Serrate/Notch Signaling Regulates the Size of the Progenitor Cell Pool in *Drosophila* Imaginal Rings

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ABSTRACT *Drosophila* imaginal rings are larval tissues composed of progenitor cells that are essential for the formation of adult foreguts, hindguts, and salivary glands. Specified from subsets of ectoderm in the embryo, imaginal ring cells are kept quiescent until midsecond larval instar, and undergo rapid proliferation during the third instar to attain adequate numbers of cells that will replace apoptotic larval tissues for adult organ formation. Here, we show that Notch signaling is activated in all three imaginal rings from middle embryonic stage to early pupal stage, and that Notch signaling positively controls cell proliferation in all three imaginal rings during the third larval instar. Our mutant clonal analysis, knockdown, and gain-of-function studies indicate that canonical Notch pathway components are involved in regulating the proliferation of these progenitor cells. Both *trans*-activation and *cis*-inhibition between the ligand and receptor control Notch activation in the imaginal ring. Serrate (Ser) is the ligand provided from neighboring imaginal ring cells that *trans*-activates Notch signaling, whereas both Ser and Delta (Dl) could *cis*-inhibit Notch activity when the ligand and the receptor are in the same cell. In addition, we show that Notch signaling expressed in middle embryonic and first larval stages is required for the initial size of imaginal rings. Taken together, these findings indicate that imaginal rings are excellent *in vivo* models to decipher how progenitor cell number and proliferation are developmentally regulated, and that Notch signaling in these imaginal tissues is the primary growth-promoting signal that controls the size of the progenitor cell pool.

KEYWORDS imaginal rings; Notch; Serrate; Delta

THE final size of an animal organ is influenced by available growth factors, environmental cues, and the size of the progenitor cell pool. For example, vertebrate liver size can increase dramatically following transplantation so that the final size is proportional to the new host. In contrast, a transplanted rat kidney only grows to a size characteristic of the donor and the final size of a mouse pancreas is dependent on the size of the progenitor cell pool, which is predetermined in the developing pancreatic bud (Potter and Xu 2001; Stanger *et al.* 2007). Abnormalities that affect the size of the progenitor cell pool have the potential to result in a large variety of developmental defects. Mice with a haploinsufficiency for EYA-1, a homolog of the *Drosophila* gene *eyes absent*, display

apoptosis in organ primordia. Consequently, the mice have craniofacial irregularities and hearing loss, while mice homozygous for the inactivated gene *Eya-1* lack ears and kidneys (Xu *et al.* 1999). During human development, the Zika virus has recently been shown to cause cell cycle arrest and cell death in neural precursor cells, leading to fetal microcephaly in mammals (Tang *et al.* 2016; Devhare *et al.* 2017).

Drosophila melanogaster, as a holometabolous organism, must go through the process of metamorphosis to finalize its transition from larva to adulthood. The majority of *Drosophila* adult tissues are differentiated from progenitor or imaginal cells that are set aside during larval stages. During metamorphosis, these imaginal cells replace larval cells and further differentiate into adult tissues, while the larval cells undergo apoptosis (Sato *et al.* 2008). Typically, imaginal cells remain quiescent until the appropriate developmental stage, when they increase in number and acquire specific fates and morphology (Beira and Paro 2016). The outer structures of the adult fly, including eyes and appendages, are formed from imaginal discs. The size and cell number of imaginal discs

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can affect the final size of the adult organ. For example, reduction of Hippo signaling in imaginal disc cells results in their overproliferation, producing oversized wings, legs, and eyes in adults (Huang *et al.* 2005; Kango-Singh and Singh 2009).

On the other hand, many organs in the *Drosophila* digestive system, such as the salivary gland, hindgut, and foregut, are produced from imaginal rings, which are located at the posterior end of the foregut, and the anterior ends of the hindgut and salivary glands during larval stages (Figure 1 and Figure S1, G–I). Imaginal ring cells are primarily diploid epithelial cells that are specified in embryogenesis and undergo expansion during larval development (Mandaravally Madhavan and Schneiderman 1977). A previous study showed that the salivary gland duct requires Serrate (Ser) signaling to specify salivary gland imaginal ring cells from salivary gland cells (Haberman *et al.* 2003). Additionally, during larval foregut formation, Notch activation expressed near the foregut imaginal ring guides cell movement to facilitate the invagination of the ectodermal foregut cells into the endodermal midgut (Fuss *et al.* 2004). Other research on the *Drosophila* hindgut system has indicated that Wingless signaling promotes proliferation of the hindgut imaginal ring (Takashima *et al.* 2008; Tian *et al.* 2016). Although vitally important, relatively little is known about the growth and development of these imaginal tissues.

In an effort to understand the development of *Drosophila* imaginal ring cells, we found that the highly conserved Notch pathway plays critical roles in multiple stages during imaginal ring development. Canonical Notch activation in *Drosophila* involves the binding of the Notch receptor with its ligands, Dl or Ser, which are expressed in adjacent cells (Lai 2004; Kopan and Ilagan 2009). This, in turn, leads to sequential proteolytic cleavage of Notch by metalloprotease-disintegrins and γ -secretase complex, which releases Notch intracellular domain (NICD) to be translocated into the nucleus (Struhl and Greenwald 1999; Hu *et al.* 2002; Lieber *et al.* 2002). Inside the nucleus, NICD interacts with Suppressor of Hairless [Su(H)] and other coactivators to activate transcription of the downstream target genes (Jarriault *et al.* 1995; Tamura *et al.* 1995). When the ligand and the receptor are expressed in the same cell, *cis*-inhibition occurs to attenuate Notch activity (Becam *et al.* 2010; del Álamo *et al.* 2011). Our studies presented here show that the *trans*- and *cis*- interactions between the ligand Ser and the Notch receptor regulate the cell number and size of the imaginal ring.

