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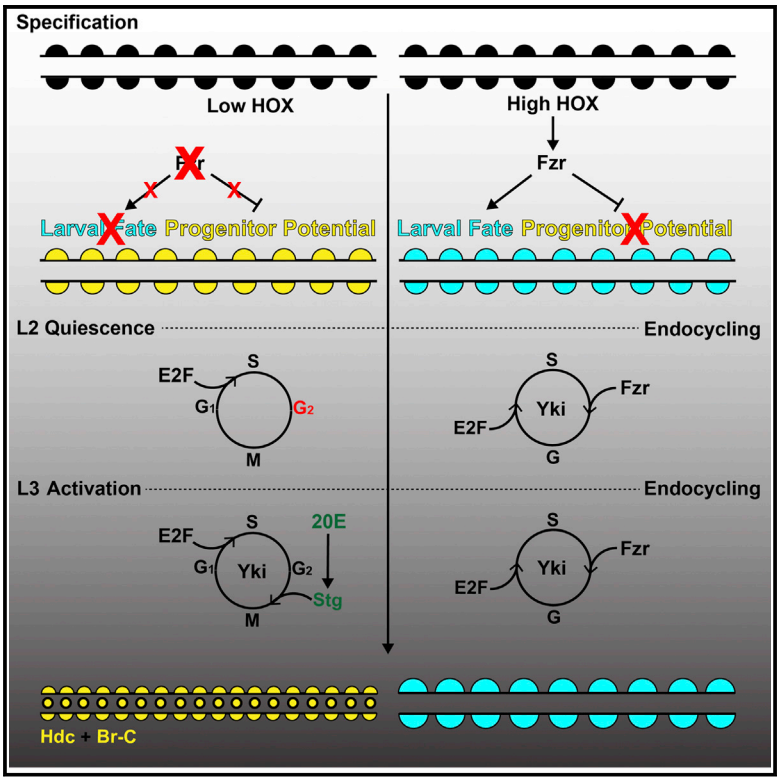
Specification of Differentiated Adult Progenitors via Inhibition of Endocycle Entry in the *Drosophila* Trachea

Graphical Abstract



Highlights

Fzr regulates cell-cycle mode and progenitor potential in the *Drosophila* trachea

Homeotic genes specify a set of differentiated tracheal cells as adult progenitors

Hormonal signaling through Ecdysone activates differentiated tracheal progenitors

Authors

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In Brief

In this paper, Djabrayan et al. describe the genetic mechanisms mediating the specification, quiescence, and activation of a subset of differentiated tracheal cells as adult progenitors in *Drosophila*. They also show that a single factor is necessary and sufficient to couple cell-cycle mode with expression of adult progenitor markers.

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Specification of Differentiated Adult Progenitors via Inhibition of Endocycle Entry in the *Drosophila* Trachea

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SUMMARY

A population of *Drosophila* adult tracheal progenitor cells arises from differentiated cells of the larval main trachea that retain the ability to reenter the cell cycle and give rise to the multiple adult tracheal cell types. These progenitors are unique to the second tracheal metamere as homologous cells from other segments, express *fizzy-related (fzr)*, the *Drosophila* homolog of CDH1 protein of the APC complex, and enter endocycle and do not contribute to adult trachea. Here, we examine the mechanisms for their quiescence and show that they reenter the cell cycle by expression of string/cdc25 through ecdysone. Furthermore, we show that preventing endocycle entry is both necessary and sufficient for these tracheal cells to exhibit markers of adult progenitors, thus modifying their genetic program. Finally, we show that Hox-mediated regulation of *fzr* expression is responsible for progenitor identity and thus specifies a group of differentiated cells with facultative stem cell features.

INTRODUCTION

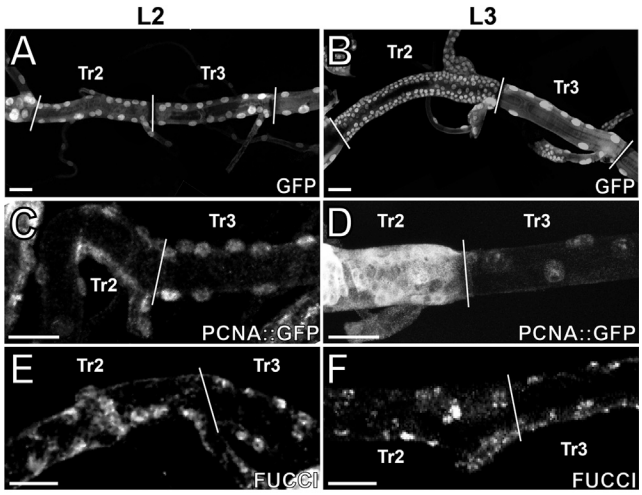
The ability of differentiated progenitor cells or facultative stem cells to re-enter the cell cycle and replace lost tissue is a major feature in development, tissue homeostasis, wound healing, and regeneration. However, it is not clear how a population of multipotent differentiated cells that forms a functional tissue is singled out and is capable of reactivation. Here, we analyze this issue by examining the transition from the larval to adult body in *Drosophila*.

