Delta-dependent Notch activation closes the early neuroblast temporal program to promote lineage progression and neurogenesis termination in *Drosophila*

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Summary

Neuroblasts in *Drosophila* divide asymmetrically, sequentially expressing a series of intrinsic factors to generate a diversity of neuron types. These intrinsic factors known as temporal factors dictate timing of neuroblast transitions in response to steroid hormone signaling and specify early versus late temporal fates in neuroblast neuron progeny. After completing their temporal programs, neuroblasts differentiate or die, finalizing both neuron number and type within each neuroblast lineage. From a screen aimed at identifying genes required to terminate neuroblast divisions, we identified Notch and Notch pathway components. When Notch is knocked down, neuroblasts maintain early temporal factor expression longer, delay late temporal factor expression, and continue dividing into adulthood. We find that Delta, expressed in cortex glia, neuroblasts, and after division, their GMC progeny, regulates neuroblast Notch activity. We also find that Delta in neuroblasts is expressed high early, low late, and is controlled by the intrinsic temporal program: early factor Imp promotes Delta, late factors Syp/E93 reduce Delta. Thus, in addition to systemic steroid hormone cues, forward lineage progression is controlled by local cell-cell signaling between neuroblasts and their cortex glia/GMC neighbors: Delta transactivates Notch in neuroblasts bringing the early temporal program and early temporal factor expression to a close.

Introduction

In most metazoans, termination of neurogenesis is an essential part of organism development, ensuring the formation of functional neural circuits and an adult brain of proper size and structure. Prolonged or ectopic neurogenesis can lead to cortical malformations and has been linked to neurodevelopmental disorders, including autism (Hazlett et al., 2011; Marchetto et al., 2017; Patzlaff et al., 2018; Casingal et al., 2020; Ossola and Kalebic, 2022). While the vast majority of neurons are generated during development, it still remains unclear how neurogenesis becomes progressively restricted and, in most cases, ends altogether after development is completed.

We use the genetically tractable model organism, *Drosophila melanogaster*, to determine how extrinsic cues, local and systemic, integrate with neural stem cell intrinsic cues to control neurogenesis timing and termination during development. The *Drosophila* CNS consists of two bilaterally symmetric brain hemispheres and a ventral nerve cord that is

functionally equivalent to the mammalian spinal cord. Each brain hemisphere contains an optic lobe and an equally sized central brain (CB) region that harbors distinctive neuropils for information processing. Neurons in the CB region are generated during development from the asymmetric cell divisions of a defined number of neural stem cells known as neuroblasts (NBs) in *Drosophila*. NBs in the CB region (referred to as CB NBs) are specified during embryogenesis and undergo stereotypic patterns of cell division (Truman and Bate, 1988; Ito and Hotta, 1992; Doe, 2008; Siegrist et al., 2010; Homem and Knoblich, 2012). Except for the mushroom body (MB) NB subset, all CB NBs enter and exit quiescence during the embryonic to larval transition and terminally differentiate or die four to five days later during early pupal stages (Truman and Bate, 1988; Ito and Hotta, 1992; Doe, 2008; Maurange et al., 2008; Siegrist et al., 2010; Yang et al., 2017).