Materials and Methods

Drosophila strains and culture

Drosophila stocks were maintained and crossed at 21–22°, unless otherwise indicated. The *w¹¹¹⁸* strain was used as a wild-type control. For mosaic clone analysis, *hs-flp*; *Act* > *CD2* > *Gal4*, *UAS-RFP* (red fluorescent protein) was applied. First-instar larvae were heat shocked for 20 min twice a day

at 37°. For mosaic analysis with a repressible cell marker (MARCM) experiments, *hsFLP*; *Gal80*, *FRT40A/Cyo*; *TubGal4*, *UAS-GFP/TM6* (40A MARCM), *hsFLP*, *UAS-GFP*; *tub-Gal4*; *FRT82B tub-Gal80/TM6B* (82B MARCM), and *FRT40A*; *FRT82B* (control) were used. First-instar larvae were heat shocked for 40 min twice a day at 37°. To control the temporal and regional gene expression targeting (TARGET) system, temperature-sensitive Gal80 (Gal80^{ts}) was used to regulate the timing of upstream activating sequence (UAS)-transgene expression. To analyze the adult lineage of imaginal ring cells, Gal4 technique for real-time and clonal expression (G-TRACE) was applied. The detailed experimental methods for TARGET and G-TRACE experiments are described in Supplemental Material, Figure S12.

The following stocks were used: *Notch Responsive Element (NRE)-GFP* (used for monitoring Notch activation) [#30727 and 30728; Bloomington *Drosophila* Stock Center (BDSC)], *Act-Gal4* (#4414; BDSC), *Retn-Gal4* (#47433; BDSC), *UAS-Notch-RNAi* (RNA interference) (#27988 and 33611; BDSC), *UAS-Dl-RNAi* [#37288 and 3720; Vienna *Drosophila* Research Center (VDRC)], *UAS-Ser RNAi* (#34700; BDSC, #108348 and 27174; VDRC), *UAS-fng-RNAi* (#25947; BDSC), *UAS-Su(H)-RNAi* (#28900; BDSC), *UAS-Psn-RNAi* (#38374; BDSC), *UAS-aph-1-RNAi* (#38249; BDSC), *UAS-nct-RNAi* (#27498; BDSC), *UAS-NICD* (Domanitskaya and Schüpbach 2012), *UAS-Ser* (#26824; BDSC), *UAS-Dl^{Myc}* (#26824; BDSC), *Notch^{ts}* (Shellenbarger and Mohler 1978), *Notch^{55e11}*, *UAS-Su(H)VP16* [14.9], *Su(H)⁴⁷*, *aph-1^{D35}*, *kuz^{ES24}*, *nct^{R46}*, *Ser-LacZ* (a gift from S. Yamamoto), and *Dl-LacZ* (#11651; BDSC).

Immunostaining and fluorescence microscopy

Larvae were dissected, fixed, and stained with antibodies as described previously (Tamori *et al.* 2016). The following primary antibodies were used: rabbit anti-phospho-histone 3 (PH3) (1:200; Millipore, Bedford, MA), mouse anti-GFP (1:200; Roche), rabbit anti-atypical protein kinase C (aPKC) (1:1000; Santa Cruz Biotechnology), rabbit anti-Dystroglycan (DG) (1:1000), and mouse anti- β -gal (1:500; Promega, Madison, WI). Alexa Fluor 488- or 546-conjugated goat anti-mouse and anti-rabbit secondary antibodies (1:400; Molecular Probes, Eugene, OR) were used. Nuclei were labeled with DAPI (1:1000). Images were captured with Zeiss ([Carl Zeiss, Thornwood, NY] LSM 510 or Zeiss LSM 800 confocal microscopes. ImageJ software was used for image analyses and processing. All quantification data were analyzed by Student's *t*-test.

Counting cell numbers for imaginal ring cells at early stages

Larvae were dissected, fixed, and stained with DAPI (1:1000). As suitable markers specifically expressed in imaginal ring cells of *Drosophila* embryos are currently unavailable, we knocked down Notch during embryonic stages 8–11 or the early first-instar larval stage [25–36 hr after egg laying (AEL)] and analyzed imaginal ring cell number until the early second-instar larval stage (54 hr AEL), at which stage the difference in

Figure 1 *Drosophila* imaginal ring. Imaginal ring cells are highlighted from other larval tissues with light orange. (A) Foregut imaginal ring is located in larval proventriculus. (B) Hindgut imaginal ring is in between the larval midgut and hindgut (ileum). (C) A pair of salivary gland imaginal rings are at the anterior of the larval salivary glands. The anterior is to the left and the posterior is to the right hereafter.

nuclear sizes between polyploid cells and diploid imaginal ring cells can be distinguished without difficulty.

Molecular cloning

Genomic DNA was extracted from NRE-GFP flies according to the standard procedure described by the VDRC. The following primers were used for PCR to amplify transcriptional regulatory fragments from NRE-enhanced GFP (eGFP) flies: 5'-AGATCTTACCTAGATTGTGTGAGAAA-3' and 5'-AGATCTGTGACTGCAGAAATTCCTGC-3'. The PCR products were further inserted at the *Bgl*III restriction enzyme site of the pBPGw vector to generate an NRE-Gal4 vector. A ϕ C31 integrase-mediated site-specific transgenesis to the (3L) 68A4 site was performed by GenetiVision.

Data availability

All strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions presented in the article are present within the article, figures, and table. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.6279545>.

