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Tracheal branch repopulation precedes induction of the *Drosophila* dorsal air sac primordium

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Abstract

The dorsal air sacs supply oxygen to the flight muscles of the *Drosophila* adult. This tracheal organ grows from an epithelial tube (the air sac primordium (ASP)) that arises during the third larval instar (L3) from a wing-disc-associated tracheal branch. Since the ASP is generated by a program of both morphogenesis and cell proliferation and since the larval tracheal branches are populated by cells that are terminally differentiated, the provenance of its progenitors has been uncertain. Here, we show that, although other larval tracheae are remodeled after L3, most tracheal branches in the tracheal metamere associated with the wing disc (Tr2) are precociously repopulated with imaginal tracheoblasts during L3. Concurrently, the larval cells in Tr2 undergo *head involution defective* (*hid*)-dependent programmed cell death. In *BX-C* mutant larvae, the tracheal branches of the Tr3 metamere are also repopulated during L3. Our results show that repopulation of the larval trachea is a prerequisite for FGF-dependent induction of cell proliferation and tubulogenesis in the ASP and that homeotic selector gene function is necessary for the temporal and spatial control of tracheal repopulation.
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Keywords: *Drosophila* air sacs; Tracheal remodeling; Programmed cell death; BX-C

Introduction

Drosophila is a holometabolous insect that employs a variety of strategies to create its adult form. Some of its organs, such as wings, legs, eyes and genitalia, do not contribute to larval functionality but develop from imaginal discs that are nourished and grow in the body cavity of the larva. Other organs, such as their gut, nervous system, salivary glands, fat body and tracheal system, serve both the larva and adult and must be recast at metamorphosis. The larval forms of these organs are mosaics, composed of terminally differentiated larval cells as well as nests of imaginal cells that do not contribute functionality until the post-larval stages. When the larval cells die, the imaginal cells use several different strategies to populate their respective organs. For example, imaginal cells in the midgut use the existing larval structure as a scaffold to build the adult organ. In contrast, imaginal cells in the salivary gland generate the adult organ de novo after the larval cells have been removed. We recently learned that the dorsal air sacs, components of the tracheal system that supply oxygen to the flight muscles of the adult, develop from a distinct tubular outgrowth called the air sac primordium (ASP). The ASP is induced by Branchless-FGF (Bnl-FGF) during the 3rd larval instar and grows by both cell proliferation and morphogenesis (Sato and Kornberg, 2002). The work presented in this paper addresses the identity of the cells that give rise to the ASP and reveals a novel program that repopulates a small region of the larval tracheae prior to the birth of the ASP. This program represents a third strategy for organ renewal in *Drosophila*.

The ASP is populated by small cells, many of which are mitotically active (Sato and Kornberg, 2002). It grows from the transverse connective, a tracheal branch that has an air-filled lumen lined with a multi-layered cuticle. The cells that make up the larval tracheae are polyploid (Makino, 1938) and presumably are terminally differentiated. They therefore would appear to be an unlikely source for the ASP progenitors. Imaginal tracheoblasts, small undifferentiated cells that will spread over the tracheae and replace the larval cells are another possible source. They are present in nests at several discrete positions in

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each metamere, as well as in the spiracular branch (Matsuno, 1990; Pihan, 1968) that extends from the transverse connective close to the location where the ASP arises. However, repopulation of larval organs with imaginal tracheoblasts is not thought to occur until metamorphosis.

In *Drosophila*, tracheoblast spreading and repopulation of the tracheal branches in the abdominal metameres take place after pupariation (Matsuno, 1990). Studies of thoracic tracheal repopulation have not been reported, but in *Calliphora*, spreading of imaginal tracheoblasts is not synchronous in all metameres. Whereas most of the metameres are repopulated after pupariation, in Tr2 tracheoblast, spreading begins earlier and is complete at, or shortly after, pupariation (Pihan, 1968).

While the replacement of larval tracheal cells by imaginal tracheoblasts has not been studied in great detail, it is likely to be regulated by ecdysteroids. Ecdysteroids coordinate and regulate many aspects of insect development, including the punctuated series of molts and metamorphosis. Major increases in the titer of ecdysteroids trigger each of these stages, and smaller fluctuations in ecdysteroid titers during embryonic and larval development may induce other events as well. At metamorphosis, ecdysteroids regulate the programmed cell death of obsolete larval cells, in particular the destruction of the larval midgut (Bodenstein, 1965) and salivary glands (Jiang et al., 2000; Yin and Thummel, 2004). Tissue death is carried out by autophagic mechanisms (Jiang et al., 1997; Lee and Baehrecke, 2001; Martin and Baehrecke, 2004) and is preceded by the induction of two key death activator genes, *reaper* and *head involution defective* (*hid*) (Jiang et al., 1997, 2000). The products of these genes inhibit a cell death repressor protein, *Drosophila* inhibitor of apoptosis 1 (Diap1), and are required for the cell death program (Goyal et al., 2000; Lisi et al., 2000; Vucic et al., 1997, 1998; Wang et al., 1999). Studies of the programs that regulate remodeling in the *Drosophila* tracheal system have not been reported.

