w4 | wt | Tr2 | 14 | 9 | 5 | |
| w9 | wt | Tr2 | 10 | 5 | 5 * | |
| w8 | wt | Tr2 | 8 | 5 | 3 | |
| w3 | wt | Tr2 | 7 | 3 | 4 | |
| w2 | wt | Tr2 | 4 | 2 | 2 | |
| w7 | wt | Tr2 | 2 | 0 | 2 | |
| w7L | wt | Tr2 | 2 | 1 | 1 | |
| w6 | wt | Tr2 | 2 | 1 | 1 | |
| | | | 92 | 45 (49%) | 47 (51%) | Total |
| b5 | btl-/- | Tr2 | 14 | 14 | 0 | |
| b6 | btl-/- | Tr2 | 12 | 12 | 0 | |
| b2 | btl-/- | Tr2 | 12 | 12 | 0 | |
| b1 | btl-/- | Tr2 | 10 | 10 | 0 | |
| b8 | btl-/- | Tr2 | 10 | 7 | 3 * | |
| b7 | btl-/- | Tr2 | 4 | 4 | 0 | |
| b4 | btl-/- | Tr2 | 4 | 4 | 0 | |
| b3 | btl-/- | Tr2 | 1 | 1 | 0 | |
| | | | 67 | 64 (95%) | 3 (5%) | Total |

ST, unicellular and multicellular stalk cells; CT, coiled tracheolar cells; *, two cells showed morphological features of CT cells but did not detectably express Blistered/DSRF; wt, wild type.

Table S3. Comparison of SB and DB tracheoblasts and other tracheal and imaginal cells

| | Non-tracheal imaginal cells | SB cells | Anterior DB stalk cells | Posterior DB stalk cells | Other tracheal cells | | Notes |
|---|---|---|---|---|---|---|---|
| General epithelial markers (eg. *arm*, *shg* E-cad, cuticle genes) | + | + | + | + | + | | This study (*18, 19*) |
| General tracheal markers (eg. *trh*) | - | + | + | + | + | | (*20, 21*) |
| *btl* expression before L2 | - | - | + | + | + | | This study (*4, 22, 23*) |
| Bud, grow, rearrange, diversify in response to Bnl | - | - | + | + | + | | (*24, 25*) |
| Patent, $O_2$-transporting tubes | - | - | + | + | + | | (*26*) |
| DB markers (eg. *knirps*) | - | - | + | + | - | | (*27, 28*) |
| Polyploid (endoreplicate), large nucleus | - | - | - | + | + | *,*** | This study (*29-33*) |
| Re-enter mitosis | + | + | + | - | - | * | This study |
| Maintain or regain developmental potential | + | + | + | - | - | | This study |
| Diploid, small nucleus | + | + | + | - | - | * | This study (*26, 33*) |
| Imaginal cell markers (*esg*, *hdc*) | + | + | - | - | - | ** | (*23, 27, 31, 33, 34*) |
| SB markers (*branch-3*) | - | + | - | - | - | *** | (*27*) |
| Closed tubes | - | +**** | - | - | - | | (*26*) |

* Except in Tr2; ** Except fusion cells; *** Except other specific cells or branches; **** Open at molts.

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