Results

The temporal pattern of imaginal ring cell proliferation

Imaginal rings are organized and arranged into tube-like structures (Figure S1, G–I). Compared with their neighboring polyploid larval tissues, both the cell and nuclear sizes of imaginal ring cells are smaller (as depicted in Figure 1). The imaginal ring cells are epithelial cells that display typical apical–basal polarity, as revealed by staining with antibodies against an apical marker, aPKC (Rolls *et al.* 2003; Morais-de-Sá *et al.* 2010), and a basal marker, DG (Deng *et al.* 2003;

Schneider *et al.* 2006). Similar to other tubes formed by epithelial cells, the apical surface of imaginal ring cells faces the lumen, whereas the basal side is away from the lumen and forms the outside surface (Figure S1, A–F).

Imaginal ring cells have been reported to undergo cell proliferation to increase the cell number during larval stages (Mandaravally Madhavan and Schneiderman 1977). To determine the precise temporal pattern of cell proliferation, we performed PH3 (an M-phase marker) antibody staining (Gurley *et al.* 1978) of imaginal rings from late first instar to early pupal stage. PH3 signal was hardly detectable during the first and early second larval instar, but became evident around the mid- to late second and early third instar. The peaks of cell proliferation in all imaginal ring cells were at 96 hr AEL. At this time, PH3 staining was present in 5.4, 6.2, and 6.1% of foregut, hindgut, and salivary gland imaginal ring cells, respectively (number of total cells observed = 5143, 4889, and 1557, respectively). The PH3 signal gradually declined when entering the pupal stage (Figure S2A). These results suggest that imaginal ring cells undergo rapid proliferation during the L3 stage. Consistent with this, we found an increase in the number of imaginal ring cells during different larval stages, which agrees with the previous study in the hindgut imaginal ring (Fox and Spradling 2009). The average numbers of foregut, hindgut, and salivary gland imaginal ring cells at the late first-instar larval stage were 51, 90, and 11 respectively. These numbers did not change substantially during the early- to midsecond instar. However, during the late third-instar larval stage the average number per imaginal ring increased to 504, 531, and 147 in foregut, hindgut, and salivary gland imaginal rings, respectively (Figure S2B). Taken together, these results suggest that imaginal ring cells are kept in a quiescent state until the midsecond-instar larval stage,

then undergo rapid cell proliferation to reach an adequate number of progenitor cells for differentiation into adult tissues.

Notch signaling is active in imaginal rings

To determine how imaginal ring development is impacted by different signaling pathways, we studied the expression pattern of several signaling pathways by screening a series of reporters and Gal4 lines. From this screen, we found that the Notch signaling reporter, *NRE-eGFP* (Housden *et al.* 2014; Zacharioudaki and Bray 2014), was expressed in all three imaginal ring tissues from first instar to late third instar (Figure 2). Although the *NRE-eGFP* pattern in young larval stages presents relatively equally in all imaginal ring cells (Figure 2, A–C), the pattern does not appear to be uniform during the third instar (Figure 2, D–F). In salivary gland imaginal rings, the middle region has the highest Notch activity, whereas the two sides have lower Notch activity. In foregut imaginal rings, *NRE-eGFP* is expressed more strongly in the most posterior region than in the anterior part. In the hindgut, posterior imaginal ring cells have stronger *NRE-eGFP* expression as well. We confirmed that Notch is active in these imaginal rings with a different Notch activity reporter line, *E(spl)-mBCD2* (Figure S3).

Lineage analysis of imaginal ring cells

Although imaginal ring cells have been described as precursors for adult salivary gland, foregut, and hindgut cells, no direct evidence has been shown. To confirm the progenitor/descendent relationship between the imaginal rings and the adult organs, we decided to use the G-TRACE system for lineage analysis, in which RFP labels Gal4 real-time-expressing cells and GFP labels the lineage cells derived from Gal4-expressing cells (Evans *et al.* 2009). However, no Gal4 lines have been reported to show the imaginal ring-specific expression pattern needed to carry out the lineage tracing study. Since Notch shows specific activity patterns in all three imaginal rings, we generated *NRE-Gal4* transgenic flies by replacing the *eGFP* sequence with *Gal4*. Crossing with a *UAS-GFP* line revealed that *NRE-Gal4* is specifically expressed in all three types of imaginal rings (Figure S4, A–C). To prevent the possibility that expression of *NRE-Gal4* in adult cells might confound this analysis, we also utilized Gal80^{ts} to control the timing of *NRE-Gal4* activation. Flies were reared at 18° first, then transferred to 29° at the late second-instar stage to inactivate Gal80^{ts} and allow functional Gal4 to mediate the G-TRACE system during the third-instar larval stage. After pupation, the flies were transferred back to 18° and dissected for further analysis (Figure S12C). We found that adult foregut (esophagus, cardia, and crop) (Figure 3, A and B), hindgut (Figure 3C), and salivary gland (Figure 3D) cells were GFP positive but RFP negative, suggesting that the above-mentioned cells are the descendants of larval *NRE-Gal4*-expressing cells and *NRE-Gal4* is indeed no longer real-time expressed because of the Gal80 function. We also noticed that a subset of adult foregut, hindgut, and salivary gland cells were not labeled with GFP. In addition, GFP-negative

cells did not show a specific pattern but presented randomly, suggesting that this phenomenon might be caused by a threshold effect of the Gal4 driver (Evans *et al.* 2009), though the possibility that cells without GFP are derived from other precursor cells cannot be ruled out. These results confirm that imaginal ring cells are progenitor cells that develop and differentiate into adult salivary glands, foreguts, and hindguts.