At the end of *Drosophila* embryogenesis just before the onset of the larval stages, most cells switch to the endocycle, a modified cell cycle that lacks an M phase, resulting in polyploid cells (Smith and Orr-Weaver, 1991; Ullah et al., 2009). Likewise, most embryonic tracheal cells switch to the endocycle, contribute to the larval trachea, and die at metamorphosis without contributing to the adult trachea (Whitten, 1957). During the third larval instar, adult tracheal progenitor cells begin to proliferate and eventually replace the larval trachea with adult structures (Guha et al., 2008; Sato et al., 2008; Weaver and Krasnow, 2008). Two classes of adult tracheal progenitor cells exist. On the one hand, the cells of the spiracular branch (SB) are specified during embryogenesis, remain quiescent and undifferentiated during larval growth as evidenced by their continuous expression of the transcription factor *escargot*, and do not form a functional organ until metamorphosis occurs (Pitsouli and Perrimon, 2010). On the other hand, a different class of adult tracheal progenitor cells comprise a population of differentiated tracheal cells in the dorsal branches (DBs) and in the dorsal trunk (DT) that during embryonic and larval life form a functioning part of the tracheal network (Guha et al., 2008; Sato et al., 2008; Weaver and Krasnow, 2008); these cells do not enter the endocycle and remain diploid according to their nuclear size, DNA content, and cell-cycle markers (Sato et al., 2008) until the third larval instar (L3) when they re-enter the mitotic cycle (Figures 1A and 1B) (Guha and Kornberg, 2005; Sato et al., 2008). Previous work has shown that the differentiated adult tracheal progenitor cells in the DT are specific to the second tracheal metamere (Tr2) and that the difference between the DT cells in Tr2 and those of the DT in other metameres is established by homeotic genes (Sato et al., 2008). However, nothing is known about how these cells remain quiescent, how they are triggered to proliferate to give rise to the adult trachea, or how they are set apart from the other tracheal cells not contributing to adult structures.

Here, we reveal how the DT tracheal adult progenitors are specified and the mechanisms that maintain their quiescence and later reactivate them. Moreover, we show that prevention of endocycle entry is both necessary and sufficient for the DT tracheal cells to exhibit markers of adult progenitors; thus, entering one or another mode of cell cycle (mitosis versus endocycle) is coupled to a switch in the cellular genetic program. These results indicate that restricting polyploidy is a means to keep a group of differentiated cells with facultative stem cell features.

RESULTS

The cells identified as adult tracheal progenitors from the DT in the Tr2 metamere (hereafter referred as Differentiated Adult



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Marker	L2 Tr2 DT	L2 Tr3 DT	L3 Tr2 DT	L3 Tr3 DT
Fzr	-	+	-	+
PCNA::GFP	+	+	+	+
FUCCI	+	+	+	+
pH3	-	-	+	-
Stg	-	-	+	-

Figure 1. A Metamere-Specific Cell-Cycle Program Sets the Timing of DAP Cell Mitosis

(A and B) Differentiated Adult Progenitor Cells of Tr2 metamere remain quiescent in L2 (A) and begin to proliferate in L3 (B). Trachea of L2 and L3 larvae expressing nuclear GFP under the control of *btlGal4* driver are shown.

(C and D) E2F responsive marker PCNA::GFP in the DT cells of Tr2 and Tr3 at L2 (C) and L3 (D). Cells in the DT of Tr2 express PCNA::GFP at much higher levels than in Tr3.

(E and F) Expression of GFP-Geminin-D box reporter in the DT cells of Tr2 and Tr3 at L2 (E) and L3 (F).

(G) Table showing the involvement of key factors in the progression of mitotic and endocycle in the DT of Tr2 and Tr3. Scale bars represent 25 μm.

Progenitor or DAP cells) remain quiescent during the initial larval stages at a point where the other DT cells grow via the endocycle; only at L3 do DAP cells reactivate mitosis and proliferate (Figures 1A, 1B, and 1G) (Guha and Kornberg, 2005; Sato et al., 2008) prior to the pupal stage and differentiate at metamorphosis. In order to understand the temporal control of DAP cell proliferation, we have characterized their progression from quiescence to proliferation by a combination of markers. As activation of E2F results in the mitotic activation of quiescent cells (Asano et al., 1996; Bernards, 1997), we first examined E2F activity and found expression of the E2F responsive reporter PCNA::GFP (Thacker et al., 2003) in DAP cells as early as L2 (Figure 1C) indicating that they have progressed to S/G2. Consistently, the GFP-Geminin-D box reporter that marks cells in S/G2/M (Sakaue-Sawano et al., 2008) confirms that DAP cells are no longer in G1 (Figure 1C). Consistent with their active growth, the same markers indicate continuous cycling of the DT cells in the other tracheal metameres during L2 and L3 (Figures 1C–1G).

