Developmental Biology

Notch signaling and Bsh homeodomain activity are integrated to diversify *Drosophila* lamina neuron types

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Abstract

Notch signaling is an evolutionarily conserved pathway for specifying binary neuronal fates, yet its mechanism remains elusive. In our accompanying paper, using the *Drosophila* lamina neurons (L1- L5) as a model, we show that the homeodomain transcription factor (HDTF) Bsh specifies L4 and L5 fates. Here we test the hypothesis that Notch signaling enables Bsh to differentially specify L4 and L5 fates. We show asymmetric Notch signaling between newborn L4 and L5 neurons, but they are not siblings; rather, Notch signaling in L4 is due to Delta expression in adjacent L1 neurons. While Notch signaling and Bsh expression are mutually independent, Notch is necessary and sufficient for Bsh to specify L4 fate over L5. With Notch signaling, L4 generates a distinct open chromatin landscape which results in distinct Bsh genome-binding loci, leading to L4-specific gene transcription. We propose that Notch signaling and HDTF function are integrated to diversify neuronal types.

eLife assessment

This paper explores how Notch activity acts together with homeodomain transcription Bsh factors to establish distinct cell fates (L4 vs L5) in the visual system of Drosophila. The findings are **important** and have theoretical or practical implications beyond a single subfield. The methods, data and analyses are **solid**, and broadly support the claims with only minor weaknesses.

Introduction

The extraordinary computational power of our brain depends on the vast diversity of neuron types characterized initially by transcription factor (TF) combinatorial codes, followed by neuron type-specific functional attributes such as cell surface molecules, neurotransmitters, and ion channels. It has been well documented how initial neuronal diversity is generated: in both invertebrate and vertebrate, spatial and temporal factors act combinatorially in progenitors to generate diverse and molecularly distinct progeny, and asymmetric Notch signaling between two newborn sister neurons further diversifies neuron types (Bayraktar and Doe, 2013; Bello-Rojas

and Bagnall, 2022; Doe, 2017; Erclik et al., 2017; Holguera and Desplan, 2018; Peng et al., 2007; Pierfelice et al., 2011; Sen et al., 2019; Spana and Doe, 1996). Yet, it remains unclear how Notch signaling controls binary neuronal fate. Notch signaling is an evolutionarily conserved pathway that controls many aspects of nervous system development, and the outcome of Notch signaling often depends on its context-dependent integration with other pathways (Bray, 2016; Louvi and Artavanis-Tsakonas, 2006). While most progenitor spatial and temporal factors are not maintained in neurons, our companion paper shows that HDTF Bsh (Brain specific homeobox) is persistently expressed in *Drosophila* lamina neurons, allowing it to couple initial fate decision to subsequent circuit formation and expression of a spectrum of neuronal functional genes, such as neurotransmitters and ion channels (Xu et al., 2023). This leads to the hypothesis that Notch signaling integrates with HDTF activity to diversify neuron types. To test this, we use the *Drosophila* lamina, the first ganglion in the optic lobe, to ask whether and how Notch signaling acts with the Bsh HDTF to diversify lamina neuron types.

The *Drosophila* lamina has only five intrinsic neuron types (L1-L5), which are analogous to bipolar cells in the vertebrate visual system (Sanes and Zipursky, 2010). During late larval and early pupal stages, lamina progenitor cells (LPCs) give rise to L1-L5 neurons (Fernandes et al., 2017; Huang et al., 1998). The cell bodies of each lamina neuron type are localized in a layer-specific manner. L2/L3 cell bodies are intermingled in the most distal layer while L1, L4, and L5 form distinct layers progressively more proximal (Tan et al., 2015; Xu et al., 2023). Each lamina neuron type expresses unique TF markers: L1, L2 and L3 neurons express Zfh1/Svp, Bab2 and Zfh1/Erm, respectively (Tan et al., 2015; Xu et al., 2023), while L4 and L5 neurons express the HDTFs Bsh/Ap and Bsh/Pdm3, respectively (Hasegawa et al., 2013; Tan et al., 2015; Xu et al., 2023). Our accompanying paper show that Bsh is initiated in LPCs and maintained in L4 and L5 neurons while Ap and Pdm3 are initiated in L4 and L5 neurons respectively (Xu et al., 2023). Based on their initiation order, we refer to Bsh as a “primary” HDTF and Ap/Pdm3 as “secondary” HDTFs (Xu et al., 2023). Bsh activates Ap and Pdm3 and specifies L4 and L5 fates (Xu et al., 2023). However, it remains unknown how a single primary HDTF Bsh activates two different secondary HDTFs and specifies two distinct neuron types (L4 and L5). Could Notch signaling distinguish L4 and L5 fates?

Here, we elucidate the role of Notch and Bsh in specifying L4 and L5 neuronal fates, resulting in the following findings. (1) Differential Notch signaling distinguishes L4 and L5 neurons. Unlike in the medulla, central brain and ventral nerve cord (Lee et al., 2020; Li et al., 2013; Mark et al., 2021), this is not due to an asymmetric partition of a Notch pathway component between sister neurons. Newborn L4 and L5 neurons are differentially exposed to the Notch ligand Delta expressed in L1, leading to asymmetric Notch activation in L4 but not L5. (2) Previously, the relationship between the primary HDTF Bsh and Notch signaling was unknown. Here we show while Notch and Bsh expression are mutually independent, they act together to differentially specify L4 and L5 fates. (3) How Notch controls binary neuronal fates has remained elusive. We hypothesize that Notch signaling might regulate chromatin accessibility. Indeed, we find that compared to $Notch^{OFF}$ L5 neurons, $Notch^{ON}$ L4 neurons exhibit distinct open chromatin landscape which shapes distinct Bsh genome-binding loci, resulting in L4-specific gene transcription. We propose that Notch signaling regulates the chromatin landscape in L4 neurons, leading to L4-distinct Bsh genomic binding, and L4-specific Bsh-dependent gene expression.

Results

Notch signaling is activated in newborn L4 neurons but not L5

Binding of a Notch ligand in one cell to Notch in an adjacent cell results in the translocation of the Notch intracellular domain (N-ICD) into the nucleus and the subsequent transcription of Notch target genes, including Hey (Monastirioti et al., 2010) (**Figure 1A**). To test whether Notch

signaling is activated in newborn lamina neurons, we stained for Hey as a reporter of active Notch signaling. We found that Hey is colocalized with Bsh+ L4 newborn neurons but not Bsh+ L5 neurons (**Figure 1B-B**”, D). We also examined the expression of another Notch target gene *E(spl)-mγ* (Almeida and Bray, 2005) and found that it was not expressed in lamina neurons (**Figure 1—figure supplement 1**). The primary HDTF Bsh activates the secondary HDTFs Ap and Pdm3 in L4 and L5, respectively (Xu et al., 2023). Here we show that Notch signaling is activated prior to the initiation of these secondary HDTFs, suggesting a potential causal relationship between asymmetric Notch signaling and differential secondary HDTF activation in L4 and L5 neurons (**Figure 1C-D**). Together, we conclude that there is differential Notch activity, with newborn L4, but not L5, expressing the Notch reporter Hey.

In most regions of the central nervous system, ganglion mother cells (GMCs) undergo asymmetric terminal divisions to make $Notch^{ON}/Notch^{OFF}$ sibling neurons with distinct cell fates (Lee et al., 2020; Li et al., 2013; Mark et al., 2021). To test whether L4 and L5 are $Notch^{ON}/Notch^{OFF}$ siblings, we used twin-spot MARCM (Yu et al., 2009) to trace two sibling neurons, which are divided from a single LPC cell, as a GFP and RFP pair. If L4 and L5 neurons are siblings generated from an LPC asymmetric division, we predict L4 and L5 to be an invariant RFP and GFP pair. In contrast, we found the GFP and RFP pair are either both L4 neurons or both L5 neurons (**Figure 1E-H**). This rules out an obligate asymmetric division to produce L4/L5 siblings, and suggests that differential Hey expression is due to differential contact with an extrinsic Notch ligand.

L1 neurons express Delta and activate Notch signaling in adjacent L4 neurons

We tested the possibility that the asymmetric Notch signaling between L4 and L5 is due to an extrinsic exposure of L4 to Delta expression, one of the two Notch ligands in *Drosophila* (Muskavitch, 1994). We found that during lamina neurogenesis, Delta is specifically expressed in L1 neurons which are adjacent to L4 neurons but not L5 (**Figure 2A-B**). Furthermore, we found that Delta is also expressed in the Tailless+ (Tll+) LPCs in the same row as the L1 neurons, suggesting that LPCs may be more heterogeneous than previously thought (Apitz and Salecker, 2014; Huang et al., 1998) (**Figure 2C-D**). To test whether the L1-specific transcription factor Svp is required to activate or maintain Delta expression in L1, we knocked down Svp expression in the lamina. We found Svp expression is indeed almost gone following Svp-knockdown (Svp-KD), yet Delta and Hey expression remains unaffected (**Figure 2—figure supplement 1**), suggesting that Delta expression in L1 neurons might be inherited from Delta-expressing LPCs instead of being activated by Svp. Together, we found that L1 neurons and a subset of LPCs express Delta, and Delta expression in L1 neurons does not depend on Svp.

Next, we asked whether Delta expression in L1 neurons is required to activate Notch signaling in adjacent newborn L4 neurons. We knocked down Delta expression in lamina using the LPC driver 27G05-Gal4 and Delta-RNAi. Indeed, Delta-KD shows the absence of Delta expression in lamina (**Figure 2E**’, F’). Importantly, reduced Delta expression abolished Hey expression in the newborn L4, showing that Delta expression in L1 neurons is required for activating Notch in L4 (**Figure 2E** - F”). Taken together, we conclude that Delta expressed in L1 neurons activates Notch signaling in L4 but not L5 neurons, making the Delta-Notch pathway a strong candidate for acting together with Bsh activity to differentially specify L4 and L5 fates (**Figure 2G**).

Bsh without Notch signaling activates Pdm3 and specifies L5 neuronal fate

To test whether the asymmetric Notch signaling between newborn L4 and L5 enables Bsh to differentially specify distinct L4 and L5 fates, we first performed Notch loss of function in the lamina using 27G05-Gal4 and UAS-Notch-RNAi. We found that this genotype caused embryonic lethality, so to preserve Notch function during early development, we performed conditional

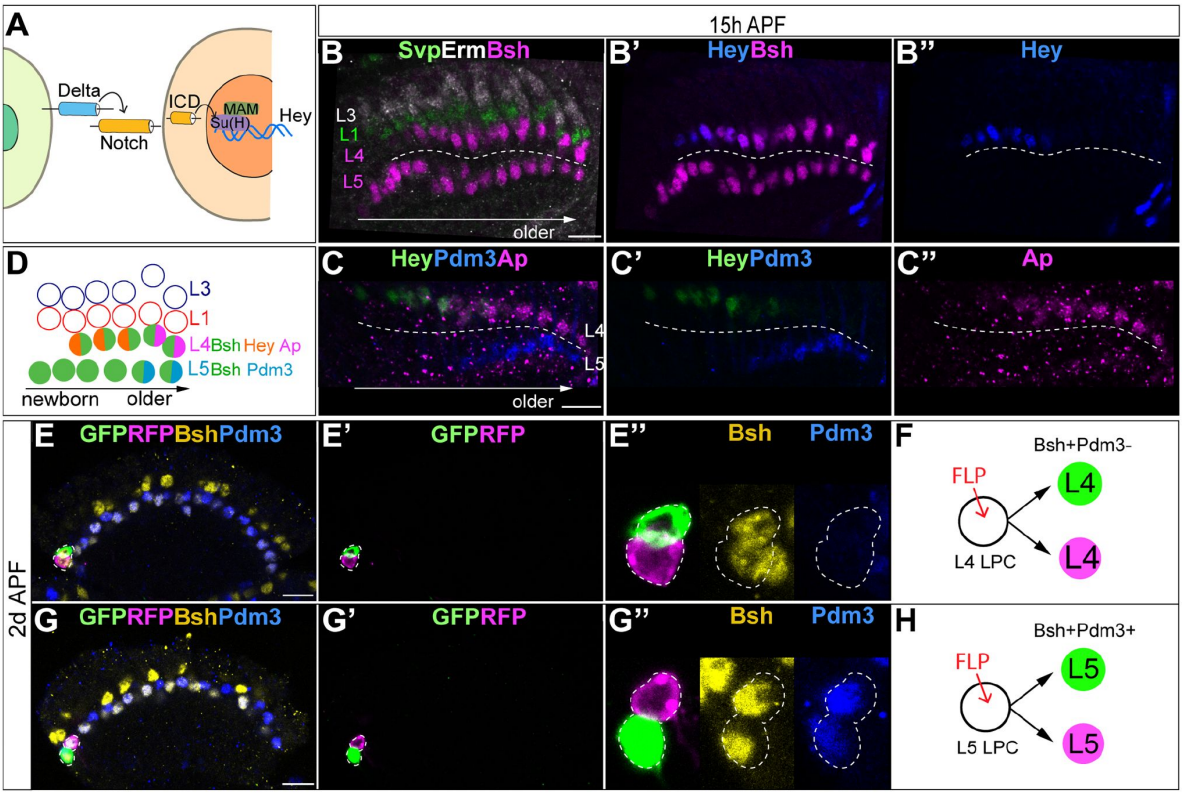

Figure 1.

Notch signaling is activated in newborn L4 neurons but not L5.

(A) Schematic of Notch signaling pathway.

(B-B") Hey as a reporter of active Notch signaling is only expressed in newborn L4 neurons but no other lamina neurons at 15h APF. Here and below, scale bar: 10 μm, n≥5 brains. Dashed line delineates the boundary between Bsh+ L4 and Bsh+ L5 neurons.

(C, D) Hey is expressed prior to the activation of the secondary HDTFs Ap and Pdm3 at 15h APF. Dashed line delineates the boundary between Bsh+ L4 and Bsh+ L5 neurons.

(E-H) Using twin-spot MARCM, two sibling neurons generated by one progenitor are traced. RFP and GFP cells are either both L4 neurons (Bsh+Pdm3-) or both L5 neurons (Bsh+Pdm3+). N=4.

Dashed line outlines RFP+ and GFP+ cell bodies.

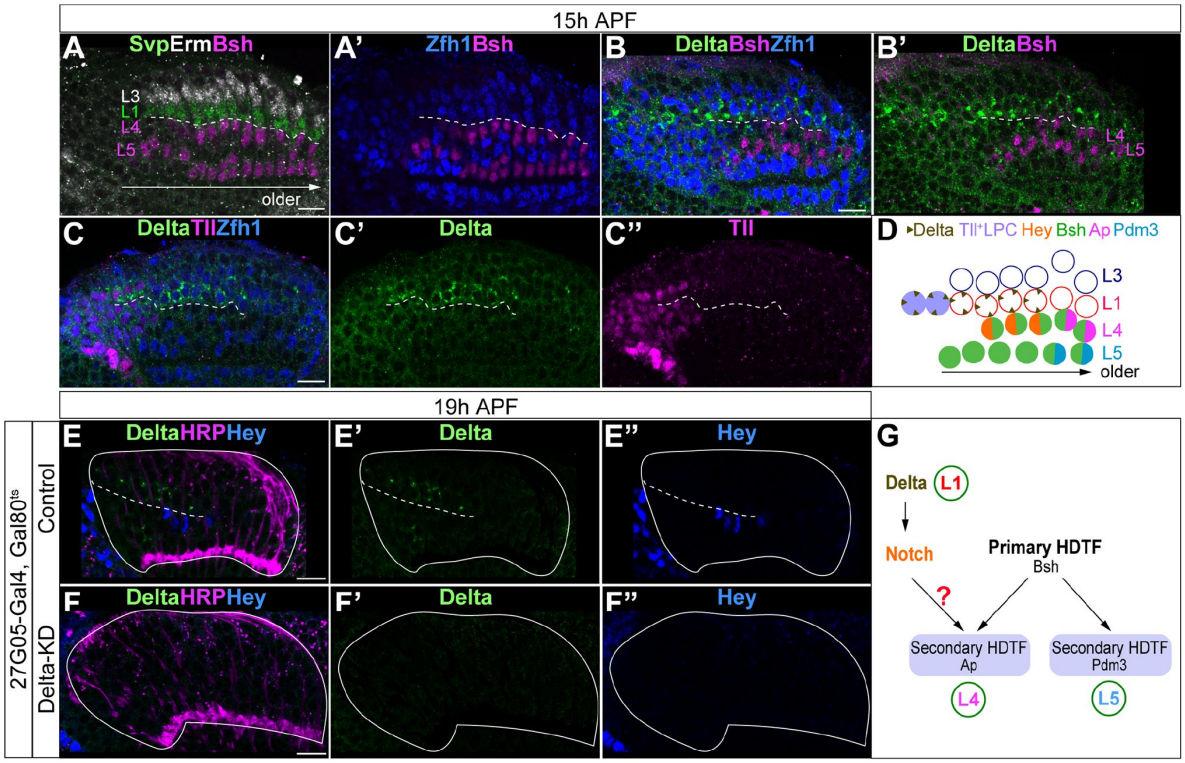

Figure 2.