Canonical Notch signaling is required for imaginal ring cell proliferation

During the third larval instar, the cell proliferation rate and Notch activity are the highest in all three imaginal rings (Figure S2A), (Figure 2, D–F). To determine the relationship between Notch signaling and imaginal ring cell proliferation, we knocked down *Notch* using the TARGET system (McGuire *et al.* 2004), which applied an *Actin5C* promoter-driven Gal4 and Gal80^{ts} to restrict the timing of *Notch-RNAi* expression only to the third-instar stage (Figure S12D; *Act-Gal4*, *Tub-Gal80^{ts}* abbreviated to *Act^{ts}* hereafter). Inactivation of Notch signaling during the third instar reduced tissue size as compared with wild-type and sibling controls (Figure 4, A–F). The ratio of PH3-positive cells to total cells counted (mitotic index hereafter) was significantly reduced in these loss-of-Notch-function imaginal rings as well (Figure 4G). To test the possibility that global dysregulation of the Notch pathway using *Act-Gal4* might result in systemic effects that in turn disrupt cell proliferation in the imaginal rings, we used two other nonubiquitous drivers, *NRE-Gal4* and *retn-Gal4* (which are expressed in imaginal rings from late second instar; Figure S4, A–C and Figure S5, A–C), to knock down Notch signaling in imaginal ring cells, and we found that the mitotic index was also dramatically diminished (Figure S4D and Figure S5D). Moreover, use of a *Notch* temperature-sensitive (*N^{ts}*) mutant resulted in decreased mitotic activity and fewer imaginal ring cells when flies were reared at the restrictive temperature (29°) during the third-instar larval stage (Figure S6, A and B and Figure S12D). To further confirm that *Notch* mutation leads to a lower cell proliferation rate, the MARCM technique (Lee and Luo 1999, 2001) was used to generate Notch mutant clones (*FRT19A N^{55e11}*). We generated MARCM clones in late second instar and dissected L3 flies to observe the clone size. The average cell number per mutant clone was about half that of the control clones (*FRT19A* only) (Figure S6C). Taken together, these results suggest that Notch signaling in imaginal ring cells is critical for cell proliferation during the late larval stage.

Furthermore, we used the MARCM and TARGET techniques to analyze whether other components of the canonical Notch pathway regulate the proliferation of imaginal ring cells. RNAi-mediated inactivation of the core transcription factor [Su(H)] and S3 Notch cleavage enzymes [Anterior pharynx defective 1 (Aph-1), Nicastrin (Nct), and Presenilin (Psn)] caused lower mitotic activity in the imaginal rings (Figure S7A). In addition, the mutant MARCM clone sizes [*Kuzbanian^{ES24}* (Kuz, S2 cleavage enzyme), *aph-1^{D35}*, *Su (H)⁴⁷*,

Figure 2 Expression of *Notch Responsive Element-enhanced GFP* (*NRE-eGFP*) during larval stages. (A–C) *NRE-eGFP* expression in imaginal ring cells in midfirst-instar larvae. (D–F) *NRE-eGFP* presents in the foregut (FG), hindgut (HG), and salivary gland (SG) imaginal rings (ImRs) of the midthird instar. (A and D) FG ImR; (B and E) HG ImR; and (C and F) SG ImR. eGFP signal was enhanced by GFP antibody staining (green). Nuclei were labeled with DAPI (blue). Bar, 20 μ m.

and *nct^{R46}*) were all significantly decreased compared with their controls (Figure S7B), indicating that loss of canonical Notch signaling impedes cell proliferation in imaginal ring cells.

We next examined whether Notch activation is sufficient to drive cell proliferation. The active form of Notch (NICD) was misexpressed in imaginal ring cells by the *Act-Gal4*, *NRE-Gal4*, and *retn-Gal4* drivers, during the third-instar larval stage (Figure S12D). Overexpression of *NICD* by either *Act-Gal4* or *retn-Gal4* drivers in salivary gland imaginal rings resulted in increased mitotic activity (13.5 and 7.5 mean number of cells per imaginal ring, respectively) as compared to control (3 and 3.1 mean number of cells per imaginal ring, respectively) (Figure 5G and Figure S5D) and oversized tissues (Figure 5, A–F). However, the number of mitotic cells was unaffected in hindgut and foregut imaginal rings. *NRE-Gal4*-driven *NICD* overexpression also caused the overproliferation phenotype in salivary gland imaginal rings [mitotic index was 8.5% compared to that of the control (5.7%)] (Figure S4D). Interestingly, *NRE > NICD* also increased

proliferation in hindgut imaginal rings [mitotic index was 6.7% compared to that of the control (5.5%)] (Figure S4D). In addition, overexpression of *Su(H)* by *Act^{ts}* led to overproliferation in the salivary gland imaginal ring (Figure S7A). These results demonstrate that overexpressed Notch signaling induces excessive cell proliferation in the salivary gland imaginal ring, as well as in hindgut imaginal ring cells, depending on which Gal4 driver is used. On the other hand, cell proliferation in the foregut imaginal ring was not affected when *NICD* was misexpressed (Figure 5, A, D, and G, Figure S4D, and Figure S5D). Future research may determine why *NICD* overexpression has no effect on the foregut imaginal ring. Taken together, these observations indicate that overexpressed Notch signaling is sufficient to promote overproliferation in salivary gland and hindgut imaginal ring cells.