In the work reported here, we investigated the dynamics of growth, proliferation and tissue renewal in the thoracic tracheae. We found that the tracheal branches associated with the wing disc undergo precocious renewal during a brief interval of L3. Tracheal renewal requires Hid-dependent programmed cell death of larval cells and concomitant replacement by dividing imaginal tracheoblasts. Following repopulation of the wing-disc-associated transverse connective, the ASP is induced, and our data suggest that imaginal tracheoblasts, not larval tracheal cells, are the progenitors for the ASP. We also found that tracheal repopulation in the wing-disc-associated tracheae precedes renewal in other tracheal metameres but that mutants defective in the *BX-C* also repopulate tracheae in the haltere-associated tracheae during L3. We conclude that the timing of cell replacement during tracheal metamorphosis is under homeotic gene control.

Results

The composition of the T2 tracheae

The larval tracheal system is a bilaterally symmetric array of interconnected tubes (Fig. 1A). Its most prominent parts are the left and right dorsal trunks that are assembled by fusion of trunk segments contributed by the 10 tracheal metameres (Tr1–Tr10). The branching patterns of Tr2–Tr9 are similar. Connecting to the dorsal trunk of each of these metameres are a dorsal branch that extends toward the dorsal midline and a transverse connective that extends ventrally. Several branches extend from the transverse connectives, the visceral and spiracular branches being the most dorsal (Fig. 1B).

In order to identify the cells that give rise to the ASP, we first determined the cell composition and dynamics of cell proliferation in the larval tracheae. In DAPI-stained specimens dissected from late L3 larvae, most of the tracheal metameres had a similar composition. Most tracheal branches were populated by cells that have large nuclei (15–20 μm diameter), although discrete clusters of densely packed cells with small nuclei (8–10 μm diameter) were also present at the junction of the transverse connective and spiracular branch. Based on the locations of these smaller cells, we presume that they are imaginal tracheoblasts. The Tr2 metamere was unique. Its branches had no large cells and instead had small cells with

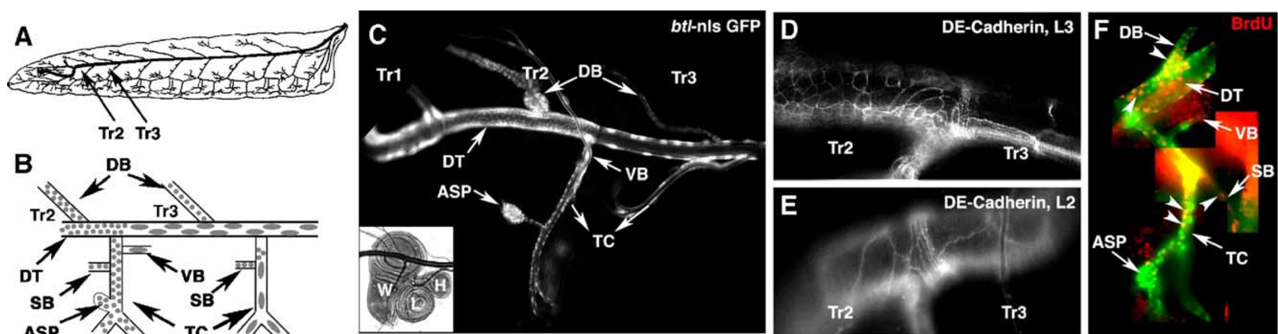

Fig. 1. Characterization of cell types in the larval tracheal branches. The 2nd and 3rd tracheal metameres are indicated by arrows in a drawing of the tracheal system in 3rd instar larvae (adapted from Manning and Krasnow, 1993) (A), and their composition is depicted in (B). A fluorescence photomicrograph of a dissected preparation from a larva expressing *btl*-nls GFP (C) with the same preparation in the inset on left at low magnification in brightfield to show the orientation of the tracheal branches relative to the wing (W), leg (L) and haltere (H) discs. Similar preparations from 2nd (D) and 3rd (E) instar larvae expressing DE-Cadherin::GFP to delineate the borders of the node cells at the junction of Tr2 and Tr3. BrdU incorporation (red) identifies replicating cells (arrowheads) in the dorsal trunk, transverse connective, ASP, spiracular branch and dorsal branch of Tr2. Abbreviations: DT, dorsal trunk; SB, spiracular branch; ASP, air sac primordium; VB, visceral branch; TC, transverse connective; DB, dorsal branch.

small nuclei throughout the Tr2 dorsal trunk, dorsal branch, transverse connective and ASP (Fig. 1B). The only tracheal branch in Tr2 not populated by cells with smaller nuclei was the visceral branch.