Although DAP cells reactivate the cell cycle in L2, they only appear to enter mitosis around 18 hr after the molt into L3 (Figures 1A, 1B, and 1G), suggesting the requirement of another activation step for the timely exit from a quiescent state. Other cell-cycle regulators such as Stg/Cdc25 and CyclinE (CycE) could contribute to E2F activity in inducing proliferation of DAP cells. Indeed, in *Drosophila* Stg and CycE are required for E2F to induce proliferation of differentiated wing and eye cells (Buttitta et al., 2010), and *stg* transcriptional activation is a necessary initiating step for histoblast proliferation (Ninov et al., 2007; Ninov and Martín-Blanco, 2009). Examination of *stg* transcription with a *stg-lacZ* reporter that reproduces its tracheal expression (Lehman et al., 1999) shows it to be absent from DAP cells in L2 and present at L3 (Figures 1G, 2A, and 2B) at the time when they enter in the mitotic cycle, indicating the activation of *stg* transcription as the final step for DAP cells to exit from a quiescent state. Accordingly, induction of Stg expression in L2 results in precocious mitosis in DAP cells (Figures 2C and 2D). Consistent with the endocycling of DT cells in other metameres, *stg* is not transcribed in them (Figures 1G and 2B), suggesting an additional factor preventing *stg* activation.

The timing of *stg* expression suggests that its transcription in DAP cells might be dependent on ecdysone, which is necessary for the transition from one larval stage to the next; in particular, a small peak of ecdysone coincides with the point at L3 where mitoses are observed in DAP cells (Riddiford, 1993). To examine this hypothesis, we knocked down the gene encoding the ecdysone receptor (EcR) specifically in the trachea. Under this condition, tracheal cells entered apoptosis as visualized with caspase (data not shown) making it impossible to analyze any effect on mitosis. We generated EcR knockdown clones in the trachea but did not recover any, suggesting either that the cells in the clones die or are outcompeted by the wild-type cells. As an alternative, we decided to ablate the prothoracic gland, the ecdysone producing organ (Riddiford, 1993) and found that individuals remain as third instar larvae without forming pupae and mitosis is severely impaired in the DAP cells (Figures 2I–2K). Furthermore, under these conditions, we also observe the loss of *stg* expression (Figures S1A and S1B). Although ablation of the prothoracic gland causes very dramatic effects, precisely because of the widespread effects of ecdysone, these results reinforce the link between ecdysone signaling and *stg* activation in the transition of DAP cells from G2 to M.

Because the major difference between DAP cells and the DT cells of other metameres is the endocycle, we wondered whether avoidance of entry into endocycle is what confers adult progenitor features to DAP cells. Endocycling in *Drosophila* is associated with *fzr* expression (Narbonne-Reveau et al., 2008; Schaeffer et al., 2004; Sigrist and Lehner, 1997). Accordingly, *fzr* is expressed in all polyploid DT tracheal cells and absent in DAP cells (Figure 3A; Sato et al., 2008). Indeed, tracheal overexpression of Fzr suppresses mitotic cycling in Tr2 (Figure 3C) and tracheal knockdown of Fzr via RNAi confers mitotic competence to the DT cells of the entire trachea (Figures 2E, 2F, and 3E–3M; Figures S2A–S2E). These observations suggest an inverse correlation between Fzr and Stg activities. Corroborating this suggestion, we observe ectopic expression of the *stg-lacZ* reporter in posterior metameres upon RNAi-mediated Fzr knockdown (Figure 2F). On the other hand, ectopic Stg