Once CB NBs reactivate from quiescence in response to dietary nutrients, they divide continuously while changing gene expression over time (Britton and Edgar, 1998; Chell and Brand, 2010; Sousa-Nunes et al., 2011; Liu et al., 2015; Syed et al., 2017; Yuan et al., 2020). These controlled transitions of gene expression over time, referred to as temporal patterning, allow for a restricted set of neural stem cells to generate a pool of molecularly and functionally diverse neuron types (Isshiki et al., 2001; Maurange et al., 2008; Bayraktar and Doe, 2013; Liu et al., 2015; Bahrampour et al., 2017; Ren et al., 2017; Syed et al., 2017; Miyares and Lee, 2019). Early larval temporal factors include the Zinc finger transcription factor, Castor (Cas), the orphan nuclear receptor, Seven-up (Svp), the RNA binding protein, IGF-II mRNA-binding protein (Imp), as well as others (Maurange et al., 2008; Liu et al., 2015; Ren et al., 2017; Syed et al., 2017). Svp expression primes NBs to respond to a systemic pulse of steroid hormone (ecdysone) during larval stages and switch temporal factor expression from early to late (Ren et al., 2017; Syed et al., 2017). Late temporal factors include the RNA binding protein, Syncrip (Syp), the steroid hormone-induced transcription factor, Eip93F (E93), as well as others (Liu et al., 2015; Syed et al., 2017; Pahl et al., 2019). Imp (early) and Syp (late) mutually inhibit each other and are expressed in opposing gradients in NBs (Liu et al., 2015; Yang et al., 2017). Imp keeps CB NBs "young" by inhibiting Syp and Mediator complex activity, whereas Syp inhibits Imp and promotes nuclear accumulation of the pro-differentiation transcription factor Prospero (Pros) in most CB NBs (Homem et al., 2014; Liu et al., 2015; Yang et al., 2017). During early pupal stages, CB NBs undergo reductive divisions and terminally differentiate, except for the MB NB subset, which divides several days longer and undergo autophagy/apoptosis prior to adult eclosion (Maurange et al., 2008; Siegrist et al., 2010; Homem et al., 2014; Pahl et al., 2019). Independent of neurogenesis timing and the mechanism by which CB NB stop divisions,

temporal patterning plays a key role in controlling numbers and types of neurons made within each of the NB lineages (Maurange et al., 2008; Tsuji et al., 2008; Bahrampour et al., 2017; Yang et al., 2017; Pahl et al., 2019).

From a targeted RNAi screen aimed at identifying genes required to terminate CB NB divisions and neurogenesis, we identified Notch and Notch pathway components. Notch is an evolutionarily conserved cell-cell signaling pathway classically known for regulating binary cell fate decisions, "A" versus "B" (Muskavitch, 1994; Cau and Blader, 2009). Here we show that Notch signaling also regulates binary temporal decisions, "early" versus "late". In *Drosophila*, there is one Notch receptor and two ligands, Delta (Dl) and Serrate (Ser). Notch receptor is proteolytically cleaved after ligand binding, first by Kuzbanian (Kuz), an ADAM metalloprotease, and then by γ-secretase. Cleaved Notch ICD (intracellular domain) relocates to the nucleus where it binds to Suppressor of Hairless [Su(H)] and Mastermind to regulate gene expression (Rebay et al., 1991; Fortini and Artavanis-Tsakonas, 1994; Pan and Rubin, 1997; Strooper et al., 1999; Mumm et al., 2000; Kitagawa et al., 2001; Kopan and Ilagan, 2009). We recently reported that Notch signaling regulates CB NB quiescence during the embryonic to larval transition (Sood et al., 2022). When Notch is knocked down, some CB NBs continue dividing during this transition. We also reported that Notch activity becomes attenuated in quiescent CB NBs because CB NBs are no longer dividing and producing Delta-expressing GMC daughters for Notch pathway transactivation. Moreover, low Notch is necessary for CB NBs to reactivate from quiescence in response to dietary nutrients (Sood et al., 2022).

Here we report that Notch signaling also regulates neurogenesis termination during pupal stages. When Notch is knocked down, CB NBs maintain early temporal factor expression longer resulting in a delay of late temporal factor expression with prolonged neurogenesis into late pupal stages and early adulthood. This defect in temporal patterning (switching from early to late) occurs well after CB NB exit from quiescence suggesting that Notch is required at multiple times throughout development in controlling CB NB proliferation decisions. Furthermore, we determine that Delta is the Notch ligand that activates Notch in CB NBs and reductions in Delta also lead to defects in CB NB temporal patterning. Moreover, we find that Delta in CB NBs, which is segregated to GMCs after cell division to transactivate Notch is regulated by CB NB temporal factors. Early factor Imp promotes Delta, whereas late factors Syp and E93 reduce Delta. Together, we report that Notch signaling positively regulates forward lineage progression by closing off the early temporal window and control of Notch pathway activity is regulated by CB NB intrinsic temporal factors.