To further characterize the cells in these tracheal preparations, we expressed nuclear GFP (nls-GFP) with the *breathless* (*btl*) promoter. *btl* encodes the *Drosophila* FGF receptor (Glazer and Shilo, 1991; Klämbt et al., 1992) and is expressed by tracheal cells except for those in the spiracular branch. We found that nls-GFP illuminated nuclei throughout the dorsal trunk, dorsal branch, transverse connective and ASP (Fig. 1C). Moreover, the correlation between DAPI-stained nuclei and GFP fluorescence in these tracheal branches was excellent (not shown), indicating that the *btl* promoter is active in all or most of the cells in these regions. In addition, the Tr2 domain of high cell density in the dorsal trunk was juxtaposed to the contrasting domains of low cell density in Tr1 and Tr3. The junctions of high and low density coincided with the specialized node cells whose cytoarchitecture differed markedly from the neighboring cells on either side (Figs. 1D, E). The annular distribution of DE-cadherin at these junctions suggests that these cells are likely to be the node cells that mediate fusion of the tracheal metameres during the assembly of the dorsal trunk (Samakovlis et al., 1996). These node cells are thought to prevent mixing of tracheal cells originating in different metameres (Pihan, 1968).

To determine how the distribution of *btl*-expressing cells correlates with mitotic activity, *btl*-nls-GFP expressing pre-wandering stage larvae were incubated in the presence of bromodeoxyuridine (BrdU). Incorporation was observed in many nuclei along the transverse connective, dorsal trunk, dorsal branch and spiracular branch of Tr2 (Fig. 1F, arrowheads). No BrdU incorporation was observed in the visceral branch, the only branch in Tr2 not populated by small cells in later L3 larvae. Using the size of the ASP as a gauge of relative age and comparing the number of $BrdU^+$ nuclei in several specimens, we observed that older larvae had larger numbers of mitotically active nuclei. We also monitored BrdU incorporation in Tr3 and observed a markedly different pattern. $BrdU^+$ nuclei were present in the dorsal branch and in the cluster of small cells at the junction of the transverse connective and spiracular branch, but nowhere else (not shown). This pattern of BrdU incorporation was also typical of other tracheal metameres, where incorporation was restricted to regions with small cells but larger cells were not labeled. These data show that the smaller cells in pre-wandering stage larvae actively synthesize DNA, and they confirm the unusual composition of Tr2.

Repopulation of Tr2 during L3

To explore the origins of the unique Tr2 composition, we examined the distribution of nuclei in larvae at selected developmental stages (Figs. 2A–H). In L2 larvae and in larvae within 0–2 h of the L3 molt, we observed 5–7 nuclei along the transverse connective. 8–10 h after the L2–L3 molt, the number of cells along the transverse connective had not

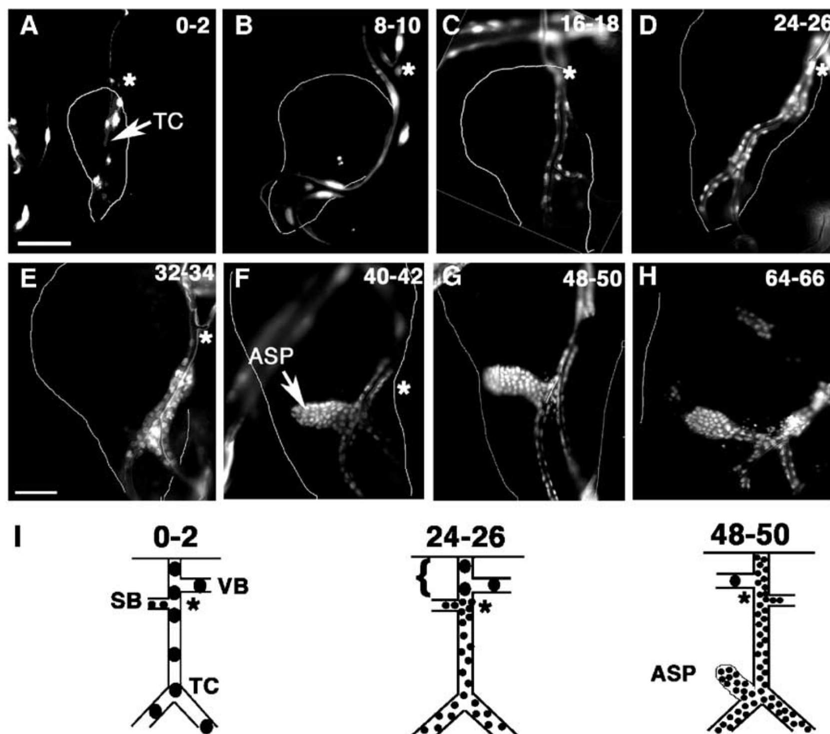

Fig. 2. Repopulation of Tr2 during the 3rd instar period. Tracheal/wing disc specimens dissected from staged larvae at the indicated hour following the 2nd to 3rd instar molt were examined under fluorescent optics to identify cells expressing *btl*-nls GFP. The outlines of the wing discs, which do not express GFP, are drawn with thin white lines. Drawings (I) depict the time course of repopulation along the transverse connective. The (*) marks the junction of the visceral branch and transverse connective. Scale bar = 50 mm.

changed, but the area and intensity of fluorescence at the nest of imaginal tracheoblasts at the junction of the transverse connective and spiracular branch had increased. A slight increase in the number and a decrease in the size of fluorescent spots along the transverse connective were evident by 16–18 h in some animals, but the most dramatic change took place during the ensuing 8 h. At 24–26 h, the transverse connective had acquired a speckled appearance with many small fluorescent nuclei distributed at regular intervals along its length. No large fluorescent nuclei were present. The density of small fluorescent nuclei continued to increase over the next 16 h. The repopulation of the entire transverse connective did not, however, occur at the same time. The dorsal-most region of the transverse connective, near its junction with the dorsal trunk (Fig. 2I), filled with small nuclei at a later time than the remainder of the branch. Although we observed the appearance of small fluorescent nuclei in this region by 40–42 h in some animals, this region was completely filled by 48–50 h.