L1 neurons express Delta and activate Notch signaling in adjacent L4 neurons.

(A, A') Svp+Zfh1+ L1 neurons are adjacent to both newborn L3 and L4 neurons. Newborn L3 neurons (Erm+) are localized strictly above (distal to) L1 neurons (Svp+Zfh1+) and L1 neurons are localized strictly above (distal to) L4 neurons. Here and below, scale bar: 10 μm, n≥5 brains. Dashed line delineates the boundary between L1 (Svp+Zfh1+) and L4 (Bsh+) cell bodies.
(B, B') Delta is expressed in Zfh1+ L1 neurons which are adjacent to Bsh+ L4 neurons. Dashed line delineates the boundary between L1 (Zfh1+) and L4 (Bsh+) cell bodies.
(C-C") Delta is also expressed in a subset of LPCs (Tll+). Dashed line highlights Delta+ cell bodies.
(D) Summary of A-C data; triangles represent Delta expression.
(E-F") Delta-KD (27G05-Gal4, tubP-GAL80[ts], UAS-Delta-RNAi) results in loss of Delta and Hey expression in lamina. HRP labels the axons of the photoreceptors which represent the lamina column. Solid white line outlines the lamina and dashed line delineates the boundary between Delta + cells and Hey+ cells.
(G) Summary

Notch-KD (N-KD) using Gal80^{ts}. We inactivated the Gal80^{ts} activity, which abrogates the suppression of 27G05-Gal4, from the beginning of the third instar stage to ensure the N-KD during lamina neurogenesis. During lamina neurogenesis, in controls, the Notch reporter Hey is expressed in newborn L4 neurons, followed by Bsh activation of Ap and Pdm3 and specification of L4 and L5 fates, respectively (Xu et al., 2023) (**Figure 3A-B**, **Figure 3—figure supplement 1B-B**"). In contrast, in N-KD, Hey expression becomes absent, and Bsh now activates Pdm3 but not Ap and specifies L5 fate (**Figure 3C-D**, **Figure 3—figure supplement 1B-C**"). During late pupal stages after the completion of lamina neurogenesis, control Bsh+ lamina neurons include both Ap^{+} L4 neurons and Pdm3^{+} L5 neurons (**Figure 3E-F**). In N-KD, however, Bsh+ lamina neurons are mainly Pdm3^{+} L5 neurons, though the number of Bsh+ lamina neurons remains unaffected, suggesting that more L5 neurons are generated at the expense of L4 in N-KD (**Figure 3E-J**). Interestingly, Bsh+ cell bodies in N-KD settle together to mix in a single layer, in contrast to Bsh+ cell bodies in controls segregating into two distinct layers with L4 distal to L5 (**Figure 3E-H**). This suggests that L4 and L5 cell bodies in controls might express different cell surface molecules to achieve their neuron type-specific settling layer.

Similarly, Delta-KD results in more L5 neurons generated at the expense of L4 (**Figure 3— figure supplement 2**). Note that ectopic Ap expression in L5 is caused by the 27G05-Gal4 line alone (**Figure 3E-G**", **Figure 3—figure supplement 2A-B**"), probably due to its genome insertion site in the Notch target gene *E(spl)m4-BFM* (**Figure 3—figure supplement 1A**), but this does not affect our conclusion that Bsh without Notch signaling activates Pdm3 and specifies L5 fate. Taken together, we conclude that in the absence of Notch signaling, the primary HDTF Bsh activates the secondary HDTF Pdm3 and specifies L5 neuronal fate (**Figure 3K**).

Bsh with Notch signaling activates Ap and specifies L4 neuronal fate

To test whether Notch signaling is sufficient for Bsh to activate Ap and specify L4 neuronal fate, we first performed Notch gain of function broadly in the LPCs and lamina neurons. We used 27G05- Gal4 and Gal80^{ts} to express the Notch intracellular domain (N-ICD; a constitutively active form of Notch) from the middle of lamina neurogenesis at 0h APF (**Figure 4—figure supplement 1A**). Following Notch misexpression, we observed ectopic Hey expression in newborn L5 neurons generated after 0h APF, showing that Notch signaling was active in L5 neurons (**Figure 4—figure supplement 1B-C**"). Similarly, Notch misexpression during late pupal stage results in ectopic Bsh+ Pdm3-L4 neurons and a loss of Bsh+ Pdm3+ L5 neurons (**Figure 4—figure supplement 1D-G**). Thus, broad activation of Notch signaling in lamina is sufficient for Bsh to activate Ap and specify L4 neuronal fate at the expense of L5 neuronal fate.

Next, we wanted to activate Notch signaling precisely in newborn L5 neurons using Bsh-Gal4. We confirmed that Bsh-Gal4 is turned on in newborn L5 and prior to Pdm3 initiation, making it the perfect driver for Notch gain of function in newborn L5 (**Figure 4—figure supplement 2A-B**"). In controls, newborn L4 neurons express Hey followed by Ap, whereas newborn L5 neurons lack Hey expression and subsequently express Pdm3 (Xu et al., 2023) (**Figure 4A-B**, **Figure 4—figure supplement 2C-C**"). In contrast, expression of N-ICD in newborn "L5 neurons" resulted in ectopic expression of Hey followed by expression of the L4 marker Ap (**Figure 4C-D**, **Figure 4—figure supplement D-D**"). The total number of Bsh+ lamina neurons remains unaffected, confirming the ectopic L4 neurons are generated at the expense of L5 neurons (**Figure 4E-J**). These data support our conclusion that Notch signaling is sufficient for Bsh to specify L4 neuronal fate at the expense of L5 neuronal fate (**Figure 4K**).

To test whether Bsh with Notch signaling specifies L4 morphology and connectivity, we took advantage of a Hey-ORF-Flp transgene (Mark et al., 2021) to trace the morphology of Hey^{+} neurons with myristoylated Tomato, and the Hey^{+} neuron presynaptic sites with Bruchpilot (Brp) using the STaR method (Chen et al., 2014; Xu et al., 2019) (**Figure 4—figure supplement**

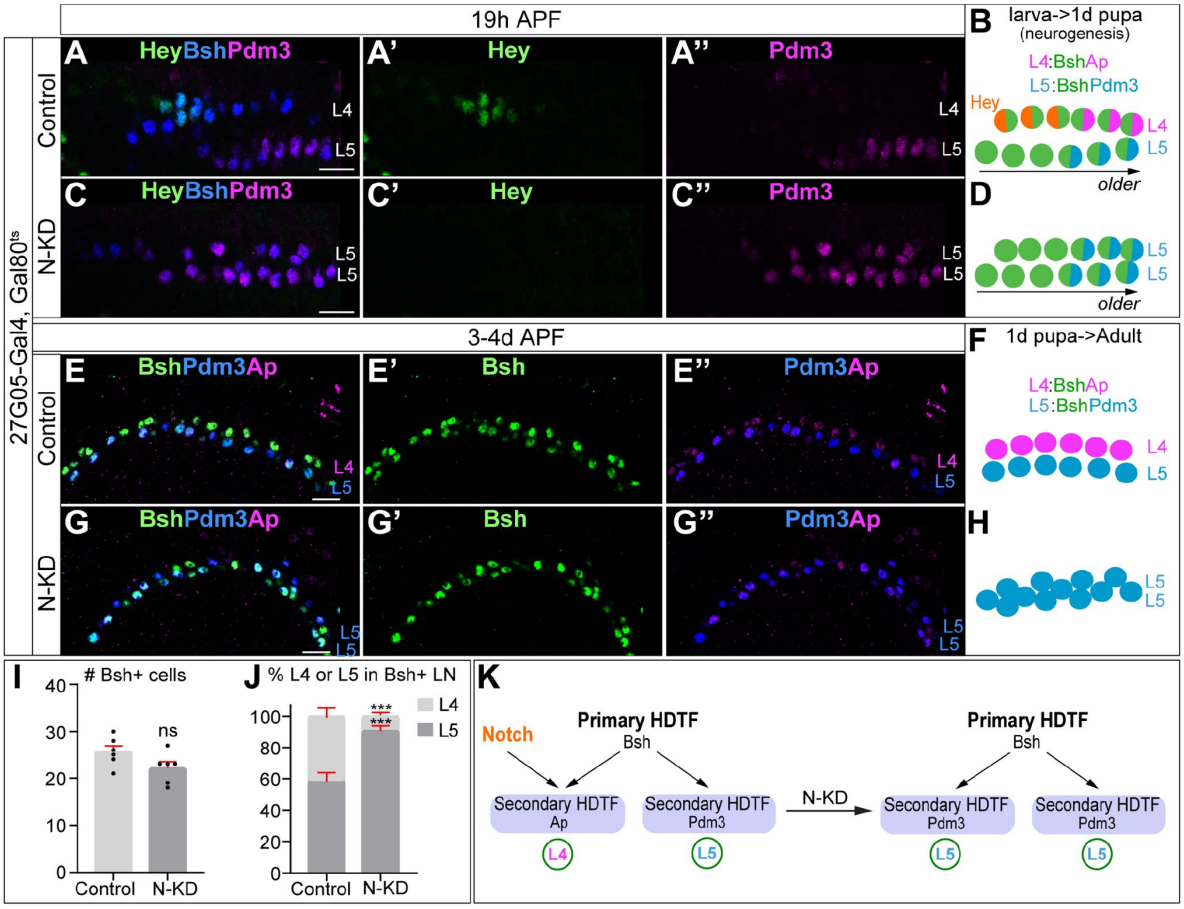

Figure 3.

Bsh without Notch signaling activates Pdm3 and specifies L5 neuronal fate.

(A-D) N-KD in lamina (27G05-Gal4, tubP-GAL80[ts], UAS-Notch-RNAi) shows Hey expression is absent, and Bsh only activates Pdm3 and specifies L5 neuronal fate during lamina neurogenesis (19h APF). Here and below, scale bar, 10 μm, n≥5 brains.
(E-J) N-KD in lamina shows loss of Ap+Pdm3-L4 neurons and gain of Pdm3+ L5 neurons at 3-4d APF. (I-J) Quantification (single optical section).
(K) Summary
Data are presented as mean ± SEM. Each dot represents each brain. n=6 brains in (I), (J).
***P<0.001, ns=not significant, unpaired t-test.

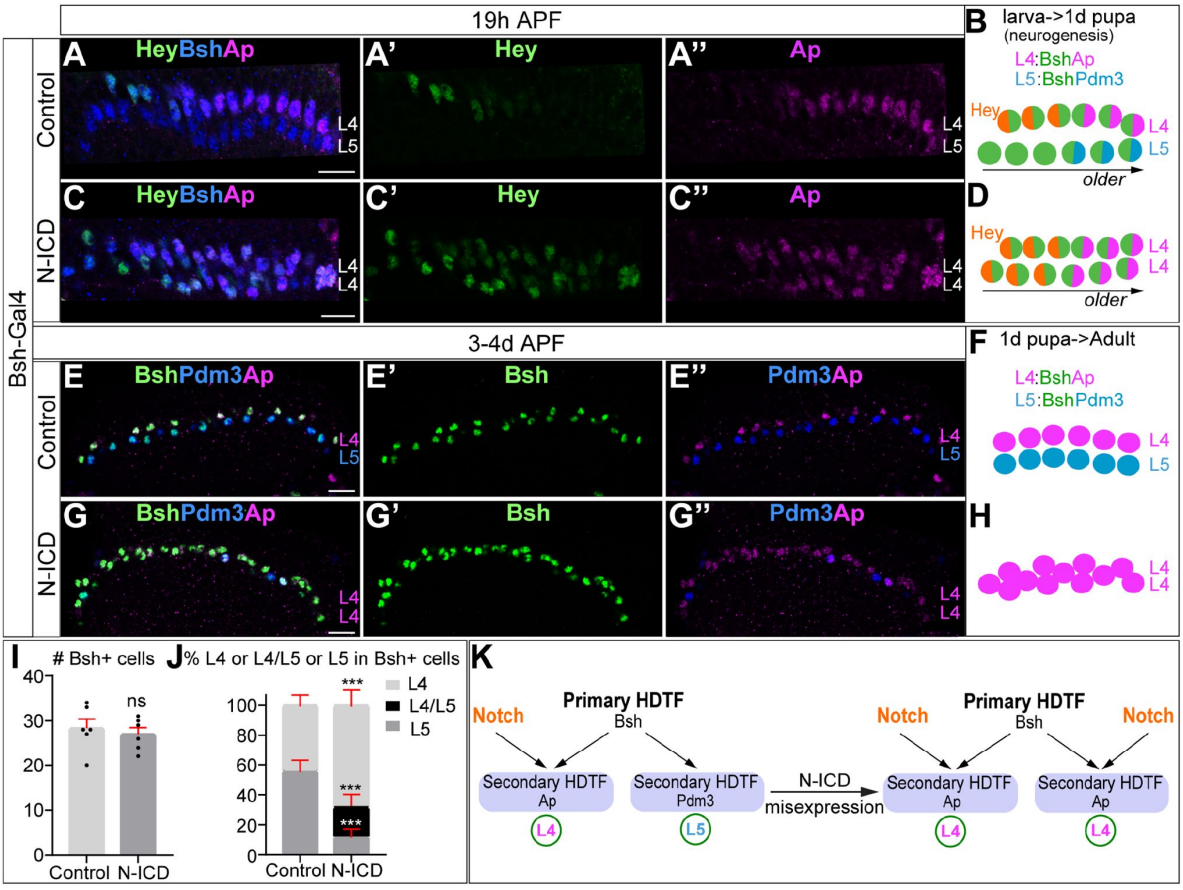

Figure 4.

Bsh with Notch signaling activates Ap and specifies L4 neuronal fate.

(A-D) Ectopic expression of N-ICD in newborn L5 neurons (Bsh-Gal4>UAS-N-ICD) results in ectopic Hey and Ap activation and an increased number of L4 neurons at 19h APF. Here and below, scale bar, 10 μm, n≥5 brains.

(E-J) N-ICD results in loss of Pdm3+ L5 neurons and gain of Ap+ L4 neurons at 3-4d APF. (I-J) Quantification (single optical section).

(K) Summary.

Data are presented as mean ± SEM. Each dot represents each brain. n=6 brains in (I), (J).