Results

Notch signaling is required for CB NB elimination and termination of neurogenesis

All central brain neuroblasts (CB NBs), except the MB NB subset (four per brain hemisphere) terminally differentiate or die during early pupal stages (Fig. 1A) (Truman and Bate, 1988; Ito and Hotta, 1992; Maurange et al., 2008; Siegrist et al., 2010; Homem et al., 2014; Yang et al., 2017). MB NBs divide several days longer and undergo apoptotic/autophagic cell death shortly before adult eclosion (Fig. 1A,B) (Siegrist et al., 2010; Pahl et al., 2019). No CB NBs remain in adult animals and no new neurons are produced (Fig. 1A,B) (Truman and Bate, 1988; Siegrist et al., 2010; Yang et al., 2017). From a targeted RNAi screen aimed at identifying genes required to terminate NB divisions and neurogenesis, we identified Notch (N) and Notch pathway components. At 48 hours APF (after pupal formation), midway through pupal stages, control animals have only the four MB NBs remaining in each brain hemisphere (Fig. 1A,B). In contrast when Notch was knocked down in NBs (*worGAL4,UAS-N RNAi #HMS00001*), on average five additional CB NBs remained (Fig. 1C,D). In one-day old *N RNAi* adults, CB NBs were also present, but not the MB NBs (Fig. 1C,D). Ectopically persisting *N RNAi* CB NBs (CB NBs at 48 hours APF and beyond) expressed the NB transcription factor Deadpan (Dpn), the S-phase indicator *pcnaGFP*, and were small on average compared to control CB NBs during earlier developmental stages (L3 control, average diameter 10-15µms) (Fig. 1B,C,E). However, at 30 hours APF when control CB NBs are still present, *N RNAi* CB NBs were larger on average (Fig. 1B,C,E). To confirm the *N RNAi* phenotype, we used MARCM to generate CB NB clones mutant for the Notch loss of function allele, $Notch^{55e11}$ (Lee and Luo, 1999; Lehmann et al., 1983). Animals were heat shocked at freshly hatched larval stages and brains assayed at 48 hours APF. More than 50% of the GFP positive clones had a single Dpn positive NB (Fig. 1F). In contrast, control clones had no Dpn positive NBs. We conclude that Notch functions in a lineage-dependent manner to eliminate CB NBs and terminate neurogenesis.

Next, we assayed other Notch pathway components. Following knockdown of *kuz*, (*worGAL4,UAS-kuzRNAi #HMS05424*), CB NBs, other than the MB NBs, remained at 48 hours APF and in adults (Fig. 1G,I,J). Following *Su(H)* knockdown (*worGAL4,UAS-Su(H)RNAi #HMS05748*), CB NBs, other than the MB NBs, also remained (Fig. 1H,J). Similar to the *N RNAi* phenotype, ectopically persisting CB NBs expressed Dpn and *pcnaGFP*, and were small (Fig. 1K). We conclude that the evolutionarily conserved Notch cell signaling pathway is required for CB NB elimination and neurogenesis termination.

Delta expressed in neighboring GMCs and cortex glia regulates Notch activity in CB NBs