Other Tr2 tracheae underwent similar transformations during L3, although the timing varied. Whereas repopulation of the dorsal branch was at approximately the same stage as the transverse connective, small cells did not colonize the dorsal trunk until approximately 40–42 h. The first signs of ASP growth were evident at 40–42 h, and the ASP continued to increase in size over the ensuing 24 h.

These observations showed that, during L3, the composition of Tr2 tracheae changes and that its larval cells are replaced. Although our analysis did not reveal the precise choreography of the transformation, the narrow time window suggests that the program of repopulation encompasses both proliferation and migration. The key points are that this program is unique to Tr2 and that it precedes the onset of ASP outgrowth. These results suggest that the presence of small cells in the transverse connective provides a substrate for ASP development, and we tested this proposal as described below.

The transverse connective gradually increased in length during L3. Between 0–2 h and 48–50 h of the L3 molt, the length of the wing disc increased 202%, and the distance along the transverse connective from the junction of the visceral branch to the distal bifurcation into the lateral branches increased 43%. These changes were gradual and are consistent with measurements of the length of the Tr5 transverse connective, which was found to increase gradually throughout larval development (Beitel and Krasnow, 2000). Yet, our observations now show that the Tr2 transverse connective has two distinct phases in L3. In the first phase (from the molt to 16–18 h), the cell number is constant, while, in the second phase, there is extensive proliferation and complete repopulation. Remarkably, the Tr2 transverse connective continues to grow and functions during this period of transformation in a manner that does not appear to be affected by its changing cellular composition.

Origin of the cells that repopulate Tr2 tracheae

Two models can explain the widespread distribution of proliferating cells in Tr2 tracheae. The first is that the tracheal branches in Tr2 are repopulated by a set of mitotically active cells that are distinct from the larval cells that comprise these branches. The second is that the mitotic cells are the descendants of the larval cells. According to this second model, tracheal cells in Tr2 grow in size over the period of larval life to facilitate tracheal growth and subsequently initiate a mitotic program. Such a program is not expected since increasing cell size during larval life correlates with endoreplication and cells that undergo endoreplication are not known to reinitiate a mitotic program.

To address the origin of the cells that repopulate Tr2 tracheae, we designed an experiment to examine the growth and lifetime of the cells that populate these branches during L2 (Fig. 3A). We exposed L2 larvae to a brief heat pulse to express a myristylated GFP ubiquitously. We examined the distribution of GFP fluorescence shortly after the heat shock and observed it to be uniform in both Tr2 and Tr3 (Fig. 3B). However, in late L3 larvae, the transverse connective, dorsal trunk, dorsal branch and ASP of Tr2 lacked detectable fluorescence, and

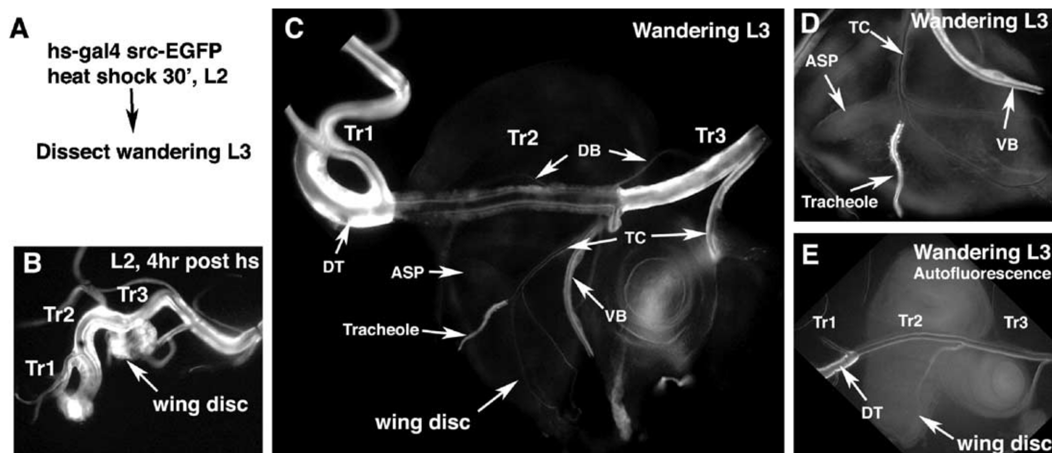

Fig. 3. Temporal regulation of tracheal cell proliferation. Scheme for labeling cells with GFP was accomplished by heat shock during the 2nd instar (A), and specimens were isolated for analysis from 2nd instar, which had uniform GFP fluorescence (B), or late 3rd instar larvae (C), whose Tr2 tracheal branches, with the exception of the disc-associated tracheole and visceral branch (D), lost most of their fluorescence. Tr1 and Tr3 branches retained fluorescence intensity, although Tr1 fluorescence is partially caused by autofluorescence (E).

only the wing-disc-associated tracheole and visceral branch of Tr2 retained GFP (Figs. 3C, D). In contrast, GFP fluorescence in most Tr3 tracheae was uniform, and only the dorsal branch and the portion of the transverse connective adjacent to the spiracular branch lacked fluorescence.