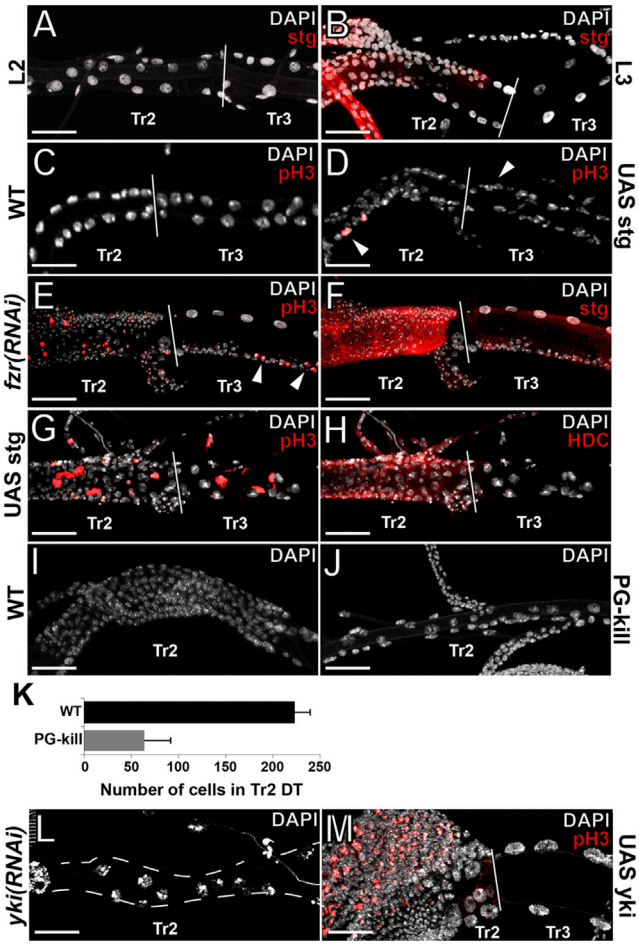

Figure 2. Regulation of *stg* Activity Triggers Mitotic Entry of DAP Cells during L3

(A and B) A tracheal preparation from a wandering stage third instar larva stained with DAPI and *stg-lacZ* during L2 (A) and L3 (B) reveals that *stg* is expressed in L3 only in the dorsal trunk of Tr2.

(C and D) Precocious mitosis in DT cells of Tr2 and aberrant mitosis in DT cells of Tr3 following Stg overexpression. Arrowheads point to pH3 (red) -positive cells in Tr2 (41 cells in *UAS stg* versus 18 in wild-type [WT]) and extra nuclei from aberrant division in Tr3 (22 cells in *UAS stg* versus 18 in WT).

(E and F) Ectopic mitoses via RNAi. (E) Ectopic mitoses in Tr3 labeled with pH3 (in red) are observed after Fzr knockdown. Arrowheads point to pH3 (red) -positive cells in Tr3. (F) Ectopic expression of *stg* (in red) in Tr3 is observed when *fzrRnai* is overexpressed in the trachea under control of *btlGal4*. See also Figure S2.

(G) Ectopic mitoses following tracheal overexpression of Stg as shown by pH3 (red).

(H) Same trachea as (G) showing normal expression pattern of Hdc (red).

(I and J) Reduction in cell number of Tr2 DT after PG (I) ablation compared to WT (J).

(K) Cell numbers in DT of Tr2 in WT and PG-kill larvae. Error bars signify SD from the mean. t test: $p < 0.0001$. See also Figure S1.

(L) Abrogation of cell division after knockdown of Yki via RNA in the trachea using btlgal4.

(M) Overexpression of Yki under the control of *btlGal4* increase mitoses in DT of Tr2 marked with pH3 (in red) and endocycling in Tr3. See also Figure S3. In all panels, nuclei are labeled with DAPI (gray).

Scale bars represent 25 μm.

expression causes the DT cells in other metameres to undergo mitotic divisions (Figure 2D), suggesting that high levels of ectopic Stg can overcome the Fzr-mediated downregulation and cause cells to switch from the endocycling to mitosis. A hypothesis consistent with the observation that most ectopic mitotic divisions occur in Tr3 where Fzr activity is lower than in the more posterior metameres (data not shown). If progenitor potential is coupled to regulation of the cell-cycle mode, escape from Fzr-mediated endoreplication should be sufficient to confer adult progenitor behavior to DT cells from any segment. Indeed, knockdown of Fzr also causes the ectopic expression of markers associated with adult progenitor cells such as Headcase (Hdc) and the Broad Complex (Br-C) (Karim et al., 1993; Weaver and White, 1995) (Figures 3F–3I). Conversely, ectopic expression of Fzr not only promotes endocycle in DAP cells but also inhibits Hdc expression (Figure 3D) further indicating that endocycling is sufficient to divert DT tracheal cells from the adult progenitor fate.

The above reported results indicate that adult progenitor markers are negatively regulated by *fzr*. However, it is possible that *fzr* may inhibit progenitor potential independently from its role in the endocycle. To distinguish between these two possibilities, we examined Hdc expression upon ectopic activation of *stg*. In artifactual situations where general tracheal expression of *stg* can induce mitosis in the DT in metameres other than Tr2 even in the presence of *fzr* activity, Hdc expression in the DT remained confined to Tr2 (Figures 2G and 2H), indicating two independent programs both regulated by *fzr*.