Next, we assayed the expression of Delta (Dl) and Serrate (Ser), two Notch ligands that activate Notch signaling when expressed on neighboring cells. Using a *Delta-GFP* protein trap line, we found that Delta was expressed in CB NBs and their recently born Prospero (Pros) positive GMC progeny during larval stages, consistent with previous reports (Fig. 2A,B) (Kooh et al., 1993; Sood et al., 2022). Delta was also expressed in cortex glia, a glial subset that ensheathe CB NBs and their GMC progeny, but levels were relatively low (Fig. 2C) (Hayashi et al., 2002; Yuan et al., 2020). Using a *Serrate-GFP* protein trap line, we found that Serrate was expressed in cortex glia, but not in CB NBs nor their GMC progeny (Fig. 2D-F). Next, we knocked down each of the Notch ligands to determine which Notch ligand from what cell type regulates Notch activity in CB NBs. We used the NB specific *E(spl)mγ-GFP* reporter to assay Notch activity (Furriols and Bray, 2001; Almeida and Bray, 2005; Zacharioudaki et al., 2012). In controls, CB NBs express *E(spl)mγ-GFP* and following knock down of Notch, *E(spl)mγ-GFP* was not expressed (Fig. 2G,H). Next, we used *worGAL4* to knock down Delta in CB NBs and because GAL4 is inherited after CB NBs divide, neighboring GMC progeny as well. Following knock down of Delta (*worGAL4,UAS-Dl RNAi #HMS01309*), *E(spl)mγ-GFP* was not detected in CB NBs (Fig. 2I). Next, we used a pan-glial GAL4 line to knock down either Delta or Serrate in cortex glia. Following knock down of Delta (*repoGAL4,UAS-Dl RNAi #HMS01309*), *E(spl)mγ-GFP* was reduced compared to controls (Fig. 2J,K). In contrast, following Serrate knock down (*repoGAL4,UAS-SerRNAi #HMS01179*), *E(spl)mγ-GFP* was not affected (Fig. 2L). We conclude that Delta is the primary Notch ligand expressed in CB NBs and their GMC progeny, while both Delta and Serrate are expressed in neighboring cortex glia. Moreover, Delta regulates CB NB Notch activity.

Delta-dependent Notch activation is required for CB NB elimination and termination of neurogenesis

Next, we knocked down each of the Notch ligands in neighboring cell types and assayed CB NB number during pupal stages. When Delta was knocked down in NBs and GMC progeny (*worGAL4,UAS-Dl RNAi #HMS01309*), ectopically persisting CB NBs were found in brains at all stages examined and in young adults (Fig. 3A-C). When Delta was knocked down in cortex glia (*NP0577GAL4,UAS-Dl RNAi #HMS01309*), one, occasionally two ectopically persisting CB NBs were found at mid pupal stages (Fig. 3E,F). Moreover, CB NBs that ectopically persisted tended to be larger than control CB NBs at early stages, consistent with the notion that cell size correlates with timing of termination (Fig. 3D,G) (Maurange et al., 2008; Siegrist et al., 2010; Homem et al., 2014; Yang et al., 2017). Next, although Serrate was not detected in CB NBs nor

their GMC progeny, we still assayed CB NB number and size after Serrate knock down in both NBs and their GMC progeny (*worGAL4,UAS-SerRNAi #HMS01179*). No differences were observed compared to controls (Fig. 3H,I). Next, we knocked down Serrate in glia (*repoGAL4,UAS-SerRNAi #HMS01179*) and again no differences were found compared to controls (Fig. 3J). We conclude that Delta, but not Serrate, is required for CB NB elimination and termination of neurogenesis.

Early Notch signaling is required to terminate neurogenesis during pupal stages

Next, we used a temperature sensitive GAL80 to determine when during development Notch signaling is required to eliminate CB NBs and terminate neurogenesis. Animals were raised at 29˚C (GAL80 inactive, GAL4 active) until 72 hours ALH and then switched to 18˚C (GAL80 active, GAL4 inactive) or the converse (Fig. 4A,B). In control animals at 48 hours APF, after either temperature shift regime, only the four MB NBs were present in each brain hemisphere (Fig. 4C and data not shown). When Notch or Delta knock down animals were raised at 29˚C (Notch pathway inactive) and then switched to 18˚C late (Notch pathway active), a significant number of persisting CB NBs were found at 48 hours APF (Fig. 4A,C). In contrast, when animals were raised at 18˚C (Notch pathway active) and then switched to 29˚C late (Notch pathway inactive), no or significantly fewer persisting CB NBs were found (Fig. 4B,C). Absence or presence of Notch pathway activity under each temperature shift regime was verified using *E(spl)mγ-GFP* reporter expression (Fig. S1A,B). We conclude that early Notch pathway activity is required to eliminate CB NBs and terminate neurogenesis.