This experiment demonstrates that the cells that populate most of the Tr3 tracheae do not change significantly during L2 and L3, while most of the cells that populate Tr2 in L2 are no longer present in the same form in late L3. Based on the absence of GFP fluorescence in the wing disc, we suggest that the dividing tracheoblasts that repopulate the Tr2 tracheae had reduced fluorescence either due to fluorophore dilution or instability of the GFP. We infer the former since the fluorescence of Tr3 tracheae remained, but the L3 wing and haltere discs, whose cells divided several times during L3, had reduced GFP fluorescence. While this experiment did not allow us to discriminate between the two models described above, we are able to conclude that the cells that originally populate Tr2 in L2 are no longer present after repopulation is complete. The original cells that populate Tr2 tracheae in L2 either undergo division or are eliminated.

hid-dependent programmed cell death of larval cells in Tr2

We distinguished between these possible models by examining the role of programmed cell death. We inhibited cell death using the method developed by Yin and Thummel (2004) by inducing expression of a *hid* double-stranded RNA transgene (dsRNA) 0–2 h after the L3 molt. Under normal conditions at 24–26 h, the transverse connective is populated by many cells with small nuclei (Fig. 4A). However, after the regimen of *hid* dsRNA expression, two differences were apparent: the number of nuclei along the transverse connective was less, and some of the nuclei were unusually large (15–25 μm, Fig. 4B). Despite the decrease in cell density, the transverse connective had grown in length during the post-induction period and was normal in size. We counted the number of discs in which one or more large nuclei (>15 μm) were present and found that 63% (n = 12/19) of the specimens had this phenotype. Although we detected some variation in nuclear size among wild type preparations, no 15–25 μm nuclei were observed. We conclude that *hid*-dependent programmed cell death is necessary for the loss of larval cells in Tr2 and for the normal distribution of imaginal tracheoblasts.

We noticed that nests of cells with small nuclei were present at the base of the spiracular branch and in the lateral branch (Fig. 4C). These nests may be populated by diploid cells that normally spread into the branches of Tr2 but do not spread in the absence of larval cell apoptosis. The continued growth of the transverse connective and the abnormally large size of the larval cells that survived *hid* dsRNA inhibition of cell death suggest that these larval cells continue a program of endoreplication-based growth under these conditions.

Repopulation is regulated by the BX-C and is a prerequisite for FGF-dependent ASP outgrowth

As noted in Introduction, studies of tracheal system development in *Calliphora* by Pihan (1968) revealed that replacement of larval cells begins in Tr2 prior to metamorphosis and that replacement of larval cells in and remodeling of tracheal branches in Tr3–9 occurs after pupariation. Although imaginal tracheoblasts do not spread throughout the *Calliphora* Tr2 metamere until after pupariation, “precocious metamorphosis” is most prominent in Tr2. Since we found that the program of tracheal development is similar in *Drosophila*, we sought to investigate its genetic basis.

As described above, the dorsal trunk and transverse connective of Tr2 are populated by many cells that have small nuclei (approximately 150 and 70, respectively), but the dorsal trunk and transverse connective of Tr3 have fewer cells with larger nuclei (approximately 14 and 8, respectively; Figs. 5A, B). Since tracheal development in the embryo has been shown previously to be controlled by the *BX-C* (Lewis, 1978), we examined the trachea in L3 larvae of *Ubx* mutants to determine if the contrasting distribution and sizes of tracheal cells in Tr2 and Tr3 are a consequence of differential homeotic gene regulation. Dissected tracheae from bx^3 $pbx^1/Ubx^{6.28}$ wandering stage L3 larvae were labeled with DAPI to distinguish the number and size of their nuclei. Tr2 tracheae appeared to be normal (Fig. 5C), but, in the transformed Tr3, there was a dramatic increase in the number of small nuclei along the