***P<0.001, ns=not significant, unpaired t-test.

3A). L4 neurons are the only lamina neurons that express Hey (**Figure 1B**'). Indeed, in controls, Tomato+ neurons, which trace Hey+ neurons, exhibit L4-specific morphology and connectivity in lamina: neurites and presynaptic Brp in the proximal lamina (**Figure 4—figure supplement 3B-C**). Following N-ICD misexpression in newborn L5, Tomato$^+$ neurons, which trace the endogenous and ectopic Hey$^+$ neurons, have L4-like morphology and connectivity: neurites and presynaptic Brp in the proximal lamina (**Figure 4—figure supplement 3D-E**). The slight deviations from control L4 morphology following N-ICD misexpression in newborn L5 might be due to a higher Notch signaling level in N-ICD misexpression compared to control. Taken together, we conclude that in the presence of Notch signaling, the primary HDTF Bsh activates the secondary HDTF Ap and specifies L4 TF marker, morphology and connectivity (**Figure 4K**).

Notch signaling and Bsh expression are mutually independent

Notch signaling is widely used in newborn neurons to specify binary neuronal fates and HDTFs are broadly expressed in neurons (Hobert, 2021; Jukam and Desplan, 2010; Louvi and Artavanis-Tsakonas, 2006; Spana and Doe, 1996), yet the relationship between Notch signaling and HDTF expression has remained elusive. Here we find that the secondary HDTFs Ap and Pdm3 are activated in a Notch-dependent manner: Notch signaling upregulates Ap expression and downregulates Pdm3 expression (**Figure 3**, 4). To test the relationship between Notch signaling and the primary HDTF Bsh expression, we performed conditional N-KD during lamina neurogenesis. This knockdown was highly effective, with Hey becoming undetectable, yet the number of Bsh+ cells remain unaffected (**Figure 5A-C**). Thus, Bsh expression is independent of Notch signaling. Conversely, we used 27G05-Gal4 and Bsh-RNAi to knock down Bsh in LPCs. This resulted in Bsh becoming undetectable, yet Hey expression was unaffected (**Figure 5D-F**). Thus, Notch signaling and Bsh expression are independent. Taken together, we conclude that while the expression of the secondary HDTFs depends on Notch signaling, the expression of primary HDTF Bsh and Notch signaling are mutually independent.

Notch signaling without Bsh specifies L3 neuron type

Our observation that Notch signaling remains unaffected following loss of Bsh raises an interesting question: what is the identity of the Hey$^+$ neurons in the absence of Bsh. To address this question, we used genetic methods (schematic in **Figure 5G**) to trace Hey$^+$ lamina neurons with myristoylated GFP. L4 neurons are the only lamina neurons expressing Hey. Indeed, in controls, GFP+ cells, which trace Hey+ neurons, express the L4 marker Bsh but not L3 markers Zfh1 or Erm (**Figure 5H**- H"). In Bsh-KD, Bsh expression is lost and GFP+ cells instead express the L3 markers Zfh1 and Erm (**Figure 5I-I**"). Zfh1 is required for L1 and L3 fates, and Bsh suppresses Zfh1 to inhibit ectopic L1 and L3 fates (Xu et al., 2023). Together, we conclude that in the absence of Bsh, Notch signaling with ectopic Zfh1+ specifies L3 neuronal fate.

We next tested whether Hey$^+$ neurons in Bsh-KD adopt L3 morphology. In controls, GFP$^+$ neurons express L4 marker Ap and exhibit L4-specific morphology: neurites in the proximal lamina and axon targeting two layers in medulla (**Figure 5J**). In Bsh-KD, GFP$^+$ neurons express the L3 marker Erm and adopt L3-specific morphology: one-direction (comb-like) neurites in lamina and axon targeting in single layer in medulla (Xu et al., 2019) (**Figure 5K**, L). We conclude that Notch signaling without Bsh in lamina determines L3-specific TF marker expression and morphology.

To test whether Hey$^+$ neurons in Bsh-KD adopt L3 connectivity, we examined the location of presynaptic sites of Hey+ neurons. We took advantage of Hey-ORF-Flp (Mark et al., 2021) to label the presynaptic marker Brp of Hey+ neurons with the STaR method (Chen et al., 2014; Xu et al., 2019) (schematic in **Figure 5M**). In controls, Tomato$^+$ neurons in lamina exhibit L4-specific morphology and connectivity, with neurites and presynaptic Brp in the proximal lamina (**Figure 5N**). In contrast, following Bsh-KD the Tomato$^+$ neurons adopt L3-specific morphology and

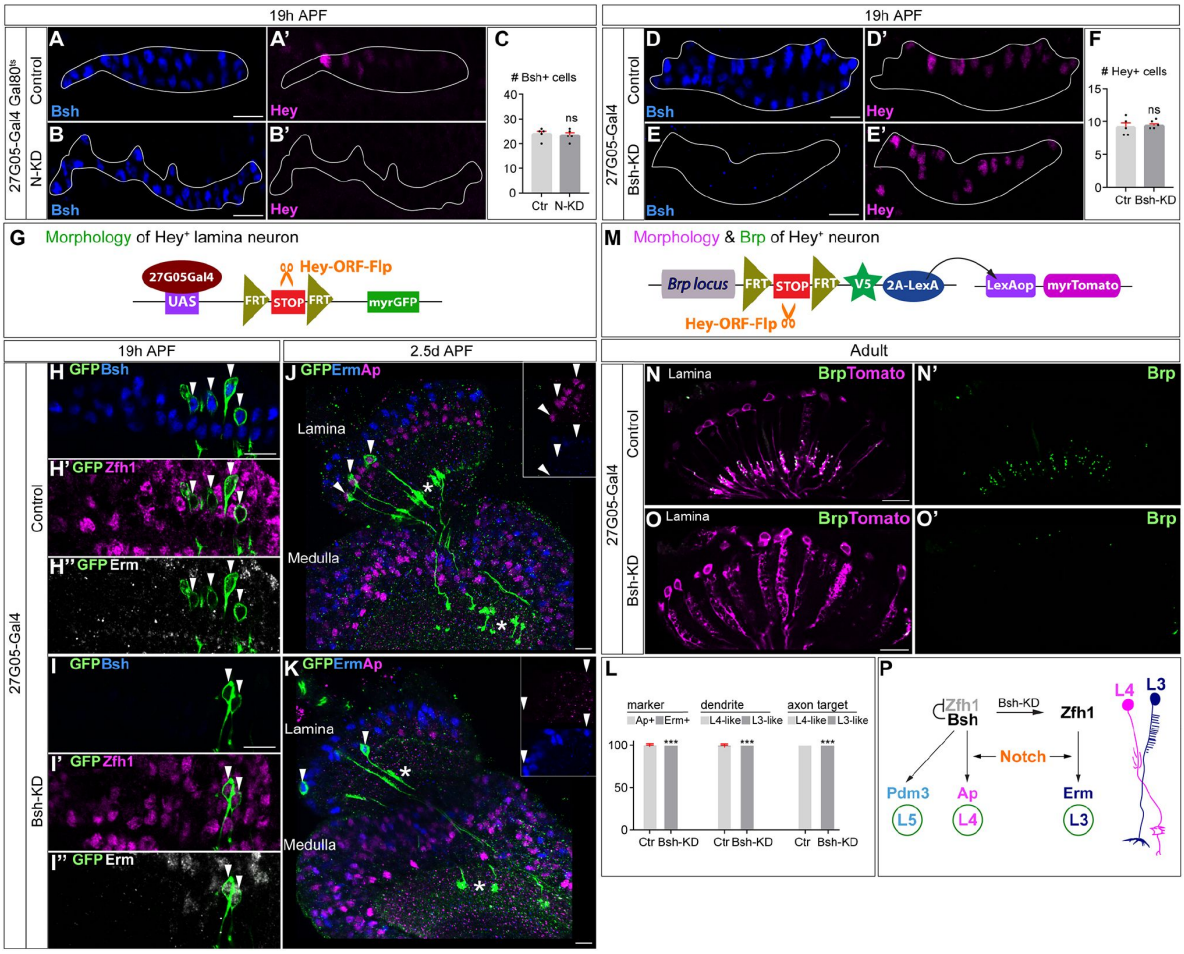

Figure 5.

Notch activation and Bsh expression are mutually independent; Notch signaling without Bsh specifies L3 neuron type.

(A-F) Notch activation and Bsh expression are mutually independent. (A-C) N-KD in lamina (27G05-Gal4, tubP-GAL80[ts], UAS-Notch-RNAi) results in loss of Hey expression without affecting Bsh expression. (C) Quantification (single optical section). Here and below, scale bar, 10 μm, n≥5 brains. (D-F) Bsh-KD in lamina (27G05-Gal4>UAS-Bsh-RNAi) results in loss of Bsh expression without affecting Hey expression. (F) Quantification (single optical section).

(G-L) Notch signaling without Bsh specifies L3 neuron type. (G) Schematic of the genetics used to trace the morphology of Hey+ lamina neurons. (H, I) In controls, GFP+ neurons express the L4 marker Bsh (white arrowhead) at 19h APF. In Bsh-KD, Bsh expression becomes absent in lamina and GFP+ cells now express L3 markers Zfh1 and Erm (white arrowhead). (J-L) In controls, GFP+ cells express the L4 marker Ap (white arrowhead) and have L4 morphology (asterisk) at 2.5d APF. In Bsh-KD, GFP+ cells express L3 marker Erm (white arrowhead) and adopt L3 morphology (asterisk). (L) Quantification for (J) and (K) (single optical section).

(M-O) (M) Schematic of the genetics used to trace the morphology and presynaptic sites of Hey+ neurons. (N, O) In controls, Tomato+ neurons have L4 morphology and presynaptic sites (Brp) in lamina. In Bsh-KD, Tomato+ neurons adopt L3 morphology and connectivity which lacks presynaptic localization in lamina (Xu et al., 2019).

(P) Summary.

Data are presented as mean ± SEM. Each dot represents each brain. n=5 brains in (C), (F) and (L).

***P<0.001, ns=not significant, unpaired t-test.

connectivity: comb-like neurites in lamina and no significant Brp in lamina (Xu et al., 2019) (**Figure 5O**). Taken together, we conclude that in the absence of Bsh, Notch signaling with ectopic Zfh1 specifies L3 TF marker, morphology, and connectivity (**Figure 5P**).

The Notch^{ON} L4 has correlated open chromatin, Bsh-bound loci and transcription profile that is distinct from the Notch^{OFF} L5

Bsh specifies two distinct neuron types: Notch^{ON} L4 and Notch^{OFF} L5. How are Notch signaling and Bsh activity integrated to specify L4 neuron type over L5? Bsh binds numerous L4 feature genes: ion channels, synaptic organizers, cytoskeleton regulators, synaptic recognition molecules, neuropeptide/receptor and neurotransmitter/receptor (Xu et al., 2023). This raises the hypothesis that Notch signaling might generate L4-distinct open chromatin landscape which determines the accessibility of Bsh target genes, resulting in L4-specific Bsh-dependent gene transcription. To determine if L4 and L5 have differing open chromatin regions and Bsh genome-binding sites, we used Targeted DamID (TaDa) (Marshall et al., 2016; Southall et al., 2013) to profile both open chromatin (Dam-alone binding) (Aughey et al., 2018) and Bsh genome-binding loci (Dam:Bsh binding) (**Figure 6A**). We performed these experiments with precise spatial and temporal control: only in L4 neurons (using L4-Gal4) which we reported in our accompanying paper (Xu et al., 2023) or L5 neurons (using L5-Gal4) (Luo et al., 2020; Nern et al., 2008) at the time of synapse formation (46-76h APF; **Figure 6—figure supplement 1A-B**"). We confirmed the function of the Bsh:Dam fusion protein previously (Xu et al., 2023). Here for L5 neurons, we performed three biological replicates which had high reproducibility (**Figure 6—figure supplement 1C**).

Our data show that the Notch^{ON} L4 neurons, compared to the Notch^{OFF} L5, have distinct open chromatin regions, which is consistent with the known role of Notch signaling driving histone acetylation, a marker of active enhancers, in cultured *Drosophila* and mammalian cells (Oswald et al., 2001; Skalska et al., 2015; Wang et al., 2014) (**Figure 6B**, Supplementary File 1). Indeed, we found the canonical binding motif of Suppressor of Hairless (Su(H)), the DNA-binding partner of Notch (Bray and Furriols, 2001), is more likely to be enriched in L4-disinct open chromatin regions (**Figure 6—figure supplement 2A**). To test whether L4-distinct open chromatin regions result in L4- specific gene transcription, we took advantage of recently published lamina neuron single cell RNA sequencing (scRNA-seq) data from the same developmental stage (Jain et al., 2022). Indeed, our data show the significant enrichment of L4-specific transcribed genes in L4-distinct open chromatin regions (**Figure 6B**, Supplementary File 2). Similarly, we found L5-specific transcribed genes are enriched in L5-distinct open chromatin regions (**Figure 6—figure supplement 2B**, Supplementary File 3). Together, our data suggest that Notch signaling directly or indirectly generates an L4-distinct open chromatin landscape which correlates with L4-specific gene transcription (**Figure 6D**).

Next, we tested the hypothesis that L4-distinct open chromatin landscape leads to L4-distinct Bsh genome-binding loci, which in turn results in L4-specific gene transcription. We found that L4 and L5 neurons indeed show distinct Bsh genome-binding loci (**Figure 6C**, Supplementary File 4). Furthermore, L4-distinct Bsh-bound loci, similar to L4-distinct open chromatin, are enriched for L4- specific transcribed genes (**Figure 6C**, Supplementary File 2). This suggests that L4-distinct open chromatin, via L4-distinct Bsh-bound loci, leads to L4-specific gene transcription. Interestingly, we did not see the significant enrichment of L5-specific transcribed genes in L5-distinct Bsh-bound loci, suggesting another unknown factor might be required for L5-specific gene transcription (**Figure 6— figure supplement 2C**, Supplementary File 3). Taken together, we propose that Notch signaling creates a distinct open chromatin landscape leading to distinct Bsh-bound loci in L4 neurons, and thus generating L4-specific Bsh-dependent gene transcription (**Figure 6D**).

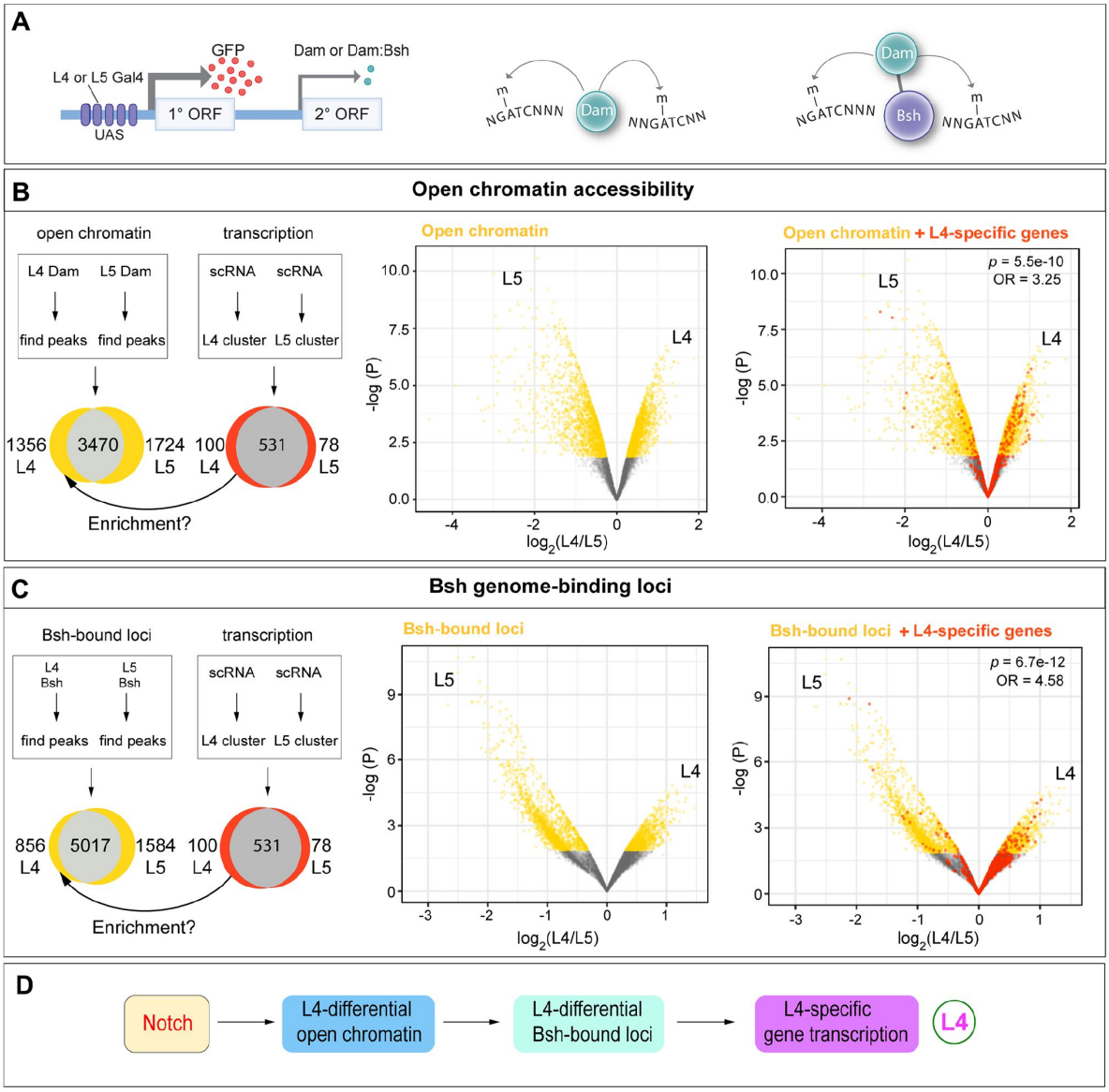

Figure 6.