Delta-dependent Notch activation refines temporal boundaries by closing the early CB NB temporal window

During development, CB NBs sequentially express a series of intrinsic factors over time to generate a diversity of neuron types (Fig. 5A). Defects in intrinsic temporal factor expression lead to changes in the molecular composition of neuron types produced and defects in timing of CB NB elimination and neurogenesis termination (Isshiki et al., 2001; Maurange et al., 2008; Liu et al., 2015; Ren et al., 2017; Syed et al., 2017; Yang et al., 2017; Pahl et al., 2019). Because Notch signaling is required early to eliminate CB NBs, we assayed expression of early temporal factors, Cas and Svp. In freshy hatched control larvae (0 hours ALH), approximately 50% of CB NBs expressed Cas and 5% expressed Svp (Fig. 5B). Over time, the percentage of Cas expressing CB NBs declined, while Svp expressing CB NBs modestly increased (Fig. 5B). Less than 1% of CB NBs co-expressed Cas and Svp at any stage and expression of both factors was

absent by 48 hours ALH (Fig. 5B,C). This is consistent with work published previously (Isshiki et al., 2001; Tsuji et al., 2008; Chai et al., 2013; Maurange et al., 2008; Ren et al., 2017; Syed et al., 2017). When Notch or Delta were knocked down in CB NBs and their GMC progeny, approximately 50% of CB NBs expressed Cas at freshly hatched larval stages (0 hours ALH), same as controls, and slightly more expressed Svp (Fig. 5B). Over time, the percentage of Cas expressing CB NBs declined, while Svp expressing CB NBs remained relatively unchanged or reduced (Fig. 5B). At 48 hours ALH, in contrast to controls, Cas and Svp were still expressed in CB NBs in both Notch and Delta knockdown animals (Fig. 5B,D,E). This suggests that Notch signaling is required for the cessation of early temporal factor expression. Moreover, early temporal defects could lead to defects in later temporal factor expression.

Next, we looked at later developmental time points. In control animals at 72 hours ALH, some CB NBs still expressed Imp, but most had transitioned to expressing Syp in response to steroid hormone signaling, and by 96 hours ALH, all expressed Syp and E93 (except MB NBs) (Fig. 5F,I,J and Fig. S2A,D). This is consistent with work published previously (Liu et al., 2015; Ren et al., 2017; Syed et al., 2017; Yang et al., 2017). When Notch or Delta were knocked down in NBs and their GMC progeny, we found that CB NBs still expressed Imp at both 72 and 96 hours ALH (Fig. 5G-I and Fig. S2B-D). Coincident with prolonged Imp expression, we found a reduction in CB NBs expressing Syp and many co-expressed both Imp and Syp, a phenotype not seen in control animals (Fig. 5G-I and Fig. S2B-D). Next, we assayed E93, whose expression is dependent on ecdysone signaling and in the MB NB lineage, Syp (Syed et al., 2017; Pahl et al., 2019). We generated RFP expressing CB NB clones that co-express *Dl RNAi* and found that E93 protein levels were reduced more than 20% compared to controls (Fig. 5J). We conclude that Delta-dependent Notch activation is required to sharpen the boundaries of temporal factor expression. Moreover, these defects in temporal factor boundaries could lead to defects in CB NB elimination.

Defects in timing of temporal transitions could be due to defects in cell cycle progression, although embryonic NBs still transition independent of cell division (Grosskortenhaus et al., 2005). We used PH3 to assay CB NB mitotic activity. In Delta knock down animals, the percentage of PH3 positive CB NBs was reduced compared to control (Fig. S2E). At 48 h APF however, Delta knock down CB NBs were still dividing based on PH3 expression (Fig. S2F). To determine whether CB NBs ectopically persist due to defects in cell cycle rate, we co-expressed *dp110* to constitutively activate PI3-kinase in Delta knock down animals. A significant number of *pcnaGFP* expressing, Dpn positive CB NBs were still observed,

suggesting that defects in cell cycle timing and growth rates alone cannot account for ectopic persistence of CB NBs into later developmental stages and adulthood (Fig. S2G).