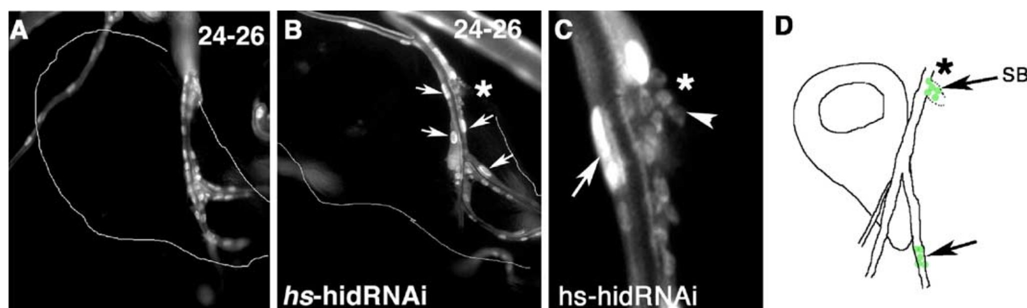

Fig. 4. Hid-dependent cell death in larval tracheae. The Tr2 transverse connective associates with the wing disc (outlined with white line), shown in (A), imaged 24–26 h following the L2–L3 molt by epifluorescence illumination of cells expressing *btl*-nls GFP. Many cells with small nuclei are distributed throughout the transverse connective, but, after heat-shock-induced expression of *hid*RNAi (B), several cells with very brightly fluorescent large nuclei were also present (arrows). A higher magnification view of the region at the junction with the spiracular branch (*) shows the juxtaposition of large and small nuclei (C). The drawing in (D) illustrates the positions where nests of small nuclei are present.

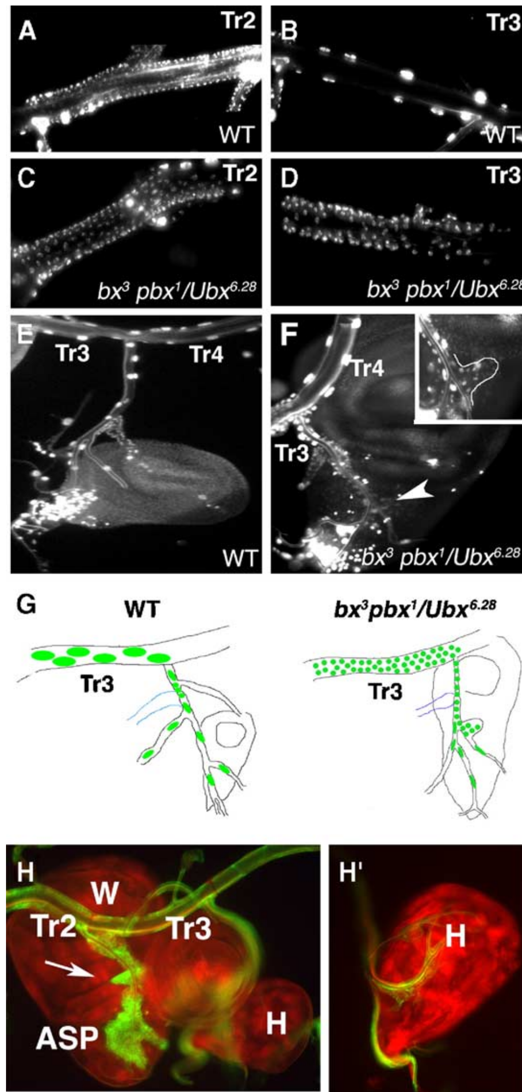

Fig. 5. The BX-C controls the program of cell proliferation in the 3rd instar tracheae. A tracheal preparation from a wandering stage 3rd instar larva stained with DAPI reveals that the dorsal trunk of Tr2 is densely populated with small nuclei (A) but is sparsely populated with several large nuclei in Tr3 (B). Similar preparations from *bx*^{3} *pbx*^{1}/*Ubx*$^{6.28}$ larvae reveal a similar pattern of nuclei densely populating both Tr2 and Tr3 dorsal trunks (C, D). Tracheal morphology and manner of contacting the haltere disc are also transformed in the mutant animals (E–G). The Tr3 transverse connective (arrow in E) contacts the wildtype haltere disc at only the dorsal-most aspect but has more extensive contacts similar to Tr2 in the mutant (F). A bud reminiscent of the ASP was present in the mutants (F (arrowhead), G); it is outlined in the higher magnification inset in (F). Ectopic expression of Bnl-FGF in clones (dark areas) induced outgrowths in Tr2 (H, arrow) and induced the ASP to enlarge, but not in Tr3, no change in the morphology of the haltere-associated tracheae was evident (H′).

dorsal trunk (Fig. 5D) and along the transverse connective (Figs. 5E, F). The size and density of nuclei in the Tr3 dorsal trunk of mutant larvae closely resembled that of the Tr2 dorsal trunk of the wild type. In addition, the morphology of the Tr3 trachea was transformed. In *bx*^{3} *pbx*^{1}/*Ubx*$^{6.28}$ mutants, the transverse connective was transformed to a Tr2-like morphology (Fig. 5G). We conclude that the distribution of tracheoblasts in the tracheae, a consequence of repopulation during L3, is regulated by *Ubx*.

As noted above, the "precocious" repopulation of Tr2 with imaginal tracheoblasts during L3 takes place prior to the induction of the ASP and leads us to suggest that the mitotically active cells in the Tr2 transverse connective are substrates for Bnl-FGF induction. Two observations support this view. First, we induced Bnl-FGF expression at ectopic sites. As reported previously, tracheoblasts in the transverse connective respond to expression of *bnl-FGF* at ectopic sites by forming new branches (Sato and Kornberg, 2002). We confirmed these observations by inducing clones of *bnl-FGF* expressing cells in L1 and L2 larvae and monitoring the morphology of the Tr2 transverse connective in late L3 larvae. Wing discs with multiple sources of Bnl-FGF were found to be associated with tracheae with many outgrowths (Fig. 5H). In contrast, no outgrowths were observed in haltere discs with ectopic Bnl-FGF expressed (Fig. 5H′). These results show that Bnl-FGF is sufficient to induce outgrowths from Tr2 tracheae but cannot induce outgrowths from Tr3 branches. Second, we observed that, in some *bx*^{3} *pbx*^{1}/*Ubx*$^{6.28}$ mutant larvae, the transformed Tr3 transverse connective had an outgrowth characteristic of an ASP (Fig. 5F, inset; Fig. 5F). The transformed transverse connectives in these larvae had been "precociously" repopulated with imaginal tracheoblasts.