Polyploidy has been widely believed to be irreversible, although it has recently been described that polyploid cells in the *Drosophila* hindgut exit endocycle and reenter mitosis (Fox et al., 2010). In this sense, entering the polyploid state may act as an irreversible lock preventing the later reactivation of larval tracheal cells. On the other hand, disruption of the endocycle may be sufficient to convert polyploid cells to a progenitor state. Interestingly, polyploid larval tracheal cells reenter mitosis upon knockdown of Fzr at L3 (Figures 3J and 3K). Furthermore, DT cells exiting endocycle and reentering mitosis by knockdown of Fzr also begin to express the imaginal cell marker Hdc (Figures 3L and 3M), confirming the relationship between escape from polyploidy and specification of adult progenitor fate in DT cells.

The above results show that absence of Fzr in DAP cells specifies their adult progenitor fate and that they reenter mitosis upon ecdysone induction of *stg* transcription. However, the Hippo pathway and its target the Yorkie (Yki) transcription factor also regulate cell division. We therefore investigated whether they have a role in this process. Interestingly, we find that, whereas DAP cells do not proliferate upon RNAi Yki inactivation (Figure 2L), the deregulation of the Hippo pathway by either knockdown of Hippo via RNAi or Yki overexpression does not induce precocious divisions (Figure S3B). Instead, we observe faster rates of mitosis in the already dividing DAP cells during L3 as well as an increased rate of endocycling in polyploid DT cells (Figure 2M; Figures S3A–S3D). These results indicate that Yki is required for the division of DAP cells and sets their rate of proliferation, but it does not have a role in controlling their exit from quiescence and progression into mitosis.

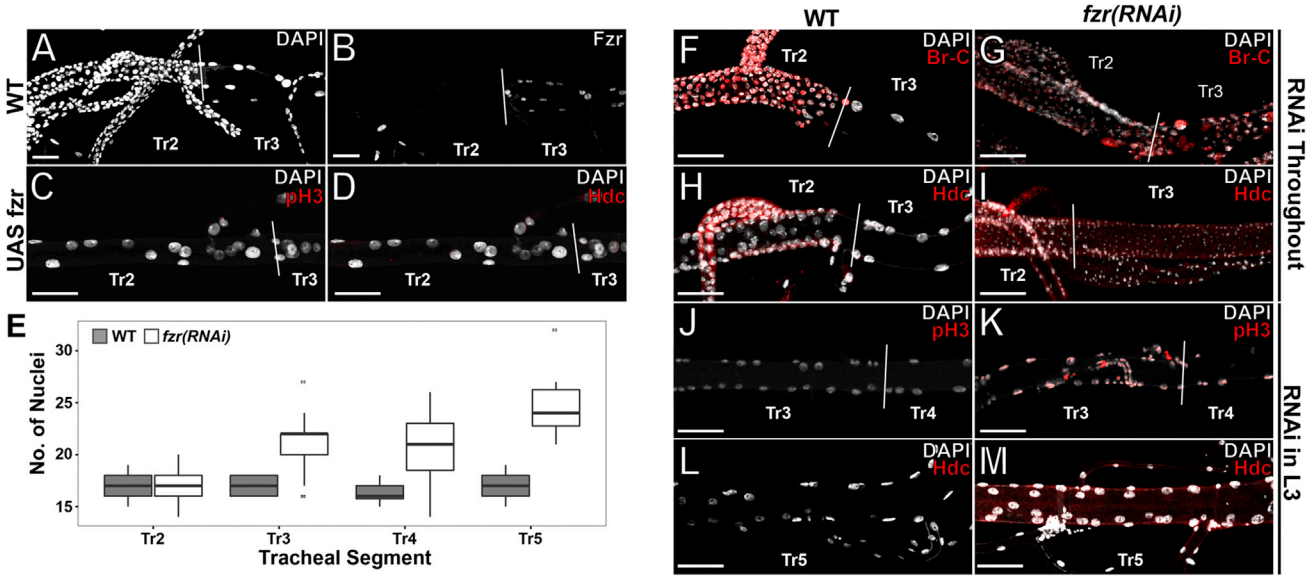

Figure 3. Avoidance of the Endocycle Determines Imaginal Potential of Tracheal Cells

(A and B) Expression of *fzr*-lacZ in DT of Tr2 and Tr3 L3 WT larvae.

(C and D) Loss of mitotic potential visualized with pH3 (in red) (C) and downregulation of imaginal marker Hdc (in red) (D) in DAP cells upon overexpression of Fzr under the control of *btlgal4* driver. Compare hdc expression in (D) with wt (H).