The Notch^{ON} L4 has correlated open chromatin, Bsh-bound loci and transcription profile that is distinct from the Notch^{OFF} L5.

(A) Schematic of the Targeted DamID method. Upon GAL4 induction, low-levels of either Dam or Bsh:Dam are expressed, allowing genome-wide open chromatin and Bsh binding targets to be identified.

(B) L4 and L5 neurons show distinct open chromatin regions (yellow), and L4-specific transcribed genes (red) are enriched in L4-distinct open chromatin regions.

(C) L4 and L5 neurons show distinct Bsh-bound loci (yellow), and L4-specific transcribed genes (red) are enriched in L4-distinct Bsh-bound loci. *P* values from Fisher's exact test; odds ratios (OR) expressed as L4/L5 in all cases.

(D) Model.

Discussion

Notch signaling is an evolutionarily conserved pathway for controlling binary cell fates in worms, flies, and mammals (Artavanis-Tsakonas et al., 1999; Bray, 2016; Ehebauer et al., 2006; Jukam and Desplan, 2010; Louvi and Artavanis-Tsakonas, 2006; Pierfelice et al., 2011). Here we identified a pair of Notch^{ON} and Notch^{OFF} Bsh+ newborn neurons. Bsh and Notch signaling together specifies L4 fate while Bsh alone specifies L5 fate. With Notch signaling, L4 neurons generate distinct open chromatin landscape, allowing distinct Bsh target loci, leading to L4-specific gene transcription. Notch signaling and Bsh expression are mutually independent. In the absence of Bsh, the Notch^{ON} and Notch^{OFF} neurons with ectopic HDTF Zfh1 adopt L3 and L1 (Xu et al., 2023) fates respectively (**Figure 7**). Our findings propose a model that the unique combination of HDTF and open chromatin landscape (e.g. by Notch signaling) specifies distinct neuron types.

Despite the important roles of Notch signaling in development, its function in lamina development has remained unknown. Here we discovered that L1 neurons express Delta and activate Notch signaling in adjacent newborn L4 but not in the more distant L5. L3 is also adjacent to the Delta+ L1; might L3 be a Notch^{ON} neuron? If so, it would use a different effector, as Hey is not expressed in newborn L3 neurons. Yet, it seems likely for several reasons: (1) like L4, newborn L3 neurons are strictly adjacent to Delta+ L1; (2) Asymmetric Notch signaling between L3 and L1 would allow Zfh1 to differentially specify L3 and L1 neuronal fates; (3) in Bsh-KD, Notch signaling with ectopic Zfh1 specifies ectopic L3 neuron type. Besides lamina neurons, we also detected Delta expression in a small pool of LPCs in the L1 row, suggesting that LPCs are more heterogeneous than previously thought (Apitz and Salecker, 2014; Fernandes et al., 2017). It would be interesting to characterize the molecular heterogeneity of LPCs using scRNA-seq and explore the potential role of Notch signaling in this population.

It is well-known that Notch signaling controls binary cell fates. Yet, its mechanism remains unclear. We found that Notch signaling and primary HDTF Bsh activity are integrated to specify two distinct neuron types: L4 and L5. Notch signaling directly or indirectly generates L4-distinct open chromatin landscape which specifies L4-distinct Bsh-bound loci, resulting in L4-specific Bsh-dependent gene transcription. This is consistent with *in vitro* findings of Notch function. In cultured mammalian cells, upon Notch activation, Notch transcription complexes recruit p300 which acetylates H3K27 and produce large increase in H3K27 acetylation levels, an active enhancer marker, across the entire breadth of super-enhancers (Oswald et al., 2001; Wang et al., 2014). In cultured *Drosophila* cells, Notch activation induces a robust increase in H3K56 acetylation (Bray, 2016; Skalska et al., 2015). One intriguing future direction would be testing whether Notch signaling directly generates open chromatin landscape *in vivo*.

Our brain function depends on the vast diversity of neuron types. The role of TFs in specifying neuron types has been well studied. For example, each temporal transcription factor (TTF) acts transiently in progenitors to generate specific neuron types during its expression window (Doe, 2017; Isshiki et al., 2001; Li et al., 2013). While most TTFs are not maintained in neurons, their function is likely maintained by another TF, e.g. HDTF, which is persistently expressed in neurons. Indeed, HDTFs function as terminal selectors and control the expression of neuronal identity genes (Cros and Hobert, 2022; Hobert, 2021; Howell et al., 2015; Reilly et al., 2022, 2020). The primary HDTF Bsh specifies L4 and L5 neuronal fates (Xu et al., 2023). However, the role of open chromatin in specifying neuron type is less well characterized. *In vitro*, during programming of mouse embryonic stem cells to neurons, proneural factors Ascl1 and Neurogenin2 establish distinct chromatin landscapes, resulting in induced neurons with different subtype identities (Aydin et al., 2019; Wapinski et al., 2013). Here, we found that, with Notch signaling, L4 generates distinct open chromatin landscape which correlates with distinct Bsh genome-binding loci, and subsequent L4- specific gene transcription. Furthermore, in Bsh-KD, L3 and L1 are ectopically generated at the expense of L4 and L5 respectively (**Figure 7**). This

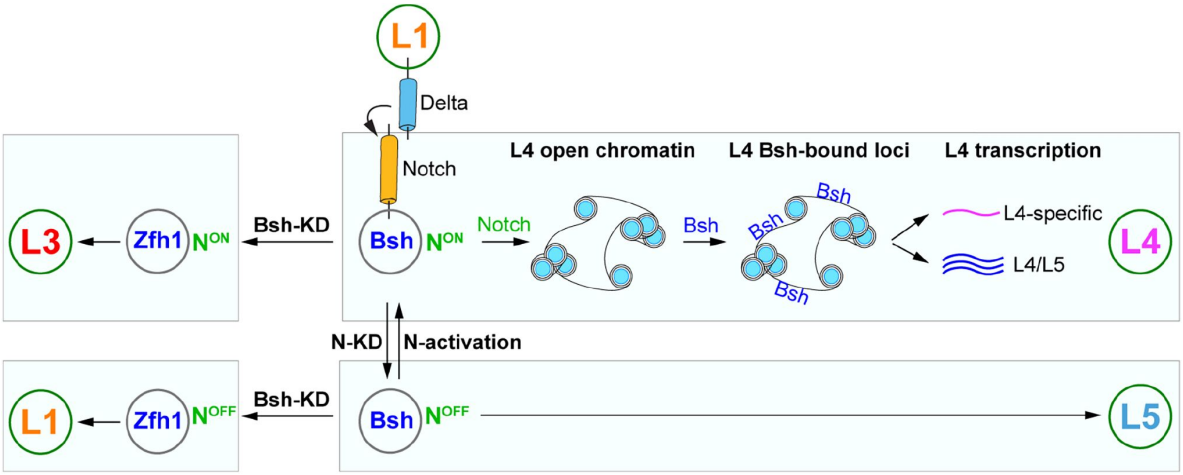

Figure 7.

Model; see the discussion section for details.

elegant one-to-one fate switch (L4>L3; L5>L1) raises an open question: would newborn L4 and L3 share the open chromatin landscape which is distinct from the one shared by newborn L5 and L1? Our findings provide a testable model that the unique combination of HDTF and open chromatin landscape specifies distinct neuron types.

Acknowledgements

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Author contributions

CX: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Resources; Writing—original draft; Writing—review & editing; Visualization; Project administration TBR: Validation; Formal analysis; Investigation; Resources; Writing—review & editing OM: Software; Formal analysis; Resources; Data curation; Writing—review & editing CQD: Methodology; Resources; Writing—review & editing; Visualization; Supervision; Project administration; Funding acquisition

Declaration of interests

The authors declare no competing financial or non-financial interests.

Data Availability Statement

All resources will be provided upon request.

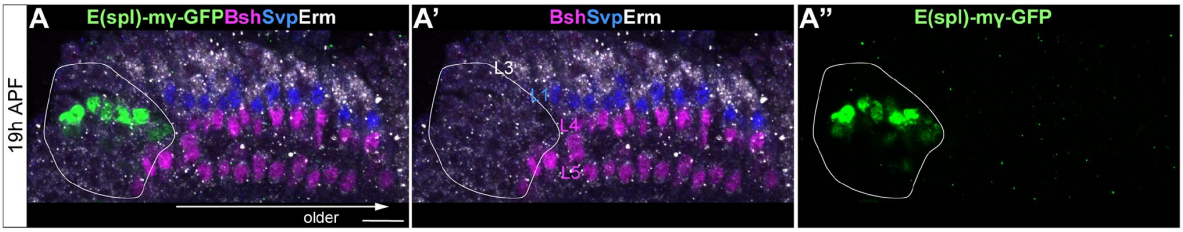

Figure 1—figure supplement 1.

E(spl)-my is not expressed in lamina neurons.

(A-A") E(spl)-my-GFP is expressed in LPC region but not lamina neurons. Erm labels L3; Svp labels L1; Bsh labels Bsh+ LPCs, L4 and L5. Solid white line outlines LPC region.

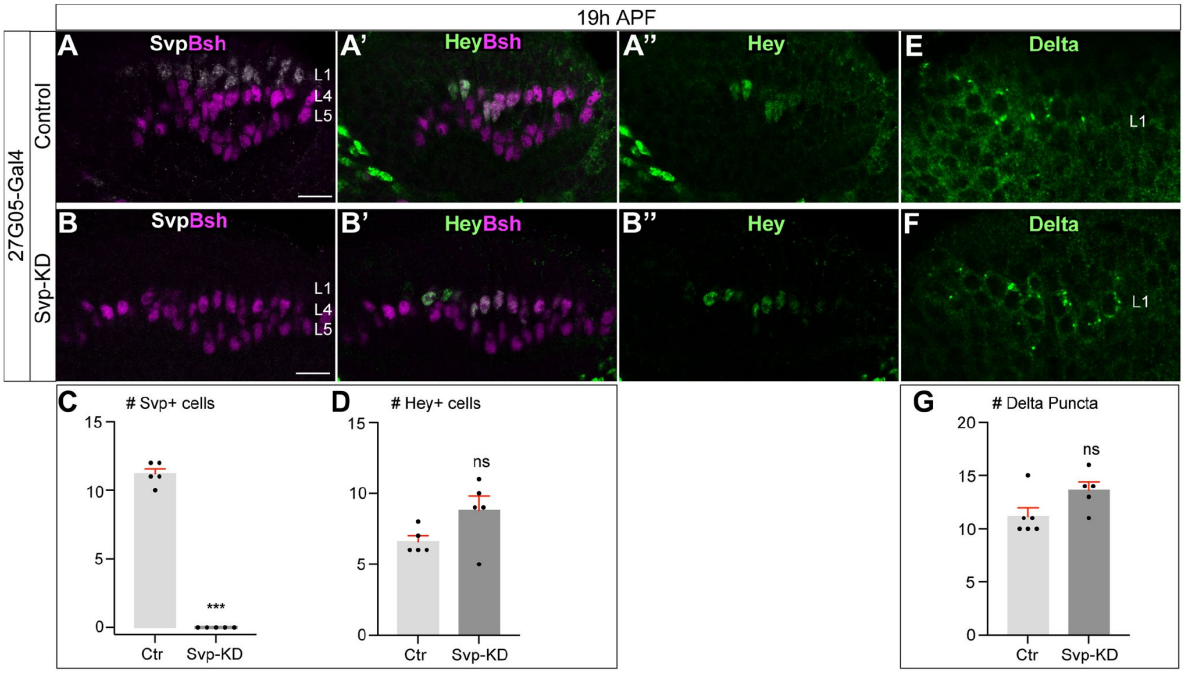

Figure 2—figure supplement 1.

Svp is not required to initiate or maintain Delta expression in L1 neurons.

(A-D) In Svp-KD (27G05-Gal4>UAS-Svp-RNAi), there is a loss of Svp while Hey expression remains unaffected at 19h APF. (C-D) Quantification (single optical section). Here and below, scale bar, 10 μm, n≥5 brains.

(E-G) In Svp-KD (27G05-Gal4>UAS-Svp-RNAi), Delta expression remains unaffected at 19h APF.

(G) Quantification (single optical section).

Data are presented as mean ± SEM. Each dot represents each brain. n=5 brains in (C) and (D). n=6 brains for control and n=5 brains for Svp-KD in (G). ***P<0.001, ns=not significant, unpaired t-test.

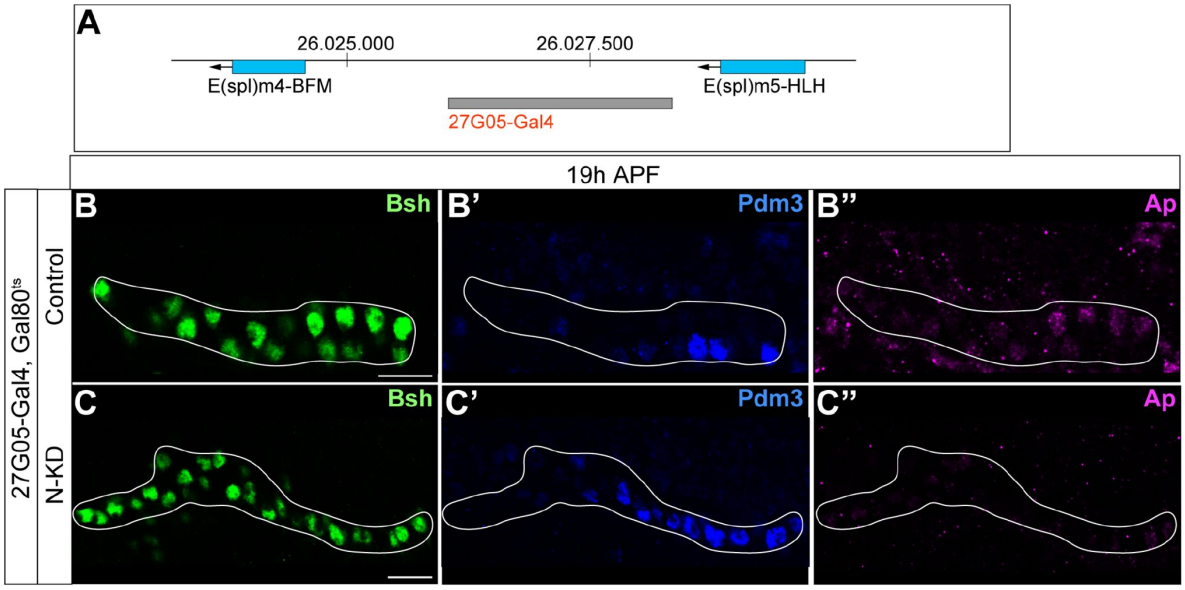

Figure 3—figure supplement 1.