Notch signaling controls the initial size of imaginal rings during embryonic and young larval stages

In addition to its expression during the third instar, *NRE-eGFP* is also expressed in imaginal ring cells of young larvae

Figure 3 Imaginal ring cells form adult foregut, hindgut, and salivary gland. G-TRACE on *NRE^{ts}* for lineage tracing study. eGFP-labeled progeny cells (green) in adult esophagus, cardia (A), crop (B), hindgut (C), and salivary gland (D). Real-time RFP expression is not detected. Nuclei were labeled with DAPI (blue). Bar, 20 μ m. eGFP, enhanced GFP; G-TRACE, Gal4 technique for real-time and clonal expression; *NRE^{ts}*, temperature-sensitive Notch responsive element; RFP, red fluorescent protein.

(Figure 2, A–C). Previous studies showed that Notch and its ligands, *Dl* or *Ser*, are expressed in the developing larval foregut and salivary gland at middle embryonic stages (Haberman *et al.* 2003; Fuss *et al.* 2004). These indicate that Notch might play a role in the development of imaginal ring cells before they highly proliferate in late larval stages. To test this, we knocked down *Notch* during different developmental

stages using the *Act^{ts}* system described above. Loss of *Notch* during early embryonic stages (stages 2–8) resulted in complete a loss of salivary glands and no imaginal ring cells were detected, confirming the critical role of Notch in the specification of ectodermal epithelia (Hartenstein *et al.* 1992).

The *Act^{ts}* system allowed us to bypass this earlier role of Notch signaling by inducing *Notch-RNAi* during embryonic

Figure 4 Notch is required for imaginal ring (ImR) cell proliferation during the third-instar larval stage. (A–F) Cell proliferation was labeled by phosphohistone 3 (PH3, red) in controls and loss-of-*Notch* ImRs. (A and D) foregut (FG) ImR; (B and E) hindgut (HG) ImR; and (C and F) salivary gland (SG) ImR. Nuclei were labeled with DAPI (green). Bar, 20 μ m. (G) Quantification of mitotic index. * $P < 0.05$ and ** $P < 0.01$. Error bars, mean $\pm$ SEM.

stages 8–11 (Figure S12A). We found that the average numbers of foregut, hindgut, and salivary gland imaginal ring cells were dramatically decreased as compared with wild-type controls reared in the same conditions (Figure 6, A–F and M). For example, normally, the salivary gland imaginal ring has ~10–11 cells in early L2, but *Notch* knockdown in embryonic stages 8–11 resulted in only three to four salivary gland imaginal ring cells. Consistently, when *NRE^{ts}* was used to induce *Notch-RNAi* expression specifically in Notch-active cells during embryonic stages 8–11, the number of imaginal ring cells was also significantly reduced (Figure S4E). Furthermore, using the *Act^{ts}* or *NRE^{ts}* driver to knock down Notch

during the first-instar larval stage (Figure S12B), the number of cells in all three imaginal rings was lower; for instance, there were only six to seven salivary gland imaginal ring cells when *Notch* was knocked down as compared to 11 cells in the control condition (Figure 6, G–M and Figure S4E). Since cell proliferation in imaginal rings is hardly detected before the early second instar (Figure S2A), these results suggest that Notch signaling during the embryonic to first-instar larval stages is probably involved in maintaining the size of the imaginal ring cell pool during this period of development.

To determine the cause for the reduced early imaginal ring cells upon Notch knockdown, we first tested whether cell

Figure 5 Notch is sufficient to induce overproliferation in salivary gland imaginal rings (ImRs) during the third instar. (A–F) Cell proliferation was labeled by phospho-histone 3 (PH3, red) in controls and overexpressed Notch intracellular domain (NICD) ImRs. (A and D) Foregut (FG) ImR; (B and E) hindgut (HG) ImR; and (C and F) salivary gland (SG) ImR. Nuclei were labeled with DAPI (green). Bar, 20 μ m. (G) Quantification of mitotic index. *** $P < 0.001$. Error bars, mean $\pm$ SEM.

death occurred. The expression of DCP1, an effector caspase that induces programmed cell death (Song *et al.* 1997), was assessed, but no signal was detected in imaginal rings or nearby regions when *Notch* was knocked down (Figure S8), suggesting that the cells lacking Notch activation did not undergo apoptosis. Another possibility for the reduced cell

number is that the imaginal ring cells become polyploid cells, as in a previous study in which salivary gland imaginal ring cells switched from diploidy to polyploidy when *Ser* was disrupted during embryogenesis (Haberman *et al.* 2003). Indeed, loss of *Notch* during late embryonic stages increased the numbers of polyploid cells slightly but significantly in the

Figure 6 Notch maintains the number of imaginal rings (ImRs) during young larval stages. *Notch-RNAi* (RNA interference) was induced by *Act^{ts}* (*Act-Gal4*, *Tub-Gal80^{ts}*) during the midembryonic stage (A–F) or early first-instar larval stage (G–L). Nuclei were labeled with DAPI (white). (A, D, G, and J) foregut (FG) ImR; (B, E, H, and K) hindgut (HG) ImR; and (C, F, I, and L) salivary gland (SG) ImR. Dashed lines indicate ImR cells. Bar, 10 μ m. (M and N) Quantification for cell numbers. E-Ctrl, embryonic control; E-loss 1, embryonic loss of *Notch* by *Notch-RNAi* (BL27988); E-loss 2, embryonic loss of *Notch* by *Notch-RNAi* (BL33611); L-Ctrl, larval control; L-loss 1, larval loss of *Notch* by *Notch-RNAi* (BL27988); and L-loss 2, larval loss of *Notch* by *Notch-RNAi* (BL33611). Embryonic experimental design was based on Figure S12A; Larval experimental design was based on Figure S12B. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Error bars, mean $\pm$ SEM.

regions of the proventriculus, hindgut, and salivary gland (Figure 6N and Figure S4F), whereas some groups of neighboring polyploid cells increased slightly, but with statistical significance, when Notch signaling was reduced in L1 (Figure 6N and Figure S4F). These findings suggest that a small number of imaginal ring cells close to the boundaries between the imaginal rings and neighboring polyploid tissues become polyploid cells when Notch signaling is not maintained during embryonic and early larval stages in imaginal rings.