(E) Number of nuclei in L2 trachea from WT and *fzr-RNAi* larvae. Data represent nuclei from trachea seen to have extra cells (75.56% n = 45 in *fzr-RNAi* versus 0% n = 33 of WT larvae). See also Figure S2.

(F and H) Expression of the imaginal markers Br-C (F) and Hdc (H) in DT cells of Tr2 and Tr3 in WT L3 larva.

(G and I) Ectopic expression of Br-C (G) and Hdc (H) larva upon expression of *fzr-RNAi* larvae with *btlgal4*. Under this condition ectopic expression of Br-C and Hdc is induced. Also note the higher number of nuclei in Tr3 compare to WT (H).

(J–M) Mitotic activity visualized with pH3 (K) and expression of imaginal marker Hdc (M) following Fzr knockdown specifically during L3 using *tubgal80ts*. See also Figure S2. In all panels, nuclei are labeled with DAPI (gray).

Scale bars represent 25 μm.

Given the finding that disruption of Fzr alone can confer progenitor markers to DT tracheal cells, it is likely that specification of progenitor identity is through regulation of Fzr expression. It has been shown that Ubx plays a role in the activation of Fzr expression in the Tr3 DT cells. However, Ubx is expressed in both Tr2 and Tr3 albeit at higher levels in Tr3 (Figures 4A and 4B; Sato et al., 2008), as well as in more posterior metameres. Indeed, lowering Ubx levels in the trachea via RNAi confers mitotic behavior to Tr3 DT cells (Figures 4C and 4E), as was also shown for clones lacking Ubx expression (Sato et al., 2008). Conversely, Ubx overexpression abrogates mitotic activity of DAP cells (Figures 4C and 4G). These results indicate that the amount of Ubx protein can account for the difference in DT cell behavior between Tr2 and Tr3. DT cells in metameres posterior to Tr3 also endocycle, suggesting that the homeotic proteins AbdA and AbdB (Akam, 1987) expressed in these segments might play a similar role to Ubx. Consistent with this hypothesis, we find that ectopic expression of AbdA also abrogates mitotic behavior in DT cells in Tr2 (Figures S4A and S4B). Mitotic activity triggered by overexpression or lowering of Ubx protein is associated with changes of *fzr* expression (Figures 4D, 4F, and 4H), and the *Drosophila* modENCODE has shown direct binding of Ubx protein to the *fzr* promoter (Celniker et al., 2009) providing a mechanistic link accounting for this shift between endocycling and mitotic activity.

DISCUSSION

The mechanism unveiled here links positional information along the body axis with specification of a pool of progenitor cells with facultative stem cell features (Figure 4I). In the *Drosophila* trachea, a pool is set aside in the DT by escape from the endocycle pathway, which is the general fate of larval cells. A similar mechanism could operate in other contexts. For instance, in the *Drosophila* intestine, stem cells are diploid and surrounded by differentiated cells that are mostly polyploid, except for some diploid differentiated cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006); it remains an open possibility whether these diploid cells could act as facultative stem cells under particular stress conditions. In higher organisms, the functions of Hox genes in tissue homeostasis and regeneration are just being elucidated. In mice, Hox genes impinge on the potential of progenitor cells during bone regeneration (Leucht et al., 2008), and some homeobox-containing genes are key regulators of adult neural stem cells and muscle regeneration (Makarenkova and Meech, 2012; Shimozaki et al., 2013). Therefore, in higher organisms a "hox code" may also establish specific pool of adult stem and progenitor cells via avoidance of irreversible differentiation (Liedtke et al., 2010) and retention of responsiveness to injury induced growth signals. In the *Drosophila* trachea, we have unveiled how DT cells in Tr2 are kept as adult progenitors