27G05-Gal4 is inserted in between two Notch target genes; Bsh without Notch signaling activates Pdm3 and specifies L5 morphology.

(A) Schematic of 27G05-Gal4 insertion site in between two Notch target genes (E(spl)m4-BFM and E(spl)m5-HLH).
(B-C") Bsh without Notch signaling activates Pdm3 and specifies L5 morphology. In N-KD (27G05- Gal4, tubP-GAL80[ts], UAS-Notch-RNAi), Bsh activates Pdm3 but not Ap and specifies L5 neuronal fate during lamina neurogenesis (19h APF). White line outlines Bsh+ lamina cells. Scale bar, 10 μm, n≥5 brains.

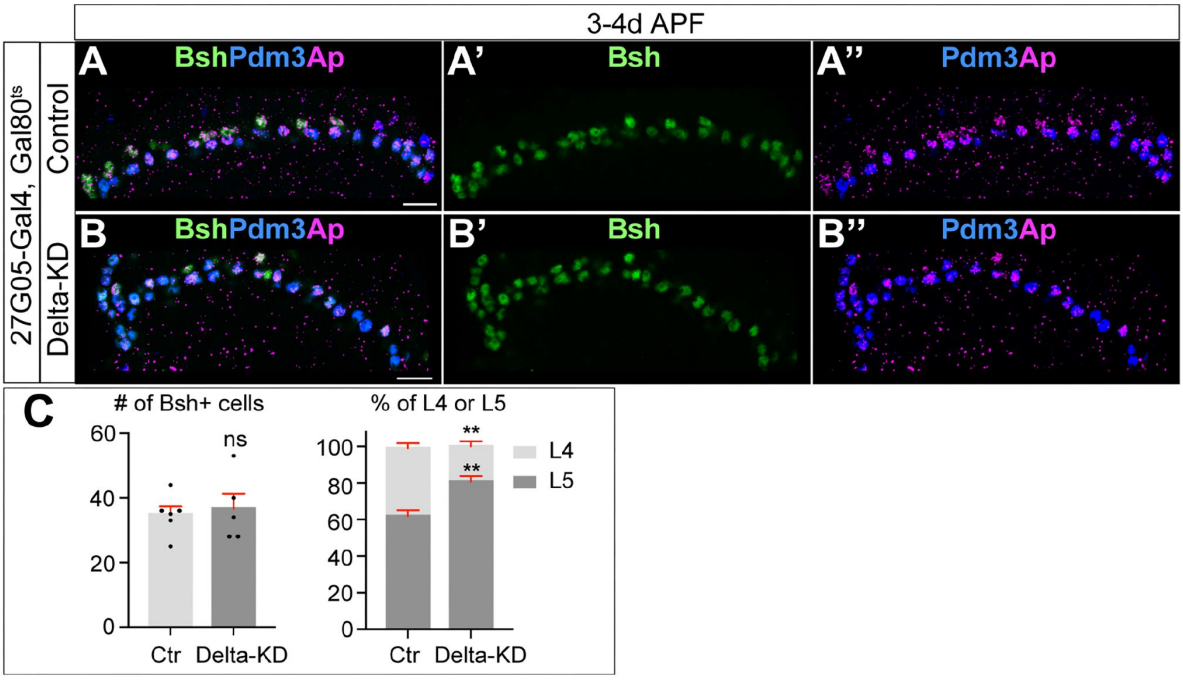

Figure 3—figure supplement 2.

Bsh specifies L5 neuronal fate over L4 following Delta-KD.

(A-C) Delta-KD (27G05-Gal4, tubP-GAL80[ts], UAS-Delta-RNAi) in lamina shows the decrease of Ap+ Pdm3-L4 neurons and increase of Pdm3+ L5 neurons at 3-4d APF. (C) Quantification (single optical section). Scale bar, 10 μm.
Data are presented as mean ± SEM. Each dot represents each brain. n=5 brains. ***P*<0.01, ns=not significant, unpaired t-test.

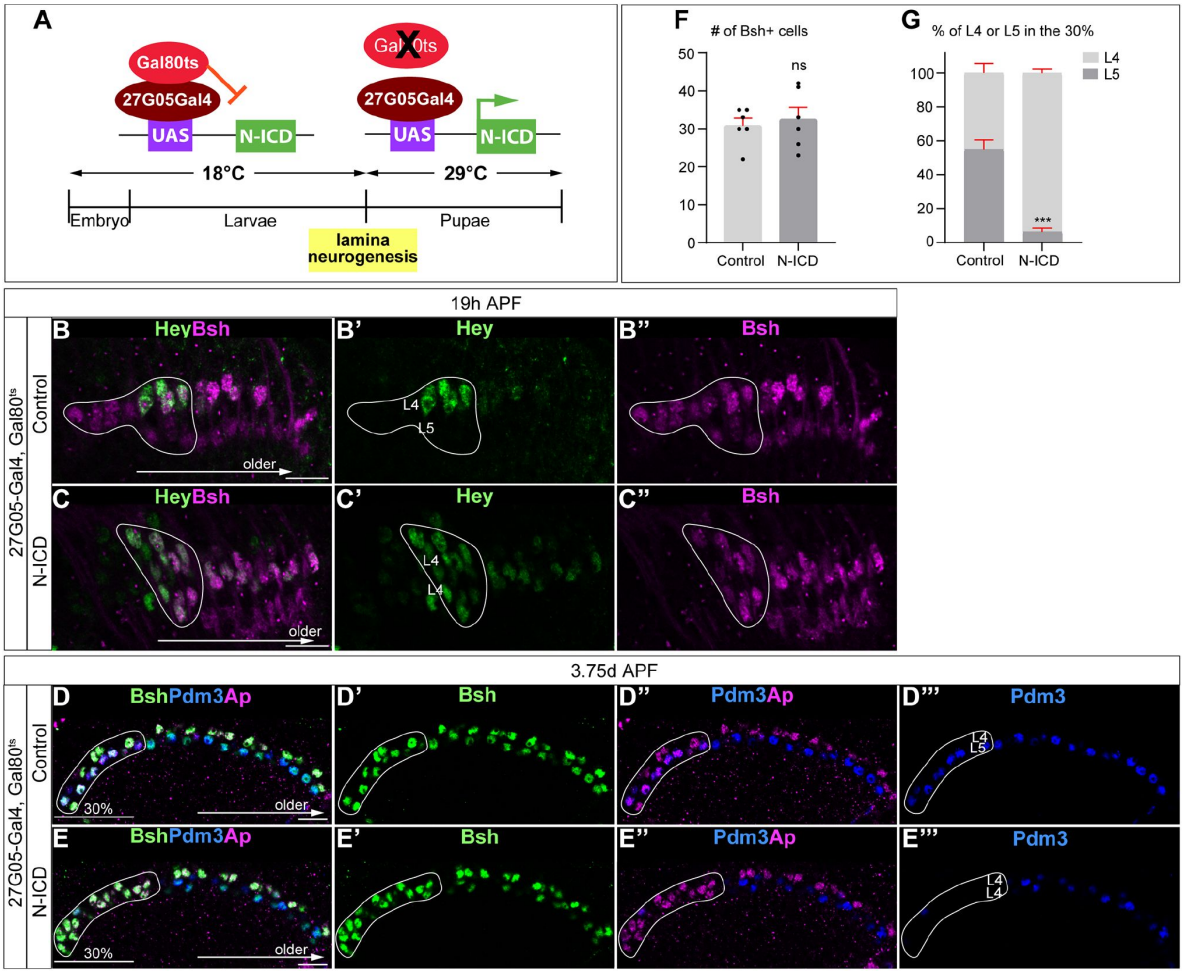

Figure 4—figure supplement 1.

Temporally-restricted activation of Notch signaling by 27G05- Gal4 enables Bsh to specify L4 neuronal fate over L5.

(A) Schematic of the genetics for temporally-restricted activation of Notch signaling.
(B-C") Activation of Notch signaling from 0h APF (affected region circled) results in ectopic Hey expression at 19h APF. Here and below, scale bar, 10 μm, n≥5 brains.
(D-G) Activation of Notch signaling from 0h APF (affected region circled) results in no change to Bsh expression, loss of Pdm3+ L5 neurons and gain of Ap+Pdm3-L4 neurons at 3-4d APF. (F and G) Quantification (single optical section).
Data are presented as mean ± SEM. Each dot represents each brain. ***P<0.001, ns=not significant, unpaired t-test.

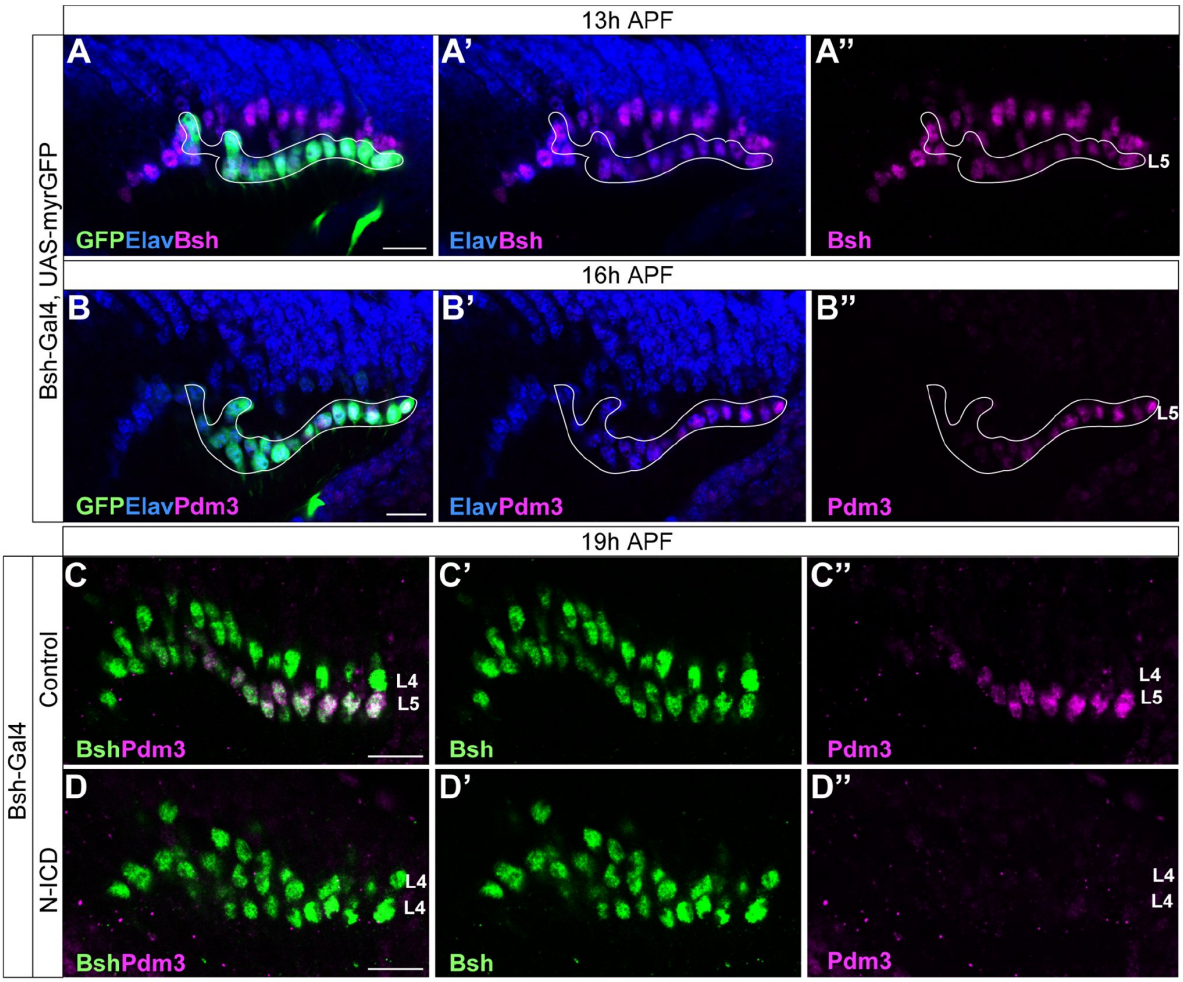

Figure 4—figure supplement 2.

Bsh-Gal4 is expressed in newborn L5 neurons; Bsh does not activate L5 marker Pdm3 when Notch signaling is activated in newborn L5 by Bsh-Gal4 and UAS-N-ICD.

(A-B") Bsh-Gal4 is expressed in newborn L5 neurons. (A-A") GFP+ cells (white line circle; Bsh-Gal4>UAS-myrGFP) label L5 neurons which are in the bottom (proximal) row of Bsh+ cells during lamina neurogenesis (13h APF). Here and below, scale bar, 10 μm, n≥5 brains. (B-B") GFP+ cells (white line circle; Bsh-Gal4>UAS-myrGFP) label Pdm3+ newborn L5 and early born L5 neurons during lamina neurogenesis (16h APF).

(C-D") Bsh does not activate L5 marker Pdm3 when Notch signaling is activated in newborn L5 by Bsh-Gal4 and UAS-N-ICD. Pdm3 expression is initiated in L5 neurons in control but not in N-ICD (Bsh-Gal4>UAS-N-ICD).

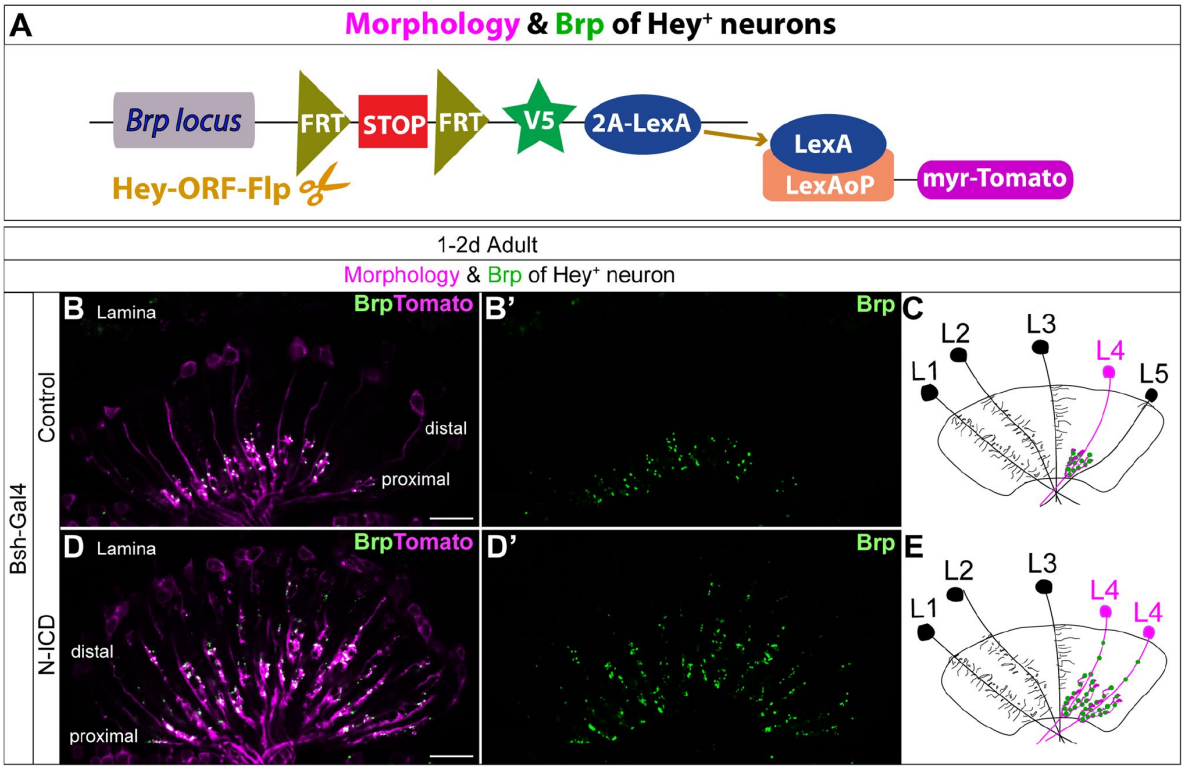

Figure 4—figure supplement 3.