CB NBs with reduced Notch pathway activity persist into adulthood due to temporal patterning defects

Next, we assayed temporal factor expression in CB NBs that ectopically persisted into late pupal stages. We generated *Notch*^{55e11} MARCM CB NB clones as described previously and found that ectopically persisting CB NBs expressed either Imp alone, co-expressed both Imp and Syp, or expressed Syp alone similar to CB NBs at earlier larval stages (Fig. 6A, n=14 clones). Similar expression profiles were observed in ectopically persisting CB NBs in Delta knock down animals (*worGAL4, UAS-Dl RNAi #HMS01309*) (Fig. 6B). Next, we tested whether temporal patterning defects account for the ectopic persistence of CB NBs with reduced Notch pathway activity. First, we knocked down Imp. Knocking down Imp alone leads to premature CB NB loss due to premature expression of late temporal factors (Fig. S3A,B) (Yang et al., 2017). When Imp was knocked down together with Delta (*worGAL4, UAS-Dl RNAi #HMS01309, UAS-Imp RNAi#HMS01168*), CB NB number was significantly reduced compared to Delta knockdown alone (Fig. 6C,D,G). CB NB size was also reduced, consistent with previous work demonstrating the importance of the early factor Imp in promoting growth (Fig. 6H) (Yang et al., 2017). Next, we constitutively expressed the late temporal factor E93, since E93 levels were reduced in *Dl RNAi* CB NB clones. When E93 was constitutively expressed in Delta knock down animals, a significant reduction in CB NB number was observed (Fig. 6E-H). We conclude that ectopically persisting CB NBs in animals with reduced Notch pathway activity is due to defects in CB NB temporal patterning: Imp expression is prolonged and E93 levels are reduced.

The early temporal factor Imp positively regulates Delta expression

To better understand how Notch signaling controls CB NB temporal factor expression, we mined publicly available datasets. The datasets that we mined include (1) results from Notch genetic, molecular, and biochemical interaction studies (https://flybase.org/reports/FBgn0004647) (2) results from RIP-seq (RNA immunoprecipitation) experiments using Imp or Syp as bait (McDermott et al., 2014; Samuels et al., 2020), and (3) RNA-sequence data from isolated, pooled AL (antennal lobe) NBs at early and late timepoints (Liu et al., 2015). First, we identified genes that were differentially expressed (DEGs) in AL NBs early (24 hours ALH) versus late (84 hours ALH). We identified 1861 genes (adj. p-value<0.1 and log fold change>1), including known temporal factors (Fig. 7A). Next, we asked which if any were present in datasets from

Notch genetic, molecular, and biochemical interaction studies. We identified 59 genes (Fig. 7B,C). Next, we determined that 23 of these 59 genes were found in the Imp-RIP dataset (Fig. 7B, orange highlight) and 6 in the Syp-RIP dataset (Fig. 7B, purple highlight). To our surprise, Delta was one of the genes on this gene list. Delta transcript levels were expressed high early, and Delta mRNA co-immunoprecipitated with Imp in RIP-seq experiments. This raised the possibility that Delta, a known Notch target gene, is regulated by NB temporal factors (Zhang et al., 2021). To test this possibility, we knocked down Imp in CB NBs (*worGAL4,UAS-Imp-RNAi #HMS01168*) and assayed expression of the protein trap, *Delta-GFP*. At 24 hours ALH, *Delta-GFP* levels were reduced compared to controls (Fig. 7D,E). This suggests that Imp positively regulates Delta.

Next, we asked whether late temporal factors would also regulate Delta, since Delta transcript levels decrease over time. We assayed *Delta-GFP* in CB NBs following constitutive Syp or E93 expression. We examined brains at 24 hours ALH, a time when Syp and E93 are normally not expressed and found reduced *Delta-GFP* expression in NBs and their GMC progeny compared to controls under both conditions (Fig. 7F,G). Together with the reduction in Delta transcript levels during late larval stages (Fig. 7B), these results suggest that Delta and Delta-dependent Notch transactivation are regulated by CB NB intrinsic temporal factors.