***Ser* is the ligand that promotes Notch activation in imaginal ring cells**

Dl and *Ser* are two major ligands for Notch signaling in *Drosophila* (Zeng *et al.* 1998). To determine the Notch ligand in

the imaginal ring, we first used *Dl* and *Ser* antibodies to examine their expressions in imaginal rings, but they were undetectable. Alternatively, we used *Dl-lacZ* (Spradling *et al.* 1999; Zeng *et al.* 2010) and *Ser-lacZ* (Yamamoto *et al.* 2012) reporters to investigate their activation in imaginal rings, and found that both were expressed in imaginal ring cells (Figure S9). To determine which ligand promotes Notch-induced cell proliferation in imaginal ring cells, *Ser* or *Dl* was knocked down using *Act^{ts}* individually during the third larval instar. *Ser* but not *Dl* knockdown resulted in reduced tissue sizes and lower mitotic activity in all three imaginal rings (Figure 7A), suggesting that *Ser* is the ligand for Notch signaling in imaginal rings. To confirm this, we knocked down *fringe* (*fng*), which encodes a glycosyltransferase that promotes

Figure 7 The interaction of Notch and its ligands. Cell proliferation of imaginal rings (ImRs) during the third instar were labeled with phosphor-histone 3 (PH3). (A) The quantification of mitotic index for knockdown of Ser, Dl, or fng by *Act^{ts}* (*Act-Gal4*, *Tub-Gal80^{ts}*). (B) The quantification of mitotic index for overexpression of Ser and Dl^{MyC} driven by *Act^{ts}*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Error bars, mean $\pm$ SEM. FG, foregut; HG, hindgut; SG, salivary gland.

Dl–Notch interaction (Brückner *et al.* 2000; Sasamura *et al.* 2003), and found that *fng* knockdown did not reduce cell proliferation in the imaginal rings (Figure 7A), further suggesting that Dl does not facilitate Notch activation in imaginal rings during the third-instar larval stage.

We further examined the Ser source for Notch activation in imaginal rings. Direct cell-to-cell contact between ligand- and receptor-expressing cells is essential for the activation of the Notch pathway (Lai 2004; Kopan and Ilagan 2009). Although foregut imaginal rings contact with outer proventricular cells and inner esophagus cells, the tissue structures of hindgut and salivary gland imaginal rings are only single layers. Therefore, we proposed that the ligands for Notch activation in imaginal ring cells are provided from the neighboring imaginal ring cells. To test this hypothesis, we used the Flip-out Gal4 driver (Pignoni and Zipursky 1997) to generate *Ser* or *Dl* knockdown mosaics and monitor *NRE-eGFP* expression levels. We found that the *NRE-eGFP* expression level in wild-type cells surrounded by *Ser* knockdown cells was significantly reduced as compared to wild-type *NRE-eGFP* expression (Figure 8, A–C and G), indicating that *Ser* knockdown clone cells were unable to induce Notch activation in their neighboring wild-type cells. These results suggest that the receptor–ligand interaction between two neighboring imaginal ring

cells underlies Notch activation. However, in contrast, *Dl* knockdown clones did not reduce *NRE-eGFP* expression in their neighboring cells (Figure 8, D–G), which confirms that Dl is not the ligand to activate Notch signaling in imaginal ring cells.

Cis-inhibition modulates Notch activity in imaginal ring cells

From the above *Ser* and *Dl* knockdown mosaic studies, we found that *NRE-eGFP* expression was increased in the clones of a single cell layer (Figure S10), indicating that loss of ligands in the same cell results in higher Notch activation. When the ligands of Notch are expressed in the same cells as the Notch receptor, they can induce *cis*-inhibition, which reduces Notch activation (Sprinzak *et al.* 2010; del Álamo *et al.* 2011). This result prompted us to further examine whether Notch signaling is also regulated by *cis*-inhibition in the imaginal rings. To determine this, we applied the Flip-out Gal4 system to generate clones with overexpressed Dl or Ser ligand and examined *NRE-eGFP* expression levels in imaginal ring cells, and found that the expression of *NRE-eGFP* in clone cells was dramatically decreased compared to wild-type *NRE-eGFP* expression (Figure S11), suggesting that overexpression of both Dl and Ser ligands is sufficient to induce *cis*-inhibition to reduce Notch activation. We next overexpressed

Flip-out-Gal4 (Act>CD2>Gal4); NRE-eGFP

Figure 8 Ser is provided from neighboring cells. Flip-out Gal4-generated mosaic ImR with clones expressing *Ser-RNAi* (A–C) or *DI-RNAi* (D–F). Third-instar larvae were observed. (A and D) FG ImR; (B and E) HG ImR; and (C and F) SG ImR. RNAi-expressing cells were labeled with RFP expression (red). *NRE-eGFP* indicated Notch activation (green). Nuclei were labeled with DAPI (blue). Dashed lines indicate nonclone cells. Bar, 20 μ m. (G) The quantified ratio of eGFP intensity. The *NRE-eGFP* intensities in the WT cells that are completely surrounded by *Ser* or *DI* loss-of-function clones and WT clones was measured. The intensities of eGFP in above nonclone cells were further divided by the intensities of eGFP from WT *NRE-eGFP* flies. *** $P < 0.001$. Error bars, mean $\pm$ SEM. eGFP, enhanced GFP; FG, foregut; HG, hindgut; ImR, imaginal ring; NRE, temperature-sensitive Notch responsive element; RFP, red fluorescent protein; RNAi, RNA interference; SG, salivary gland; UAS, upstream activating sequence; WT, wild-type.