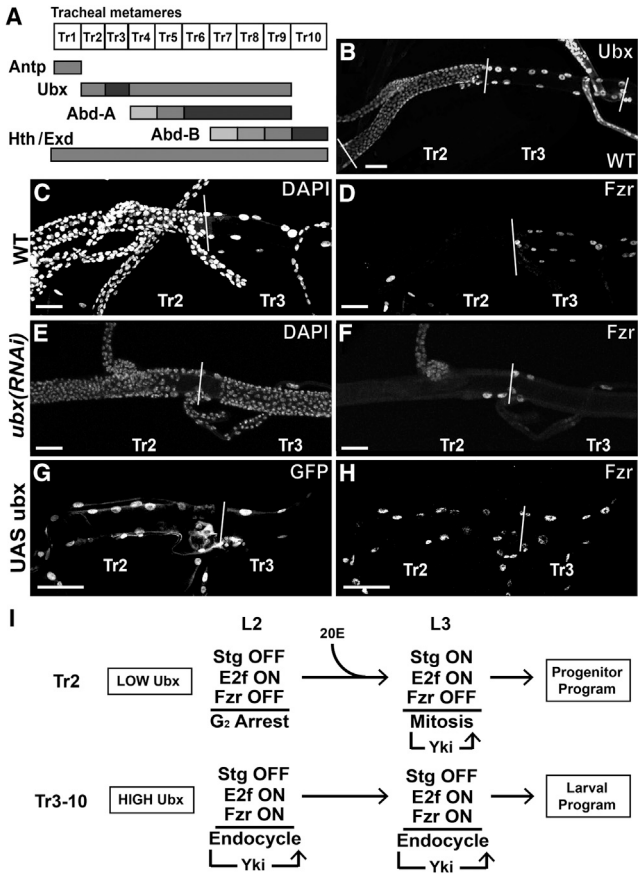

Figure 4. Level of Ubx Protein Determines DAP Cell Potential via Regulation of Fzr

(A) Diagram of expression of Hox proteins in the *Drosophila* body and correspondence with the tracheal metameres. Ubx expression is higher in the DT of Tr3 than in Tr2.

(B) Expression of Ubx protein in WT L3 trachea. Note that Ubx expression is higher in Tr3.

(C) Wild-type trachea visualized by DAPI.

(D) Wild-type expression of *fzr-lacZ* reporter.

(E and F) Mitotic activity (E) marked with nuclear marker DAPI and loss of *fzr* (F) expression in DT of Tr3 after reduction of *Ubx* via RNAi. Expression of *fzr* in the dorsal branch of Tr2 as cells differentiate to form pupal tracheoles appears to be under a different regulatory mechanism.

(G and H) Loss of mitotic potential of DT cells visualized by nuclear GFP in Tr2 (G) and induction of *fzr* expression (H) upon *Ubx* ectopic expression under the control of *btl*gal4 driver. See also Figure S4.

(I) Proposed model of DAP specification and mitotic activation. In DAP cells, relatively low amounts of Ubx prevent the activation of *fzr*. As larvae grow, the absence of Fzr allows for mitotic activation and eventual execution of the progenitor genetic program at metamorphosis. Conversely, the presence of Fzr in larval cells ensures the execution of the larval genetic program at metamorphosis.

Scale bars represent 25 μm.

by escaping the endoreplication pathway; as mentioned above, progenitor cells also exist in other tracheal branches specified by alternative genetic programs, and thus might escape endoreplication by different mechanisms. Polyploidy is widespread among living beings (De Veylder et al., 2011; Lee et al., 2009), although its benefits are not fully understood (Davoli and de Lange, 2011), and few examples of a biological function are specifically attributed to endopolyploidy (Fox and Duronio, 2013). Indeed, inhibition of endoreplication has been reported without gross effect (Chen et al., 2012; Pandit et al., 2012), raising the suggestion that polyploidy could be dispensable in some cases (Meserve and Duronio, 2012). In other cases, however, an effect is clear: preventing entry into endocycle is required for oocyte identity in *Drosophila* (Hong et al., 2003), whereas a block in endoreplication aborts leaf trichome fate in *Arabidopsis* (Bramsiepe et al., 2010). Notably, here we show a switch between entering mitosis or endocycling to trigger the switch between the genetic programs associated with either larval or adult progenitor cells. Moreover, we also show that the switch between mitosis and endocycle is associated with the transcriptional regulation of *stg*. All these data suggest that Fzr, involved in protein degradation (Sigrist and Lehner, 1997), probably regulates cell behavior and cell fate by downregulation of specific transcription factors. Indeed, our results indicate that Fzr regulates the expression of adult progenitor markers and the expression *stg* independently. Interestingly, CDH1, the mammalian *fzr* homolog, can downregulate particular transcription factors (Li et al., 2008; Ping et al., 2012; Stegmüller et al., 2006). This is particularly relevant not only for the control of developmental processes but also regarding the link between polyploidy and cancer. For instance, whereas some studies suggest that polyploidy might suppress cancerous growth (Celton-Morizur et al., 2010), others argue that polyploidy followed by mitosis might contribute to the origin of some cancers (Fox et al., 2010), because polyploidy would render cells inherently less able to divide faithfully (Ganem et al., 2007). Our data suggest a further outcome as the switch between polyploidy and diploidy could also influence tumor progression by altering the transcriptional profile of cells, conferring proliferative and progenitor potential. Thus, as is the case of the *Drosophila* tracheal adult progenitors, also in other systems a shift between the endocycle and mitotic modes of division may be linked to a change in the genetic programs of cells in development and in disease.