Discussion

The assemblage of organs that comprise the *Drosophila* adult includes some that are unique to the adult stage, such as the legs, wings and halteres. These organs derive from monolayered epithelial invaginations that grow into discrete imaginal discs during larval development. The imaginal discs do not contribute to larval function. Other organs, such as the gut, salivary glands and tracheal system, are remodeled from larval tissues that are essential to larval life, and their formation entails replacing larval cells with imaginal cells that had been sequestered in discrete regions of the larval organs. Metamorphosis coordinates the implementation of these diverse strategies of organogenesis and involves both the selective removal of larval cells as well as the growth and morphogenesis of their replacements. The work described here was designed to establish the identity and origins of the cells that give rise to the adult dorsal air sacs. We determined that the progenitors of the ASP are imaginal tracheoblasts that proliferate and displace the larval tracheal cells of Tr2 prior to metamorphosis. These results indicate that the ASP is not a mosaic of larval and imaginal cells but forms after the larval cells of Tr2 have been replaced. The "precocious" repopulation of Tr2 during the 3rd instar revealed that remodeling can occur while the tracheae remain functional and also suggests that the signal that initiates the program of larval cell death and imaginal cell repopulation can act in a spatially restricted manner. These findings do not appear to conform to the commonly accepted paradigm that the conversion from larval to adult form is regulated systemically and is initiated at metamorphosis. Moreover, these findings provide evidence for a strategy of tissue renewal distinct from the programs that remodel the salivary glands and midgut. In this Discussion, we

speculate on possible mechanisms that might accommodate our findings with the current understanding of how metamorphosis is regulated.

Many transitions in *Drosophila* development are regulated by ecdysteroids, and it seems reasonable to propose that the transformation of the Tr2 metamere during the 3rd instar has a similar basis. Transient pulses that significantly increase the titer of ecdysteroids coordinate morphogenetic movements in the embryo (Kozlova and Thummel, 2003) and instigate the larval molts and metamorphosis (Borst et al., 1974; Riddiford, 1993). In addition, small fluctuations during the intervals between these major pulses may be important for timing other developmental events as well. Several such fluctuations have been identified during the 3rd instar, and it is possible that one may regulate the program that replaces the larval cells in Tr2. Previous work has shown that responses to ecdysteroids are both stage- and organ-specific. At the end of the 3rd instar, a transient increase in ecdysteroid titer triggers the formation of the prepupa, and, 10–12 h later, another transient increase triggers the formation of the pupa. The first pulse induces imaginal disc evagination and changes in salivary gland transcription. It also instigates a program of cell death in the larval midgut cells (Jiang et al., 1997; Lee and Baehrecke, 2001). The second pulse activates a cell death program in the larval salivary gland cells. In both the midgut and salivary gland, tissue renewal accompanies larval cell destruction, although again the mechanisms differ. Imaginal cells are sequestered in a ring at the proximal end of the salivary gland but are dispersed in the midgut, and whereas cell death in the salivary gland is synchronous, it is not in the midgut (Jiang et al., 1997; Lee and Baehrecke, 2001).

Our finding that death of the larval cells in the Tr2 metamere requires Hid, a protein that is an essential component of the cell death regulatory pathway, shows that regulation of Tr2 development shares the use of this pathway with the larval salivary gland and midgut. Although we have not determined precisely how the death and removal of the Tr2 cells is orchestrated, repopulation in the different branches of Tr2 is not simultaneous, and we have identified two distinct sources of imaginal cells in the Tr2 transverse connective. These properties suggest that the process that recasts the composition of Tr2 is more similar to the larval midgut than the salivary glands. The unique features of Tr2 are that renewal takes place during the 3rd instar period and is largely restricted to a single metamere in the tracheal system.

It is assumed, of course, that small lipophilic hormones such as the ecdysteroids disperse throughout the animal and that they equilibrate such that all tissues are exposed to the same concentrations at essentially the same time. Different responses are presumably a consequence of intrinsic properties of the different cells. We found that Ubx function is not required in the Tr2 metamere for tissue renewal but that it is required in the Tr3 metamere to delay the replacement of its larval cells. These findings indicate that the contrasting behavior of Tr2 and Tr3 cells is a consequence of their intrinsic differences that are established by *BX-C* functions. Although finding a role for the *Ubx* gene in larval tracheae was not unexpected, Lewis previously showed that the *BX-C* is essential for normal tracheal development in the embryo (Lewis, 1978), an indication that the *BX-C* regulates developmental timing. We are intrigued by fact that cell death and repopulation in the Tr3 cells is negatively regulated by the *BX-C* since this implies that the "ground state" from which other tracheal metameres diverged is one in which tissue renewal precedes metamorphosis.