Bsh-Gal4 activation of Notch signaling in newborn L5 neurons specifies L4-like morphology and presynaptic sites.

(A) Schematic of the genetics used to trace the morphology and presynaptic site (Brp) of Hey+ neurons.
(B-E) In controls, Tomato+ neurons in lamina have L4 morphology and presynaptic sites labeled by Brp-V5. In Bsh-KD, Tomato+ cells, which trace the endogenous and ectopic Hey+ neurons, have L4-like morphology and presynaptic sites labeled by Brp-V5 in the lamina. Scale bar, 10 μm. n≥5 brains.

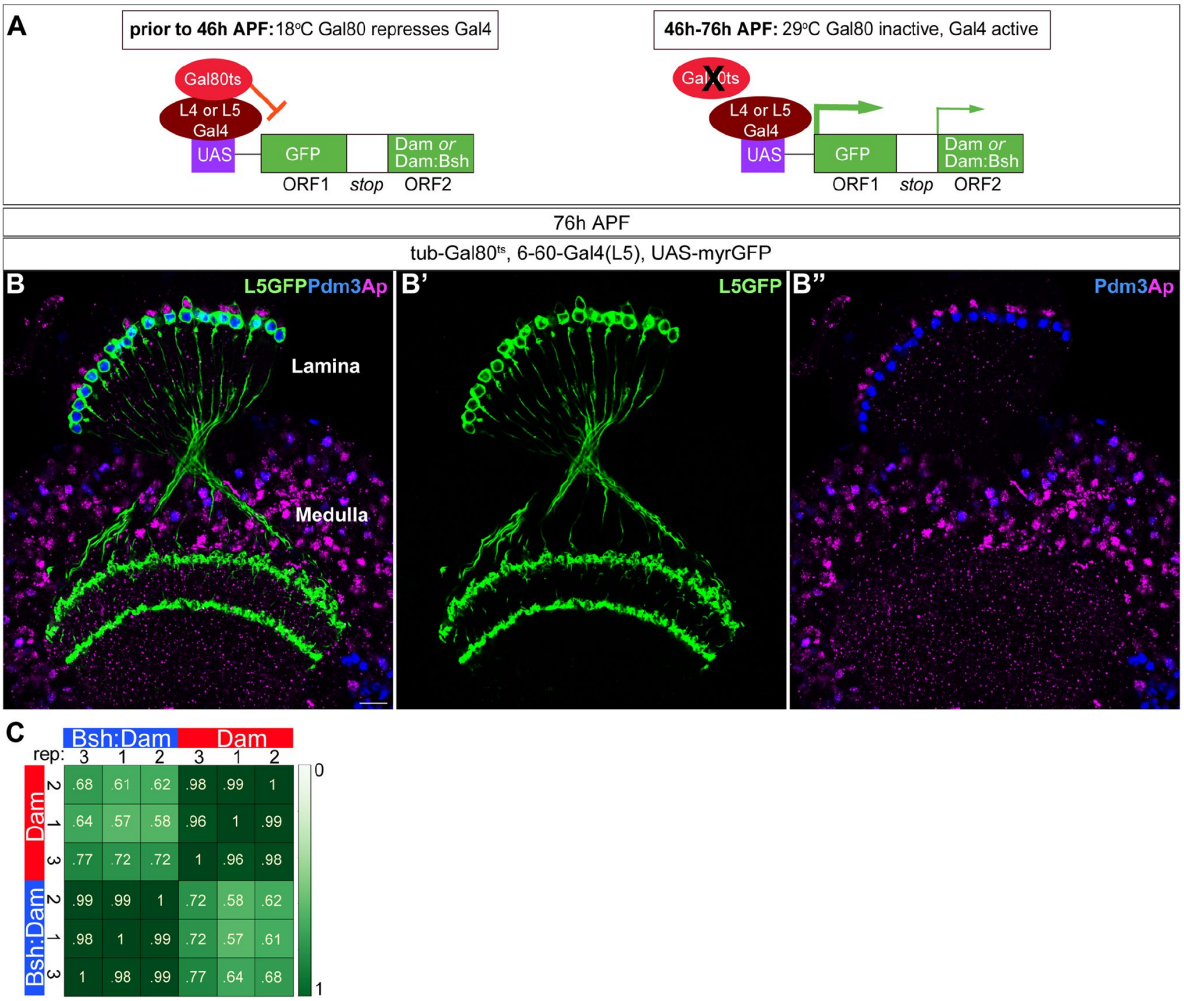

Figure 6—figure supplement 1.

6-60-Gal4 used for TaDa experiment is specifically expressed in L5 neurons at 46h-76h APF.

(A) Schematic of the genetics used to perform targeted Dam ID experiments (see **Figure 6**).
(B-B") 6-60-Gal4, which is expressed at 46h-76h APF, drives GFP expression in all Pdm3+ L5 neurons and weak GFP expression in some Ap+ L4 neurons. Scale bar, 10 μm. n≥5 brains.
(C) Both Dam and Bsh:Dam show high Pearson correlation coefficients among their three biological replicates, with lower Pearson correlation coefficients between Dam and Bsh:Dam. Heat map is generated using the multiBamSummary and plotCorrelation functions of deepTools.

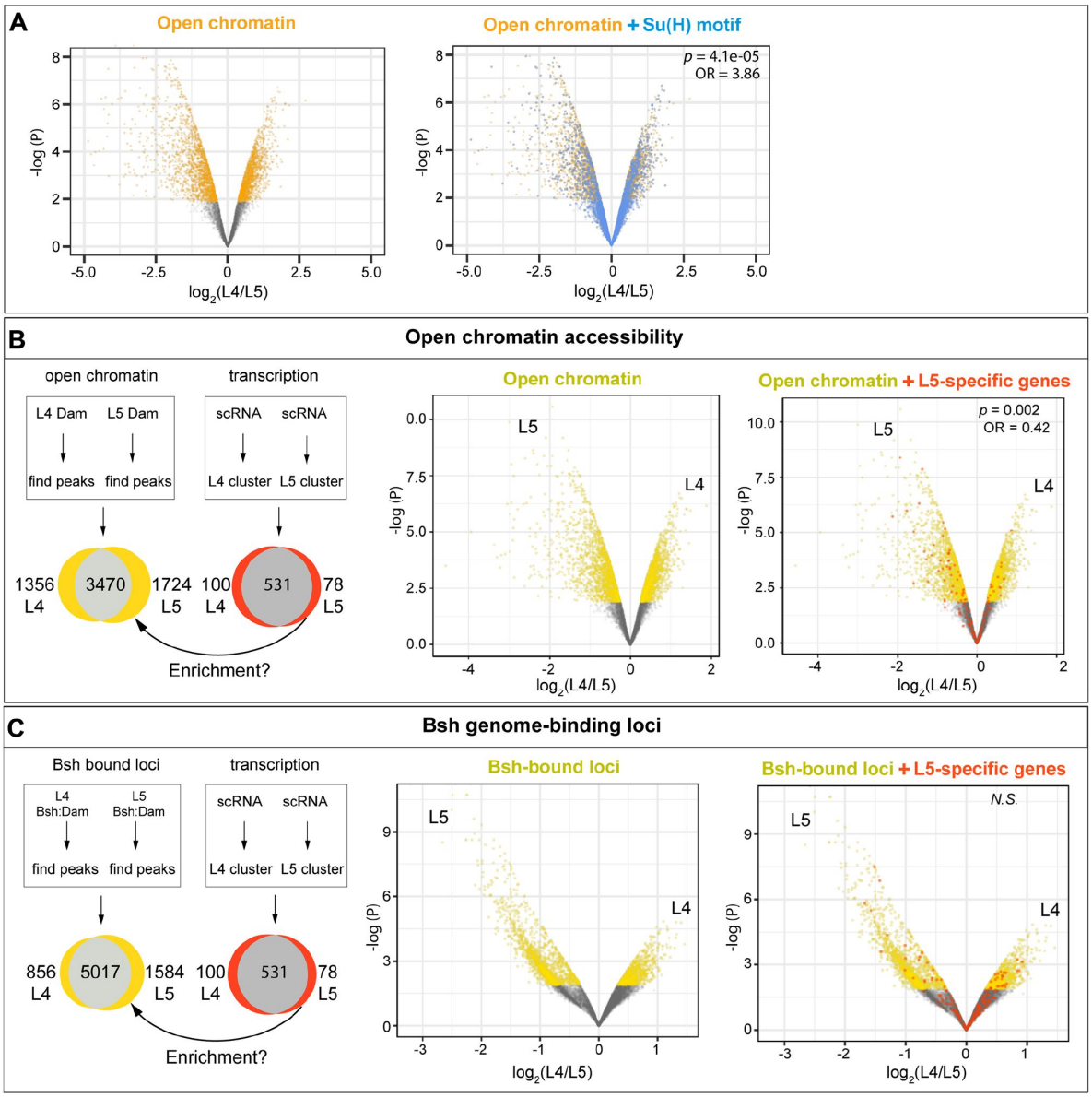

Figure 6—figure supplement 2.

L4-disinct open chromatin regions are more likely to contain the Su(H) binding motif; L5-specific transcribed genes are correlated with L5-distinct open chromatin regions but not L5-distinct Bsh-bound loci.

(A) L4 and L5 neurons show distinct open chromatin regions (yellow), and the Su(H) binding motif (blue) is more likely to be present in L4-distinct open chromatin regions.

(B) L4 and L5 neurons show distinct open chromatin regions (yellow), and L5-specific transcribed genes (red) are enriched in L5-distinct open chromatin regions.

(C) L4 and L5 neurons show distinct Bsh-bound loci (yellow); L5-specific transcribed genes (red) and L5-distinct Bsh-bound loci do not show correlation. *P* values from Fisher's exact test; odds ratios (OR) expressed as L4/L5 in all cases.

Materials and Methods

Key resources table

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
strain, strain background (*D. melanogaster*)	10xUAS-IVS-myristoylated-GFP	Bloomington Drosophila Stock Center	RRID: BDSC_32199	w[1118]; P{y[+t7.7] w[+mC]=10XUAS-IVS-myr::GFP}su(Hw)attP5
strain, strain background (*D. melanogaster*)	*R27G05*GAL4	Bloomington Drosophila Stock Center	RRID: BDSC_48073	w[1118]; P{y[+t7.7] w[+mC]=GMR27G05-GAL4}attP2
strain, strain background (*D. melanogaster*)	UAS-Bsh-RNAi	Bloomington Drosophila Stock Center	RRID: BDSC_29336	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02498}attP2

strain, strain background (*D. melanogaster*)	yw; UAS-mCD8-GFP, UAS-rCD2i, FRT40A/CyO; TM3, Sb/TM6B, Tb	Bloomington Drosophila Stock Center	RRID: BDSC_56185	y[1] w[*]; P{y[+t7.7] w[+mC]=UAS-mCD8.GFP.UAS-rCD2i}attP40 P{ry[+t7.2]=neoFRT}40A/CyO; TM3, Sb[1]/TM6B, Tb[1]
strain, strain background (*D. melanogaster*)	yw, hsFlp122; frt40a, UAS-CD2-RFP, UAS-GFP-miRNA	Gift from Claude Desplan Lab		yw, hsFLP; uas-CD2::RFP, UAS-GFP-miRNA/cyo; tm2/tm6b,tb
strain, strain background (*D. melanogaster*)	UAS-Notch-RNAi	Bloomington Drosophila Stock Center	RRID: BDSC_33611	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00001}attP2
strain, strain background (*D. melanogaster*)	UAS-Notch-ICD	(Struhl and Greenwald, 2001)		;UAS-Notch-ICD; +/+
strain, strain background (*D. melanogaster*)	Hey-ORF-T2A-FlpD5	(Mark et al., 2021)		w; Hey-ORF-T2A-FLP(RFP+)/cyo, wg-LacZ; +/+
strain, strain background (*D. melanogaster*)	UAS-FRT-STOP-FRT-myrGFP	Bloomington Drosophila Stock Center	RRID: BDSC_55810	w[1118]; P{y[+t7.7] w[+mC]=10XUAS(FRT.stop)GFP.Myr}su(Hw)attP5

strain, strain background (*D. melanogaster*)	w; Brp-FRT-STOP-FRT-smGdP-v5-T2A-LexA; LexAop-Tomato	(Peng et al., 2018; Xu et al., 2019)		w; Brp-FRT-STOP-FRT-smGdP-v5-T2A-LexA/cyo; LexAop-myrtdTomato/tm6b
strain, strain background (*D. melanogaster*)	UAS-Svp-RNAi	Bloomington Drosophila Stock Center	RRID: BDSC_28689	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03105}attP2
strain, strain background (*D. melanogaster*)	*6-60-Gal4*	Gift from Lawrence Zipursky		W; Bl/cyo; 6-60-Gal4/tm6b
strain, strain background (*D. melanogaster*)	Bsh-L-Gal4	Gift from Makoto Sato		;Bsh-L-Gal4/cyo; +/+
strain, strain background (*D. melanogaster*)	tubP-GAL80[ts]	Bloomington Drosophila Stock Center	RRID: BDSC_7017	w[*]; P{w[+mC]=tubP-GAL80[ts]}2/TM2
strain, strain background (*D. melanogaster*)	UAS-Zfh1-RNAi	Vienna Drosophila Resource Center	VDRC 103205	P{KK109931}VIE-260B
strain, strain backgroun	31C06-Gal4, UAS-myristoylat	Gift from Lawrence Zipursky		;Bl/cyo; 31c06-Gal4, UAS-

d (*D. melanogaster*)	ed-tdTomato			myristoylated-tdTomato/tm6b
strain, strain backgroun d (*D. melanogaster*)	E(spl)-mγ-GFP	Gift from Sarah Bray (Almeida and Bray, 2005)		w; +/+; E(spl)mγ-GFP/tm6b
strain, strain backgroun d (*D. melanogaster*)	VALIUM20-mCherry	Bloomington Drosophila Stock Center	RRID: BDSC_35785	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=VALIUM20-mCherry}attP2
strain, strain backgroun d (*D. melanogaster*)	Bsh-ORF-3XHA (86Fb)	FlyORF Webshop	Cat#F000054	M{UAS-bsh.ORF.3xHA.GW}ZH-86Fb
strain, strain backgroun d (*D. melanogaster*)	flyORF-TaDa	Bloomington Drosophila Stock Center	RRID: BDSC_91637	w[1118]; M{RFP[3xP3.PB] w[+mC]=FlyORF-TaDa}ZH-86Fb
strain, strain backgroun d (*D. melanogaster*)	hs-FlpD5; FlyORF-TaDa	Bloomington Drosophila Stock Center	RRID: BDSC_91638	w[1118]; P{y[+t7.7] w[+mC]=hs-FLPD5}attP40; M{RFP[3xP3.PB] w[+mC]=FlyORF-TaDa}ZH-86Fb
strain, strain backgroun d (*D. melanogaster*)	Bsh-TaDa	(Xu et al., 2023)		w; +/CyO; UAS-GFP-Bsh-DAM/tm6b