Discussion

Here we report that CB NBs utilize Notch signaling to progress forward through their stem cell lineages, ultimately terminating their divisions through differentiation or death (see Model Fig. 7H). Somewhat paradoxically, we find that early Notch activity is required early to terminate CB NB divisions late. This is because Notch regulates early temporal patterning and defects in early temporal patterning transmit to late temporal defects including the time at which CB NBs stop divisions. Notch curtails expression of at least three early temporal factors (Cas, Svp, and Imp), suggesting that Notch may function broadly to close the early temporal window. It is known that the early to late temporal transition is dependent on both early intrinsic temporal factors, Cas and Svp, and on extrinsic steroid hormone signaling (ecdysone) (Ren et al., 2017; Syed et al., 2017). Cas overexpression is sufficient to prolong Svp expression, but Cas is not required for Svp expression, and Svp primes CB NBs to respond to ecdysone (Ren et al., 2017). Whether Notch pathway activity curtails both Cas and Svp or just Cas remains an open question, however it has been reported that both cas and svp are associated with at least one enhancer that is responsive to Notch activity (Zacharioudaki et al., 2016). Also, it remains unknown whether Notch directly inhibits Imp or whether Notch indirectly inhibits Imp through Syp

expression in response to ecdysone or a yet unidentified factor. Notch was recently shown to regulate timing of Sloppy-paired expression in the optic lobe (Ray et al., 2022).

While some CB NBs maintained early Imp expression, others co-expressed both Imp and Syp, or expressed Syp alone. This suggests that Notch function is lineage-dependent and/or suggests that more than one pathway regulates lineage progression. While Cas is likely expressed in all CB NBs, Svp appears to be more restricted. Whether Notch inhibits early temporal progression only in Svp expressing CB NBs is not yet known. Somewhat unexpectedly, we also found a significant percentage of ectopically persisting CB NBs expressing the late temporal factor Syp. Syp promotes accumulation of nuclear Pros in CB NBs during pupal stages to induce terminal differentiation (Maurange et al., 2008; Yang et al., 2017). This suggests that either a Pros-independent mechanism exists to eliminate CB NBs and/or that Syp regulates expression of additional unknown temporal factors required for Pros nuclear accumulation. As we report here, Syp and E93 inhibit expression and localization of Delta and decreased Delta leads to ectopic persistence of CB NBs. This is consistent with the notion that once CB NBs transition from early to late temporal factor expression in response to ecdysone, late temporal factors (Syp/E93) inhibit Delta. Whether this changes Notch activity and/or transcription of Notch target genes is not yet known. Nevertheless, it will be important to identify the Notch transcriptional target genes that regulate lineage progression. One good place to start will be to follow up on the eight transcription factors we identified from data mining.

During early larval stages, CB NBs reactivate from quiescence and produce GMCs that express Delta (Zacharioudaki et al., 2012; Sood et al., 2022). This leads to the transactivation of Notch in CB NBs (Sood et al., 2022). The early temporal factor Imp positively regulates Delta in CB NBs and their GMC progeny. In developing egg chambers, Imp positively regulates Notch pathway activity by controlling Kuz localization (Fic et al., 2019). Whether Imp in CB NBs also regulates Kuz localization remains an open question. Delta is also expressed in cortex glia and regulates CB NB Notch activity. Whether Delta expression in cortex glia changes over time as is the case for CB NBs remains an open question. Hedgehog signaling in CB NBs promotes lineage progression downstream of Cas and Hedgehog ligands are produced in cortex glia and GMCs (Chai et al., 2013). Thus, CB NBs integrate cues from their GMC progeny and neighboring cortex glial cells to control temporal progression and lineage termination.