Previous studies of larval cell replacement suggest that genetically controlled susceptibility to a systemic signal is not sufficient to account for the way this process is regulated. Ablation of a cluster of imaginal tracheoblasts in the dipteran insect *Lucilia caesar* was observed to inhibit secondary tracheae growth and prolong the survival of larval cells that were destined to be replaced by these imaginal tracheoblasts (Pihan, 1970, 1971). This suggests that the cell death program of larval cells depends on a signal generated locally by the imaginal tracheoblasts. A similar observation has been made in studies of *Drosophila* abdominal histoblasts. Nests of imaginal histoblasts proliferate and displace adjacent larval cells at metamorphosis (Madhavan and Madhavan, 1980), but experimental elimination of histoblast nests by irradation resulted in the perdurance of the larval cells until adulthood (Madhavan and Madhavan, 1984). We might expect that imaginal tracheoblasts in *Drosophila* orchestrate tracheal renewal by a similar mechanism and cite an unusual aspect of ecdysteroid signaling that might be relevant. The active forms of ecdysteroids are generated by peripheral tissues from inactive steroid precursors made in the ring gland (Gilbert, 2003; Riddiford, 1993). It is therefore at least a formal possibility that imaginal tracheoblasts induce the apoptosis of adjacent larval cells by controlling ecdysteroid conversion in their local environment. The production of short-range cues that induce local apoptosis is a plausible model for the spatially restricted patterns of tracheal cell renewal.

Imaginal cell proliferation and migration must also be coordinated with larval cell apoptosis to effect tracheal renewal. Bnl-FGF is known to be an essential mitogen for the tracheal cells that generate the dorsal air sacs, and ectopic expression of Bnl-FGF induces over-proliferation within the transverse connective and dorsal trunk of Tr2 (Fig. 4H and Sato and Kornberg, 2002). Consistent with these effects of Bnl-FGF, the Btl-FGFR is expressed in most or all cells in the tracheal branches. However, despite the presence of Btl-FGFR in Tr3 cells, ectopic expression of Bnl-FGF did not induce proliferation or ectopic growth in Tr3 tracheae. Either the function of Ubx in the Tr3 cells blocked their sensitivity to Bnl-FGF or the larval cells in the Tr3 tracheae are terminally differentiated and cannot proliferate even when stimulated by FGF. We favor the latter possibility because expression of a dominant negative FGFR construct in Tr2 cells did not prevent repopulation of the Tr2 branches (A.G. and T.B.K., unpublished), but it did inhibit growth of the ASP (Sato and Kornberg, 2002). Although conclusions from the observed lack of effect on repopulation must be tempered because the effectiveness of the dominant negative protein may not be complete, this result suggests that the signal that induces the imaginal tracheoblasts of Tr2 to

proliferate and migrate is not Bnl-FGF. Work in progress addresses the role of ecdysteroids in Tr2 renewal and the identity of the factors that regulate imaginal tracheoblast proliferation.

Materials and methods

Fly strains

btl-Gal4 (Shiga et al., 1996) was used to express nuclear-localized GFP (UAS-nls GFP) and myristylated GFP (UAS GAP-GFP) specifically in tracheal cells. Clones were induced as described in Sato and Kornberg (2002). A chimeric transgene of E-Cadherin::GFP controlled by the *ubiquitin* promoter was obtained from N, Khare and was used to visualize the apical margins of tracheal cells. HS-GAL4 UAS srcGFP (myristylated GFP) was obtained from the Bloomington Stock Center; bx^3, pbx^1, $Ubx^{6.28}$ and HS-*hid*RNAi were from D. O'Keefe and C. Thummel, respectively. HS-*hid*RNAi was induced at 0–2 h after the L2–L3 molt by incubation at 37°C for 30 min.

BrdU incorporation

Feeding 2nd and 3rd instar larvae were placed on cornmeal agar medium containing BrdU (1 mg/ml) for a period of 8 h according to the method of Truman and Bate (1988). Fixation and immunostaining were performed according to *Drosophila protocols* (Sullivan et al., 2000) using primary mouse anti-BrdU antibody (1:50, Becton Dickinson) and Cy3-conjugated donkey anti-mouse secondary (1:750, Jackson Labs).

Staging larvae

1st and 2nd instar larvae were identified by the morphology of the anterior spiracle and were transferred from cornmeal agar bottles to yeasted sucrose–agar plates. Larvae on the plates were examined at defined intervals, and animals that had completed the molt to 3rd instar were identified by the transformation in the anterior spiracle. Animals that showed a transformation in both anterior spiracles were aged on yeasted cornmeal agar plates at 25°C, dissected, stained with DAPI (1 μg/ml) and examined with fluorescence optics.

Sample preparation for imaging and microscopy

All preparations, fixed and unfixed, were mounted using the "hanging drop" method (Sato and Kornberg, 2002). All samples were imaged using a deconvolution microscope system (Intelligent Imaging Innovations).

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