DI and *Ser* during the third-instar larval stage and monitored cell proliferation with PH3 staining. In both cases of *DI* and *Ser* overexpression driven by *Act^{ts}*, the mitotic index was significantly reduced (Figure 7B). Taken together, these results suggest that *cis*-inhibition of the Notch pathway results in the reduction of Notch activation, which further causes a lower cell proliferation rate in imaginal ring cells.

Discussion

Imaginal rings as models to study progenitor cell growth regulation

During imaginal ring development, cell specification, maintenance of cell identity, and cell proliferation are tightly controlled. The growth pattern of the imaginal ring provides an interesting model to understand how the number of cells in the tissue is developmentally regulated. These cells are specified during embryogenesis, quiescent during L1 and early L2, but undergo rapid expansion during the late second and third instars. In this study, we examined how developmental signals such as Notch contribute to the size of the imaginal ring. We first used lineage analysis to confirm that imaginal ring cells are the progenitor cells for adult foreguts, hindguts, and salivary glands. Our studies reveal a dependency on Notch signaling throughout imaginal ring development during the

larval stages. Insufficient Notch signaling leads to smaller imaginal rings and reduced cell division. Although our data demonstrate the important role that Notch plays in imaginal ring development, a few questions remain. Since Notch is consistently active in imaginal ring cells, the trigger for different growth rates in different developmental stages is still a mystery. Additional signaling pathways, including hormonal signaling, are likely part of the regulatory network. Similar to imaginal rings, imaginal discs arise from a cluster of precursor cells during embryonic stages and keep mitotic activities low until the appropriate larval stages. In the early first-instar stage, imaginal discs contain ~20–70 cells, which resume mitosis during the first instar and divide exponentially during the second- and third-instar stages to reach 10,000–50,000 cells in each disc (Mandaravally Madhavan and Schneiderman 1977). The balance among proliferation, patterning, and differentiation in discs is essential for a functional adult tissue. Several signaling and hormonal regulations control the developmental processes of discs. For instance, in the zone of non-proliferating cells of wing discs, Wg secretion is required for the initiation of differentiation and the cessation of proliferation, whereas ecdysone represses Wg to promote the cell cycle and proliferation (Mitchell *et al.* 2008).

Another remaining question is the different responses to overexpressed active Notch (NICD) in different imaginal

rings. Excessive Notch signaling results in overproliferation in the hindgut and salivary gland imaginal ring cells, suggesting that Notch is sufficient for cell proliferation in these tissues. However, overexpressed NICD is not able to cause foregut imaginal ring cell overproduction. In addition, different Gal4 drivers also result in different overproliferation phenotypes in imaginal rings. The distinct responses to elevated Notch signaling in different imaginal ring cells may be because a negative feedback loop only presents in foregut imaginal rings, or other components are needed to collaborate with Notch signaling to promote additional cell division. Moreover, the different expression levels, patterns, and timing of Gal4 drivers may contribute to different cell proliferation responses to overexpressed NICD.

In addition, what acts downstream of Notch signaling in controlling the imaginal ring cell number is still unknown. To address this question, we conducted an RNAi screen for 300 transcription factors by *Act^{ts}* (Table S1; 264 from JASPAR, FlyReg, and FlyFactorSurvey). However, no transcription factor showed an obvious cell proliferation phenotype in imaginal rings. It could be because of the redundancy of transcription factors or because Notch could directly regulate cell cycle genes. Indeed, Notch activates cyclin D3 and CDK4/6 to promote G1/S transition in human T-cells, which could further induce T-cell acute lymphoblastic leukemia (T-ALL) (Demarest *et al.* 2008; Joshi *et al.* 2009). Notch also upregulates cyclin A to initialize S-phase in *Drosophila* eye discs during the second mitotic wave (Baonza and Freeman 2005). On the other hand, Notch suppresses string/cdc25 to block G2/M progression in *Drosophila* follicle cells (Deng *et al.* 2001).

Notch signaling in early imaginal ring development

Our studies also showed that Notch not only promotes the growth of the cell population in all imaginal ring cells in the third-instar stage, but is also involved in the maintenance of cell number of imaginal rings during the embryonic and L1 stages. Early knockdown of *Notch* causes reduced imaginal ring cell number and increased adjacent polyploid cells. However, further studies are still needed to determine the precise role(s) of Notch signaling in this process. Due to the lack of an imaginal ring marker in embryos and young larvae, we cannot directly determine whether these lost imaginal ring cells adopt other cell fates, such as salivary gland cells. Although this study cannot confirm that Notch functions in the specification and maintenance of imaginal ring cell fate, numerous studies have shown the role of Notch signaling in cell specification. In *Drosophila*, Notch signaling participates in numerous events of cell specification over the entire life of the animal. For example, Serrate/Notch signaling functions in the cell specification of crystal cells in *Drosophila* larval lymph glands (Duvic *et al.* 2002). Serrate/Notch signaling is also required for tubular tissue specification in vertebrates: Jagged, the vertebrate ortholog of Ser, induces the lineage specification of liver and pancreas duct cells from progenitor cells (Zhang *et al.* 2017). Additionally, we cannot completely rule

out the possibility that Notch regulates cell proliferation during embryogenesis and the early larval stage. Although previous studies and our research indicate that imaginal ring cells are mitotically quiescent before early to midlarval stages, a very low level of cell proliferation is still possible. In this situation, Notch in the imaginal rings may both maintain cell fate and control cell proliferation simultaneously during early stages. Although *Notch* mutant clone analysis could be applied to confirm this possibility, depending on the heat shock duration, our MARCM clonal experiments resulted in either a lack of clone cells when the mitotic clones were induced during late embryonic to late L1 stages, or a larval lethal phenotype when clones were induced during early to midembryonic stages. The lack of a clone phenotype might be because of no or very low mitotic activity in imaginal ring cells during the late embryonic to late L1 stages. The lethal phenotype further suggests that Notch is essential for normal development during early embryonic stages.