EXPERIMENTAL PROCEDURES

Drosophila Stocks and Genetics

The following flies were obtained from the Bloomington Stock Center: btl-Gal4, *UAS-gfp*, P0206-GAL4, *UAS-yki*, *UAS-ubx*, *tubgal80ts*, *UAS-abdA*, *fzr-lacZ*, and *UAS-rpr*. *UAS-hpoRNAi*, *UAS-ykiRNAi*, *UAS-ubxRNAi*, *UAS-fzrRNAi*, and *UAS-ubx* were obtained from the VDRC. The following strains were given: *UAS-fzr* (Silies and Klämbt, 2010), the R6.4 *stg-lacZ* (Lehman et al., 1999), *UAS-stg* (Bruce Edgar), PCNA::GFP (Cayatano Gonzalez), and FUCCI (Enrique Martin-Blanco).

Immunochemistry

Larval trachea were dissected at either L2 or L3 larval instar and immunostained according to standard protocols. The following primary antibodies were used: mouse anti-Ubx FP3.38 (1:1), mouse anti-Broad-core 25E9.D7 (1:200), mouse anti-Hdc (1:1), mouse anti-DCAD2 (1:100), and mouse anti-β-galactosidase 40.1a (1:200) from the Hybridoma Bank and rabbit anti-PH3 (Ser) (1:200) from Cell Signaling Technology. Secondary antibodies labeled with Alexa 488, Alexa 555, or Alexa 683 were obtained from Molecular Probes. Micrographs were acquired with Leica SP5 confocal microscope and images were processed with either Fiji or Photoshop CS4 (Adobe).

Hox Knockdown and Overexpression

In order to knock down the desired gene, virgin *UAS-ubxRNAi* flies were crossed with *btl-Gal4 > UAS-gfp;tub-Gal80* males. In order to overexpress the desired gene throughout the trachea, virgin *UAS-ubx* and *UAS-abdA* flies were crossed with *btl-Gal4 > UAS-gfp;tub-Gal80ts* males. The resulting progeny were reared at the permissive temperature for GAL80ts (18°C) until L2, when they were shifted to the nonpermissive temperature for Gal80ts (29°C) and maintained until late L3 and assayed for cell division and marker expression.

FZR Knockdown and Overexpression

In order to knock down Fzr via RNAi throughout larval life, *UAS-fzrRNAi* virgins were crossed with *btlgal4* male flies. The resulting progeny were reared at 29°C to ensure robust expression of *fzrRNAi* until the L3, when they were assayed for cell division and expression of imaginal markers. In order to knock down FZR during L3, virgin *UAS-fzrRNAi* flies were crossed with *btl-Gal4 < UAS-gfp;tub-Gal80ts* males. The resulting progeny were reared at the permissive temperature for Gal80ts (18°C) until early L3, when they were shifted to the nonpermissive temperature for Gal80ts (29°C) and maintained for several days and assayed for cell division and expression of Hdc. In order to ectopically express FZR in the Tr2 DT, virgin *UAS-fzr* flies were crossed with *btl-Gal4 > UAS-gfp;tub-Gal80ts* males. The resulting progeny were reared at the permissive temperature for Gal80ts (18°C) until L2, when they were shifted to the nonpermissive temperature for Gal80ts (29°C) and maintained until late L3 and assayed for cell division and Hdc expression.

Prothoracic Gland Ablation

In order to assess cell division and *stg-lacZ* expression in the absence of the Prothoracic Gland, a GAL80 suppressible P0206-GAL-4 was used to drive Reaper expression in the Prothoracic Gland during the second larval instar. Virgin *UAS-rpr;tub-gal80ts* flies were mated with *stg-lacZ;P0206-Gal4* males. Larvae were maintained at the permissive temperature for Gal80ts (18°C) until the second larval instar when they were shifted to the nonpermissive temperature (29°C) and maintained there for at least 7 days at L3 before being assayed for cell division and expression of *stg-lacZ*.

Hippo Pathway

In order to disrupt the Hippo pathway, Yki and Hippo were knocked down by crossing virgin *UAS-ykiRNAi* and *UAS-hippoRNAi* flies to *btl-Gal4 > UAS-gfp;tub-Gal80ts* males. In order to overexpress Yki in the trachea, virgin *UAS-yki* flies were crossed with *btl-Gal4 < UAS-gfp;tub-Gal80ts* males. The resulting progeny were reared at the permissive temperature for Gal80ts (18°C) until L2, when they were shifted to the nonpermissive temperature for Gal80ts (29°C) and maintained until late L3 and assayed for cell division.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.09.043.

AUTHOR CONTRIBUTIONS

N.J.-V.D., J. Cruz, C.d.M., and X.F.-M. performed the experiments. N.J.-V.D., J. Cruz, X.F.-M., and J. Casanova designed the experiments. N.J.-V.D. and J. Casanova wrote the manuscript. All authors discussed the results and commented on the manuscript.

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