Antibody	Chicken polyclonal	Abcam	Cat#ab13970, RRID_300798	Anti-GFP (1:1000)
Antibody	Rabbit polyclonal	Medical & Biological Laboratories Co.	Code#PM005	Anti-RFP (1:500)
Antibody	Rabbit polyclonal	Gift from Claude Desplan (Özel et al., 2021)		Anti-Bsh (1:1000)
Antibody	Guinea pig polyclonal	Gift from Lawrence Zipursky (Tan et al., 2015) & Makoto Soto (Hasegawa et al., 2011)		Anti-Bsh (1:1000)
Antibody	Rat monoclonal	This study		Anti-Bsh (1:750); see methods section- generating Bsh antibody
Antibody	Rabbit polyclonal	Gift from Markus Affolter (Bieli et al., 2015)		Anti-Apterous (1:1000)
Antibody	Rat monoclonal	Gift from Cheng-Ting Chien (Chen et al., 2012)		Anti-Pdm3 (1:200)
Antibody	Rabbit polyclonal	Gift from Cheng-Yu Lee (Janssens et al., 2014)		Anti-Erm (1:100)

Antibody	Rat monoclonal	Gift from Jing Peng (Santiago et al., 2021)		Anti-Erm (1:70)
Antibody	Mouse monoclonal	Developmental Studies Hybridoma Bank	Cat#Seven-up D2D3, RRID_2618079	Anti-Svp (1:10)
Antibody	Rabbit polyclonal	Gift from James Skeath (Tian et al., 2004)		Anti-Zfh1 (1:1000)
Antibody	Rabbit polyclonal	Asian Distribution Center for Segmentation Antibodies	Code#812	Anti-Tailless (1:200)
Antibody	Mouse monoclonal	Developmental Studies Hybridoma Bank	Cat#Elav-9F8A9, RRID: AB_528217	Anti-Elav (1:200)
Antibody	Mouse monoclonal	Bio-Rad Laboratories	Cat#MCA1360A647, RRID: AB_770156	Anti-V5-TAG:Alexa Fluor® 647 (1:300)
Antibody	Mouse monoclonal	Developmental Studies Hybridoma Bank	Cat#nc-82, RRID: AB_2314866	Anti-Brp (1:50)
Antibody	Mouse monoclonal	Developmental Studies Hybridoma Bank	Cat#C594.9B, RRID: AB_528194	Anti-Delta (1:10)
Antibody	Mouse monoclonal	TaKaRa	Cat#632543	Anti-Cherry (1:500)

Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat# 711-475-152 AB_2340616	DyLight 405 anti-rabbit (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#703-545-155, RRID: AB_2340375	Alexa Fluor 488 anti-chicken (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#706-545-148, RRID: AB_2340472	Alexa Fluor 488 anti-guinea pig (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#711-545-152, RRID: AB_2313584	Alexa Fluor 488 anti-rabbit (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#715-545-150, RRID: AB_2340846	Alexa Fluor 488 anti-mouse (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#715-295-151, RRID: AB_2340832	Rhodamine Red-X anti-mouse (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#712-295-153, RRID: AB_2340676	Rhodamine Red-X anti-rat (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#711-295-152, RRID: AB_2340613	Rhodamine Red-X anti-rabbit (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#706-295-148, RRID: AB_2340468	Rhodamine Red-X donkey anti-guinea pig (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#711-605-152, RRID: AB_2492288	Alexa Fluor 647 donkey anti-rabbit (1:400)

Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#715-605-151, RRID: AB_2340863	Alexa Fluor 647 donkey anti-mouse (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#706-605-148, RRID: AB_2340476	Alexa Fluor 647 anti-guinea pig (1:400)
Antibody	Donkey polyclonal	Jackson ImmunoResearch Lab	Cat#712-605-153, RRID: AB_2340694	Alexa Fluor 647 anti-rat (1:400)
sequence-based reagent	Oligonucleotide	Integrated DNA technologies		DamID Adaptor (top strand): CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA
sequence-based reagent	Oligonucleotide	Integrated DNA technologies		DamID Adaptor (bottom strand): TCCTCGGCCG
sequence-based reagent	Oligonucleotide	Integrated DNA technologies		DamID Primer for PCR: GGTCGCGGCCGAGGATC
commercial assay or kit	QIAamp DNA Micro Kit	Qiagen	Cat#56304	
commercial assay or kit	PCR Purification Kit	Qiagen	Cat#28104	
chemical compound, drug	EDTA	Sigma-Aldrich	Cat#E6758	

chemical compound, drug	DpnI and CutSmart buffer	NEB	Cat#R0176S	
chemical compound, drug	DpnII and DpnII buffer	NEB	Cat#R0543S	
chemical compound, drug	MyTaq HS DNA Polymerase	Bioline	Cat#BIO-21112	
chemical compound, drug	AlwI	NEB	Cat#R0513S	
chemical compound, drug	RNase A (DNase free)	Roche	Cat#11119915001	
chemical compound, drug	T4 DNA ligase and 10x buffer	NEB	Cat#M0202S	
software, algorithm	Fiji	(Schindelin et al., 2012)	https://imagej.nih.gov/ij/download.html	
software, algorithm	FastQC (v0.11.9)	The Babraham Bioinformatics group	https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc	
software, algorithm	damidseq_pipeline	(Marshall and Brand, 2015)	https://owenjm.github.io/damidseq_pipeline/	
software, algorithm	Bowtie2 (v2.4.5)	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml	

software, algorithm	IGV (v.2.13.2)	(Robinson et al., 2011)	https://software.broadinstitute.org/software/igv/download	
software, algorithm	SAMtools (v1.15.1)	(Li et al., 2009)	http://www.htslib.org/download/	
software, algorithm	deepTools (v3.5.1)	(Ramírez et al., 2016)	https://deeptools.readthedocs.io/en/develop/content/installation.html	
software, algorithm	Find_peaks	(Marshall et al., 2016)	https://github.com/owenjm/find_peaks	

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Chundi Xu (cxu3@uoregon.edu) or Chris Doe (cdoe@uoregon.edu).

Experimental model and subject details

All flies were reared at 25°C on standard cornmeal fly food, unless otherwise stated.

Method details

Animal collections

For all RNAi knockdown experiments without tubP-GAL80[ts], crosses were kept at 25°C and the progeny were kept at 27.5°C with 16:8 hours light-dark cycle from the embryo stage until dissection.

For the experiment R27G05GAL4>UAS-Notch-RNAi, tubP-GAL80[ts], the crosses were kept at 18°C and the progeny were moved to 29.2°C after six days in 18°C. Pupae at 0h APF were staged at 29.2°C and then dissected after 15 hours at 29.2°C. Pupae 3-4d APF at 25°C were dissected once they turned dark.

For the experiment 27G05GAL4>UAS-N-ICD, tubP-GAL80[ts], the crosses were kept at 18°C and the progeny were moved to 29.2°C at 0hAPF. Pupae at 19h APF or 3.75d APF were dissected.

For the experiment Bsh-Gal4>UAS-N-ICD, the crosses were kept in 25°C and the progeny were moved to 29.2°C at early L3 stage for 15 hours (19 hours APF at 25°C) or until dark (3-4d APF at 25°C).

For twin-spot MARCM, the crosses (virgin: hsFlp122; frt40a, UAS-CD2-RFP, UAS-GFP-miRNA; Tm2/tm6b; male: w; frt40a, UAS-CD8-GFP, UAS-CD2-miRNA; 27g05Gal4/tm6b) were kept in 25°C.

Progeny at 0h APF were heat shocked at 34°C for 2 minutes and 50 seconds and dissected 24 hours after heat shock.

Generating Bsh antibody

Bsh protein and antibody were made by GenScript (Piscataway, NJ) in guinea pigs, and immunized with the following portion of the Bsh open reading frame:

MHHHHHHAMLNEASLSPADAHAHANATTPTHSKAAAMASATTMLTTKTPFSIE
HILFQNLNSASNNNNSSDTNGIAANTNNYAPKSSRNAVKSARSAFAHDNNPHKH
PSQHSHPPQSHPPASASASATATARSNQAASGYAGEDYGKSMHSTPRSNHHSRH
GTSHYNGDQISQQLGSGAAQHPPVPTTQPQPPPPPPLNGGSGASNGVLYPNAPYT
DHGFLQMTLGYLSPSSGTYKSVDPYFLSQASLFGGAPFFGAPGCVPELALGLGM
GVNALRHCRRRKARTVFSDPQLSGLEKRFEGQRYLSTPERVELATALGLSETQV
KTWFQNRRMKHKKQLRRRDNANEPVDFSRSEPGKQPGEATSSSGDSKHGKLNP
GSVGGTPTQPTSEQQLQMCLMQQGYSTDDYSDLEADSGDEDNSSDVDIVGDAK LYQLT.

Immunohistochemistry

Fly brains were dissected in Schneider's medium and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 25 min. After fixation, brains were quickly washed with PBS with 0.5% Triton X-100 (PBT) and incubated in PBT for at least 2 hr at room temperature. Next, samples were incubated in blocking buffer (10% normal donkey serum, 0.5% Triton X-100 in PBS) overnight at 4°C. Brains were then incubated in primary antibody (diluted in blocking buffer) at 4°C for at least two nights. Following primary antibody incubation, brains were washed with PBT. Next, brains were incubated in secondary antibody (diluted in blocking buffer) at 4°C for at least one day. Following secondary antibody incubation, brains were washed with PBT and mounted in SlowFade Gold antifade reagent (Thermo Fisher Scientific, Waltham, MA). Images were acquired using a Zeiss 800 confocal and processed with Image J, Adobe Illustrator (San Jose, CA) and Adobe Photoshop (San Jose, CA).

TaDa in L4 and L5 neurons at the time of synapse formation

For TaDa in L4, homozygous tubP-GAL80[ts]; 31C06-Gal4, UAS-myristoylated-tdTomato males were crossed to homozygous virgin females (FlyORF-TaDa line for Dam; Bsh-TaDa line for Bsh:Dam). For TaDa in L5, homozygous tubP-GAL80[ts]; 6-60-Gal4 UAS-myristoylated-tdTomato males were crossed to homozygous virgin females (FlyORF-TaDa line for Dam; Bsh-TaDa line for Bsh:Dam). Crosses were reared at 18°C. To perform TaDa in L4 and L5 neurons during the synapse formation window, we collected pupae with the age of 46h APF and moved them to 29°C to activate 31C06-Gal4 (L4) or 6-60-Gal4 (L5) for 24 hours (**Figure 6**—figure supplement 1). Then lamina were dissected (age equivalent at 25°C: 76h APF) in cold PBS within one hour and stored at -20°C immediately until sufficient laminae were collected for each group—about 70 lamina from 35 pupae. The published (Marshall et al., 2016) TaDa experimental pipeline was followed with a few modifications. Briefly, DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Germantown, MD) digested with DpnI (NEB, Ipswich, MA) overnight, and cleaned up using Qiagen PCR purification columns. DamID adaptors were ligated using T4 DNA ligase (NEB, Ipswich, MA) followed by DpnII (NEB, Ipswich, MA) digestion for 2 h and PCR amplification using MyTaq HS DNA polymerase (Bioline, Memphis, Tennessee). The samples were sequenced on the NovaSeq at 118 base pairs and 27-33 million single end reads per sample.

Bioinformatic analysis

DamID-seq NGS sequencing reads were processed using damidseq_pipeline v1.5.3 (Marshall and Brand, 2015). For generating Bsh binding profile bedGraphs, the default settings were used. For generating Dam-alone coverage bedGraphs, the pipeline was run on the Dam-alone sequencing

files with the --just_coverage setting. For both datasets (Bsh binding and Dam-alone), peaks were identified using find_peaks (Marshall et al., 2016) on separate replicates.

All downstream analyses were conducted using R (Team, 2016). Significant peaks from all replicates were combined using the GenomicRanges R package (Lawrence et al., 2013), and the average occupancy over each significant peak was determined for each replicate. As the overall Bsh binding profiles between L4 and L5 neurons were highly correlated, the binding profiles of all replicates were quantile normalized prior to further analysis.

Differentially-open regions in the Dam-alone dataset, and differentially-bound regions in the Bsh binding dataset, were identified using NOIseq (Tarazona et al., 2015) as previously described (Hatch et al., 2021) at a threshold of q=0.85. Separately, peaks were assigned to genes, with all genes within 1kb of a peak assigned as putative regulatory targets of that peak.

Tests for enrichment were conducted using Fisher's exact test, with contingency tables derived from differentially open or bound regions and the overlap of these with lineage-specific gene expression targets. The alternative hypothesis was that Bsh-bound targets or open chromatin would be enriched for genes expressed in the same lineage. Odds ratios were expressed as L4/L5 for all samples.

Motif searches were conducted using the core YGTGRGAAM motif, obtained as a PWM from CisBP (Weirauch et al., 2014) (Motif ID: M10415_2.00), using the R Biostrings package (Pagès et al., 2022). For motif searches, open chromatin regions were limited to a maximum size of 1kb around the center of the region. Motifs with a match score >= 85% were counted as a match.

Statistical Analysis

Statistics were performed using either Microsoft Excel or Prism (GraphPad, San Diego, California) software. Unpaired t-test was used, unless otherwise noted. Data are presented as mean ± SEM unless otherwise noted. A 95% confidence interval was used to define the level of significance. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$, ns=not significant. All other relevant statistical information can be found in the figure legends.

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Reviewer #1 (Public Review):

Like the "preceding" co-submitted paper, this is again a very strong and interesting paper in which the authors address a question that is raised by the finding in their co-submitted paper - how does one factor induce two different fates. The authors provide an extremely satisfying answer - only one subset of the cells neighbors a source of signaling cells that trigger that subset to adopt a specific fate. The signal here is Delta and the read-out is Notch, whose intracellular domain, in conjunction with, presumably, SuH cooperates with Bsh to distinguish L4 from L5 fate (L5 is not neighbored by signal-providing cells). Like the back-to-back paper, the data is rigorous, well-presented and presents important conclusions. There's a wealth of data on the different functions of Notch (with and without Bsh). All very satisfying.

I have again one suggestion that the authors may want to consider discussing. I'm wondering whether the open chromatin that the author convincingly measure is the CAUSE or the CONSEQUENCE of Bsh being able to activate L4 target genes. What I mean by this is that currently the authors seem to be focused on a somewhat sequential model where Notch signaling opens chromatin and this then enables Bsh to activate a specific set of target genes. But isn't it equally possible that the combined activity of Bsh/Notch(intra)/SuH opens chromatin? That's not a semantic/minor difference, it's a fundamentally different mechanism, I would think. This mechanism also solves the conundrum of specificity - how does Notch know which genes to "open" up? It would seem more intuitive to me to think that it's working together with Bsh to open up chromatin, with chromatin accessibility than being a "mere" secondary consequence. If I'm not overlooking something fundamental here, there is actually also a way to distinguish between these models - test chromatin accessibility in a Bsh mutant. If the author's model is true, chromatin accessibility should be unchanged.
I again finish by commending the authors for this terrific piece of work.

Reviewer #2 (Public Review):

Summary:

In this work, the authors explore how Notch activity acts together with Bsh homeodomain transcription factors to establish L4 and L5 fates in the lamina of the visual system of Drosophila. They propose a model in which differential Notch activity generates different chromatin landscapes in presumptive L4 and L5, allowing the differential binding of the primary homeodomain TF Bsh (as described in the co-submitted paper), which in turn activates downstream genes specific to either neuronal type. The requirement of Notch for L4 vs. L5 fate is well supported, and complete transformation from one cell type into the other is observed when altering Notch activity. However, the role of Notch in creating differential chromatin landscapes is not directly demonstrated. It is only based on correlation, but it remains a plausible and intriguing hypothesis.