In addition, our studies suggest that Notch plays multiple roles in the development of the imaginal rings. The involvement of Notch signaling in multiple steps during the history of a particular cell type is not unique to the imaginal ring cells. In *Drosophila* follicle cells, Notch is needed for the polar/stalk cell lineage, and then it is required for a transition from the mitotic cycle to the endocycle in main body follicle cells (Dobens and Raftery 2000; Grammont and Irvine 2001; Assa-Kunik *et al.* 2007). Also, in murine intestinal stem cells, Notch is required for stem cell maintenance and cell proliferation (Jiang and Edgar 2012).

Ligands (Ser and Dl) in cis-inhibition and trans-activation

Dl and Ser ligands can each interact with the Notch receptor in *Drosophila*. However, their interactions with Notch differ, as determined by the Notch extracellular domain (NECD) structure. The NECD is mainly composed of multiple epidermal growth factor repeats (EGFs) (Kopan and Ilagan 2009). Mutation studies on different EGFs have shown that EGFs are essential to determine the interactions of Notch and its ligands. For example, EGF-11 and EGF-12 are required for the interactions with both Ser and Dl (Rebay *et al.* 1991). On the other hand, a point mutation in EGF-8 specifically reduces the interaction with Ser, resulting in a smaller salivary gland imaginal ring (Yamamoto *et al.* 2012). Our study shows that reduced Fng expression does not lead to lower proliferation activity in imaginal rings. As Fng modifies Notch by extending O-linked fucose on the EGFs to promote Notch's interaction only with Dl, our data suggest that modification of Notch by Fng does not affect cell proliferation in the imaginal rings. In contrast, our data show that Ser-mediated Notch activity controls cell proliferation in the imaginal rings, implying that the EGFs in the NECD might bear specific modifications that promote the interaction with Ser. Thus, imaginal rings may be an excellent model for further studies of receptor–ligand interactions in the Notch pathway.

Depending on the expression patterns of the Notch ligands, the interaction between Notch and ligand can have two

outcomes: *trans*-activation or *cis*-inhibition. In *Drosophila*, both ligands, Ser and Dl, have shown *cis*-inhibition and *trans*-activation of Notch signaling. *Trans*-activation of Notch is triggered by the juxtaposed ligand in adjacent cells, which could be provided from cells in neighboring tissues or the same tissue. For example, in *Drosophila* egg chambers, germline cells (the oocyte and nurse cells) provide Dl, which activates Notch in adjacent follicle cells (Deng *et al.* 2001). On the other hand, ligands may also be produced by the tissues themselves, as the ligand Dl, for Notch activation in the *Drosophila* germline stem cell niche, is synthesized within the niche (Yang *et al.* 2013). Our study suggests that the Ser ligand for Notch-dependent cell proliferation is produced from neighboring imaginal ring cells. Although Ser/Notch-dependent cell proliferation has been shown in the precursor cells to adult flight muscles in *Drosophila*, in that context, the Ser ligands are produced from neighboring wing disc epithelium (Gunage *et al.* 2014). In contrast, imaginal rings are single-layer tissues, which makes them an attractive system to study how the expression pattern of ligands regulates the *trans*-activation of Notch signaling and cell proliferation within the same tissue. In addition to Ser-induced *trans*-activation, *cis*-inhibitions also occur in imaginal rings and can be mediated by both Dl and Ser. The combinations of the *cis*- and *trans*- interactions could induce complex patterns of Notch activation (LeBon *et al.* 2014). Indeed, we observed nonuniform patterns of Notch activation in three imaginal rings during the third instar. Therefore, the interaction among Ser-induced *cis*-inhibition and *trans*-activation and Dl-induced *cis*-inhibition might contribute to the patterns of Notch activation in the imaginal rings; it will be important to determine the significance of these complex Notch activity patterns in the imaginal rings. For these reasons, imaginal rings could be an excellent model to study the patterning of Notch signaling in tissues for the determination of different developing zones.

Potential model for tumorigenesis

In tissues where Notch signaling regulates cell proliferation or the specification of stem cells, dysfunction of Notch signaling could result in developmental defects, or tissue overproliferation and tumorous phenotypes. Notch activity has been shown to have anticancer functions in some types of cancer and cancer-promoting roles in other types of cancer (Radtke and Raj 2003; Lobry *et al.* 2014). The different roles that Notch plays in various types of cancer may depend on the endogenous roles of Notch in these tissues. If Notch is involved in promoting cell differentiation, for example, as in *Drosophila* adult intestine stem cells and in follicle cells during the M/E switch, a tumor suppressor role of Notch is expected (Jiang and Edgar 2011). In contrast, if Notch is involved in promoting cell proliferation, as in adult T-cell leukemia, a tumor-promoting role of Notch is expected if consistent Notch activity is induced. Further studies will test whether Notch-dependent tumorigenesis occurs in the imaginal rings. Due to the highly conserved nature of the Notch

pathway, our findings on the role of Ser/Notch signaling in progenitor cell proliferation and the development of the imaginal rings presents a new model for future studies of tubular organogenesis and Notch-dependent tumorigenesis.

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