Strengths:

The authors are successful in characterizing the role of Notch to distinguish between L4 and L5 cell fates. They show that the Notch pathway is active in L4 but not in L5. They identify L1, the neuron adjacent to L4 as expressing the Delta ligand, therefore being the potential source for Notch activation in L4. Moreover, the manuscript shows molecular and morphological/connectivity transformations from one cell type into the other when Notch activity is manipulated.

Using DamID, the authors characterize the chromatin landscape of L4 and L5 neurons. They show that Bsh occupies distinct loci in each cell type. This supports their model that Bsh acts as a primary selector gene in L4/L5 that activates different target genes in L4 vs L5 based on the differential availability of open chromatin loci.

Overall, the manuscript presents an interesting example of how Notch activity cooperates with TF expression to generate diverging cell fates. Together with the accompanying paper, it helps thoroughly describe how lamina cell types L4 and L5 are specified and provides an interesting hypothesis for the role of Notch and Bsh in increasing neuronal diversity in the lamina during evolution.

Weaknesses:

Differential Notch activity in L4 and L5:
● The manuscript focuses its attention on describing Notch activity in L4 vs L5 neurons. However, from the data presented, it is very likely that the pool of progenitors (LPCs) is already subdivided into at least two types of progenitors that will rise to L4 and L5,

respectively. Evidence to support this is the activity of E(spl)-mγ-GFP and the Dl puncta observed in the LPC region. Discussion should naturally follow that Notch-induced differences in L4/L5 might preexist L1-expressed Dl that affect newborn L4/L5. Therefore, the differences between L4 and L5 fates might be established earlier than discussed in the paper. The authors should acknowledge this possibility and discuss it in their model.
• The authors claim that Notch activation is caused by L1-expressed Delta. However, they use an LPC driver to knock down Dl. Dl-KD should be performed exclusively in L1, and the fate of L4 should be assessed.
• To test whether L4 neurons are derived from NotchON LPCs, I suggest performing MARCM clones in early pupa with an E(spl)-mγ-GFP reporter.
• The expression of different Notch targets in LPCs and L4 neurons may be further explored. I suggest using different Notch-activity reporters (i.e., E(spl)-GFP reporters) to further characterize these differences. What cause the switch in Notch target expression from LPCs to L4 neurons should be a topic of discussion.

Notch role in establishing L4 vs L5 fates:
• The authors describe that 27G05-Gal4 causes a partial Notch Gain of Function caused by its genomic location between Notch target genes. However, this is not further elaborated. The use of this driver is especially problematic when performing Notch KD, as many of the resulting neurons express Ap, and therefore have some features of L4 neurons. Therefore, Pdm3+/Ap+ cells should always be counted as intermediate L4/L5 fate (i.e., Fig3 E-J, Fig3-Sup2), irrespective of what the mechanistic explanation for Ap activation might be. It's not accurate to assume their L5 identity. In Fig4 intermediate-fate cells are correctly counted as such.
• Lines 170-173: The temporal requirement for Notch activity in L5-to-L4 transformation is not clearly delineated. In Fig4-figure supplement 1D-E, it is not stated if the shift to 29{degree sign}C is performed as in Fig4-figure supplement 1A-C.
• Additionally, using the same approach, it would be interesting to explore the window of competence for Notch-induced L5-to-L4 transformation: at which point in L5 maturation can fate no longer be changed by Notch GoF?

L4-to-L3 conversion in the absence of Bsh
• Although interesting, the L4-to-L3 conversion in the absence of Bsh is never shown to be dependent on Notch activity. Importantly, L3 NotchON status is assumed based on their position next to Dl-expressing L1, but it is not empirically tested. Perhaps screening Notch target reporter expression in the lamina, as suggested above, could inform this issue.
• Otherwise, the analysis of Bsh Loss of Function in L4 might be better suited to be included in the accompanying manuscript that specifically deals with the role of Bsh as a selector gene for L4 and L5.

Different chromatin landscape in L4 and L5 neurons
• A major concern is that, although L4 and L5 neurons are shown to present different chromatin landscapes (as expected for different neuronal types), it is not demonstrated that this is caused by Notch activity. The paper proves unambiguously that Notch activity, in concert with Bsh, causes the fate choice between L4 and L5. However, that this is caused by Notch creating a differential chromatin landscape is based only in correlation (NotchON cells having a different profile than NotchOFF). Although the authors are careful not to claim that differential chromatin opening is caused directly by Notch, this is heavily suggested throughout the text and must be toned down.
e.g.: Line 294: "With Notch signaling, L4 neurons generate distinct open chromatin landscape" and Line 298: "Our findings propose a model that the unique combination of HDTF and open chromatin landscape (e.g. by Notch signaling)" . These claims are not supported well enough, and alternative hypotheses should be provided in the discussion. An alternative hypothesis could be that LPCs are already specified towards L4 and L5 fates. In this context, different

early Bsh targets in each cell type could play a pioneer role generating a differential chromatin landscape.

● The correlation between open chromatin and Bsh loci with Differentially Expressed genes is much higher for L4 than L5. It is not clear why this is the case, and should be discussed further by the authors.

Author Response

> ### *Reviewer #1 (Public Review):*
>
> *Like the "preceding" co-submitted paper, this is again a very strong and interesting paper in which the authors address a question that is raised by the finding in their co-submitted paper - how does one factor induce two different fates. The authors provide an extremely satisfying answer - only one subset of the cells neighbors a source of signaling cells that trigger that subset to adopt a specific fate. The signal here is Delta and the read-out is Notch, whose intracellular domain, in conjunction with, presumably, SuH cooperates with Bsh to distinguish L4 from L5 fate (L5 is not neighbored by signal-providing cells). Like the back-to-back paper, the data is rigorous, well-presented and presents important conclusions. There's a wealth of data on the different functions of Notch (with and without Bsh). All very satisfying.*

Thanks!

> *I have again one suggestion that the authors may want to consider discussing. I'm wondering whether the open chromatin that the author convincingly measure is the CAUSE or the CONSEQUENCE of Bsh being able to activate L4 target genes. What I mean by this is that currently the authors seem to be focused on a somewhat sequential model where Notch signaling opens chromatin and this then enables Bsh to activate a specific set of target genes. But isn't it equally possible that the combined activity of Bsh/Notch(intra)/SuH opens chromatin? That's not a semantic/minor difference, it's a fundamentally different mechanism, I would think. This mechanism also solves the conundrum of specificity - how does Notch know which genes to "open" up? It would seem more intuitive to me to think that it's working together with Bsh to open up chromatin, with chromatin accessibility than being a "mere" secondary consequence. If I'm not overlooking something fundamental here, there is actually also a way to distinguish between these models - test chromatin accessibility in a Bsh mutant. If the author's model is true, chromatin accessibility should be unchanged.*
>
> *I again finish by commending the authors for this terrific piece of work.*

Thanks! It is a crucial question whether Notch signaling regulates chromatin landscape independently of a primary HDTF. We will include this discussion in the text and pursue it in our next project. We think Notch signaling may regulate chromatin accessibility independently of a primary HDTF based on our observation: in larval ventral nerve cord, all motor neurons are NotchON neurons while all sensory neurons are NotchOFF neurons; NotchON neurons share similar functional properties, despite expressing distinct HDTFs, possibly due to the common chromatin landscape regulated by Notch signaling.

Reviewer #2 (Public Review):

Summary:

In this work, the authors explore how Notch activity acts together with Bsh homeodomain transcription factors to establish L4 and L5 fates in the lamina of the visual system of Drosophila. They propose a model in which differential Notch activity generates different chromatin landscapes in presumptive L4 and L5, allowing the differential binding of the primary homeodomain TF Bsh (as described in the co-submitted paper), which in turn activates downstream genes specific to either neuronal type. The requirement of Notch for L4 vs. L5 fate is well supported, and complete transformation from one cell type into the other is observed when altering Notch activity. However, the role of Notch in creating differential chromatin landscapes is not directly demonstrated. It is only based on correlation, but it remains a plausible and intriguing hypothesis.

Thanks for the positive feedback!

Strengths:

The authors are successful in characterizing the role of Notch to distinguish between L4 and L5 cell fates. They show that the Notch pathway is active in L4 but not in L5. They identify L1, the neuron adjacent to L4 as expressing the Delta ligand, therefore being the potential source for Notch activation in L4. Moreover, the manuscript shows molecular and morphological/connectivity transformations from one cell type into the other when Notch activity is manipulated.

Thanks!

Using DamID, the authors characterize the chromatin landscape of L4 and L5 neurons. They show that Bsh occupies distinct loci in each cell type. This supports their model that Bsh acts as a primary selector gene in L4/L5 that activates different target genes in L4 vs L5 based on the differential availability of open chromatin loci.

Thanks!

Overall, the manuscript presents an interesting example of how Notch activity cooperates with TF expression to generate diverging cell fates. Together with the accompanying paper, it helps thoroughly describe how lamina cell types L4 and L5 are specified and provides an interesting hypothesis for the role of Notch and Bsh in increasing neuronal diversity in the lamina during evolution.

Thanks for the positive feedback on both manuscripts.

Weaknesses:

Differential Notch activity in L4 and L5:

● The manuscript focuses its attention on describing Notch activity in L4 vs L5 neurons. However, from the data presented, it is very likely that the pool of progenitors (LPCs) is already subdivided into at least two types of progenitors that will rise to L4 and L5, respectively. Evidence to support this is the activity of E(spl)-mγ-GFP and the Dl puncta observed in the LPC region. Discussion should naturally follow that Notch-induced differences in L4/L5 might preexist L1-expressed Dl that affect newborn L4/L5. Therefore,

> *the differences between L4 and L5 fates might be established earlier than discussed in the paper. The authors should acknowledge this possibility and discuss it in their model.*

We agree. Historically, LPCs are thought to be homogenous; our data suggests otherwise. We now emphasize this in the Discussion as requested. We are also investigating this question using single cell RNAseq on LPCs to look for molecular heterogeneities. Thanks for the great comment!

> ● *The authors claim that Notch activation is caused by L1-expressed Delta. However, they use an LPC driver to knock down Dl. Dl-KD should be performed exclusively in L1, and the fate of L4 should be assessed.*

Dl is transiently expressed in newborn L1 neurons. To knock down Dl in L1, we need to express Dl-RNAi before Dl protein is expressed in newborn L1; the only known Gal4 line expressed that early is the LPC-Gal4 that we used. There is no L1-gal4 line expressed early enough to eliminate L1 expression of Dl.

> ● *To test whether L4 neurons are derived from NotchON LPCs, I suggest performing MARCM clones in early pupa with an E(spl)-mγ-GFP reporter.*

We agree! Whether L4 neurons are derived from NotchON LPCs is a great question. However, MARCM clones in early pupa with an E(spl)-mγ-GFP reporter will not work because E(spl)-mγ-GFP reporter is only expressed in LPCs but not lamina neurons. We now mention this in the Discussion.

> ● *The expression of different Notch targets in LPCs and L4 neurons may be further explored. I suggest using different Notch-activity reporters (i.e., E(spl)-GFP reporters) to further characterize these. differences. What cause the switch in Notch target expression from LPCs to L4 neurons should be a topic of discussion.*

Thanks! It is a great question why Notch induces Espl-mγ in LPCs but Hey in new-born neurons. However, it is not the question we are tackling in this paper and it will be a great direction to pursue in future. We will add this to our Discussion.

> *Notch role in establishing L4 vs L5 fates:*
>
> ● *The authors describe that 27G05-Gal4 causes a partial Notch Gain of Function caused by its genomic location between Notch target genes. However, this is not further elaborated. The use of this driver is especially problematic when performing Notch KD, as many of the resulting neurons express Ap, and therefore have some features of L4 neurons. Therefore, Pdm3+/Ap+ cells should always be counted as intermediate L4/L5 fate (i.e., Fig3 E-J, Fig3-Sup2), irrespective of what the mechanistic explanation for Ap activation might be. It's not accurate to assume their L5 identity. In Fig4 intermediate-fate cells are correctly counted as such.*

Thanks for the comment! We will annotate Pdm3/Ap+ as L4/L5 fate in the corresponding figures.

> ● *Lines 170-173: The temporal requirement for Notch activity in L5-to-L4 transformation is not clearly delineated. In Fig4-figure supplement 1D-E, it is not stated if the shift to 29{degree sign}C is performed as in Fig4-figure supplement 1A-C.*

Thank you for catching this. We will correct it in the text.

> *● Additionally, using the same approach, it would be interesting to explore the window of competence for Notch-induced L5-to-L4 transformation: at which point in L5 maturation can fate no longer be changed by Notch GoF?*

Our data show that Bsh with Notch signaling in newborn neurons specifies L4 fate while Bsh without Notch signaling in newborn neurons specifies L5 fate. Therefore, we think the window of fate competence is during newborn neurons. We will include the data to support this.

> *L4-to-L3 conversion in the absence of Bsh*
>
> *● Although interesting, the L4-to-L3 conversion in the absence of Bsh is never shown to be dependent on Notch activity. Importantly, L3 NotchON status is assumed based on their position next to Dl-expressing L1, but it is not empirically tested. Perhaps screening Notch target reporter expression in the lamina, as suggested above, could inform this issue.*

Our data show that the L4-to-L3 conversion in the absence of Bsh and in the presence of Notch activity while the L5-to-L1 conversion in the absence of Bsh and in the absence of Notch activity. Therefore, Notch activity is necessary for the L4-to-L3 conversion. Unfortunately, currently we only have Hey as an available Notch target reporter in new-born neurons. To tackle this challenge in the future, we will profile the genome-binding targets of endogenous Notch in newborn neurons. This will identify novel genes as Notch signaling reporters in neurons for the field.

> *● Otherwise, the analysis of Bsh Loss of Function in L4 might be better suited to be included in the accompanying manuscript that specifically deals with the role of Bsh as a selector gene for L4 and L5.*

That is an interesting suggestion, but without knowing that Bsh + Notch = L4 identity the experiment would be hard to interpret. Note that we took advantage of Notch signaling to trace the cell fate in the absence of Bsh and found the L4-to-L3 conversion (see Figure 5G-K).

> *Different chromatin landscape in L4 and L5 neurons*
>
> *● A major concern is that, although L4 and L5 neurons are shown to present different chromatin landscapes (as expected for different neuronal types), it is not demonstrated that this is caused by Notch activity. The paper proves unambiguously that Notch activity, in concert with Bsh, causes the fate choice between L4 and L5. However, that this is caused by Notch creating a differential chromatin landscape is based only in correlation. (NotchON cells having a different profile than NotchOFF). Although the authors are careful not to claim that differential chromatin opening is caused directly by Notch, this is heavily suggested throughout the text and must be toned down.e.g.: Line 294: "With Notch signaling, L4 neurons generate distinct open chromatin landscape" and Line 298: "Our findings propose a model that the unique combination of HDTF and open chromatin landscape (e.g. by Notch signaling)" . These claims are not supported well enough, and alternative hypotheses should be provided in the discussion. An alternative hypothesis could be that LPCs are already specified towards L4 and L5 fates. In this context, different early Bsh targets in each cell type could play a pioneer role generating a differential chromatin landscape.*

We agree and appreciate the comment, it is well justified. We have toned down our comments and clearly state that this is a correlation that needs to be tested for a causal relationship. Thank you for requesting it!

> • *The correlation between open chromatin and Bsh loci with Differentially Expressed genes is much higher for L4 than L5. It is not clear why this is the case, and should be discussed further by the authors.*

We agree, and think in L5 neurons, the secondary HDTF Pdm3 also contributes to L5 specific gene transcription during synaptogenesis window, in addition to Bsh. We will include this in the text.