Although the exact course of temporal progression is yet to be defined in the mammalian nervous system, mammalian NSCs temporally express several factors including microRNAs, mRNA-binding proteins and transcription factors, allowing them to produce deep layer neurons (early-born neural progeny), superficial layer neurons (late-born neural progeny) and glial cells

sequentially throughout development (Okano and Temple, 2009; MuhChyi et al., 2013; Oberst et al., 2019; Telley et al., 2019). COUP-TFI and COUP-TFII, orthologs of *Drosophila* Svp, function as late temporal factors allowing NSCs to switch from producing early-born neural fates to late-born neural fates (Naka et al., 2008). This is similar to the function of Svp in the *Drosophila* brain where Svp mutants failed to switch from early Chinmo positive daughters to late Broad-complex positive daughters (Maurange et al., 2008). Similarly, mammalian NSCs temporally express RNA-binding protein Imp-1, ortholog of *Drosophila* Imp, and in Imp-1 deficient animals, NSCs are lost prematurely similar to premature loss of CB NBs seen in *Drosophila* (Nishino et al., 2013; Yang et al., 2017; Pahl et al., 2019). As in *Drosophila* NBs, temporal progression of mammalian NSCs is not completely dependent on cell-intrinsic cues but also requires cell-extrinsic cues like feedback signals from the lineage and environmental cues (Okamoto et al., 2016; Oberst et al., 2019; Zhang et al., 2020), however, very little is known about the signaling pathways regulating the transitions from early to late temporal fates. Even though Notch activity is required for the temporal switch from neurogenesis to gliogenesis in mammalian NSCs, it remains unclear whether Notch function extends to regulation of temporal progression required for the switch from early born to late born neuron subtypes (Morrison et al., 2000; Grandbarbe et al., 2003; Ohtsuka and Kageyama, 2019).

Materials and Methods

Fly Stocks: Fly stocks used in this study and their source are listed in the Supplemental Table 2.

Animal husbandry: All animals were raised in uncrowded conditions at 25˚C with the exception of animals with tub-GAL80(ts). For experiments using *tub-Gal80^{ts}* (temperature sensitive), animals were kept in uncrowded conditions at 29˚C and dissected at developmental timings to be equivalent to development at 25˚C unless otherwise stated. Animals were staged from hatching for larval dissections and from white prepupae for pupal dissections.

Induction of clones: For induction of Flp-FRT and MARCM clones, animals were heat shocked at 37˚C between 30-60 mins at L1 and dissected at the stated developmental timings.

Temperature-shift experimental paradigm: For early knockdown experiments, animals were raised at 29˚C until 72 hours ALH (equivalent to 25˚C) and then moved to 18˚C to develop until 48 hours APF (equivalent to 25˚C). For late knockdown experiments, animals were raised at

18˚C until 72 hours ALH (equivalent to 25˚C) and then moved to 29˚C to develop until 48 hours APF (equivalent to 25˚C).

Immunofluorescence and confocal imaging: Larval, pupal, and adult brains were dissected as previously described (Pahl et al., 2019). In brief, dissected tissues were fixed in 4% EM grade formaldehyde in PEM buffer for 20 mins. (larvae) or 30 mins. (pupae and adults) and rinsed in 1X PBS with 0.1% Triton X-100 (PBT). Tissues were blocked overnight at 4˚C in 10% normal goat serum in PBT followed by antibody staining. Primary antibodies used are listed in the Supplemental Table 2. To detect primary antibodies, Alexa-Fluor conjugated secondary antibodies (Thermo-Fisher) listed in the Supplemental Table 2 were used. Images encompassing the entire brain hemispheres were acquired using a Leica SP8 laser scanning confocal microscope equipped with a 63x/1.4 NA and 40x/1.3 NA oil-immersion objectives and analyzed using Fiji software. All images were processed using Fiji and Adobe Photoshop software and figures assembled using Adobe Illustrator software. NBs were identified based on Dpn expression and superficial location. The Fiji “cell counter” plugin was used to count and track number of Dpn positive NBs. NB size was calculated by averaging the lengths of two perpendicular lines through the center of the NB in Fiji. Quantification of fluorescence was performed in Fiji. Nuclear E93 levels were quantified as follows: CB NB nuclei labeled with Dpn were manually traced and the average E93 fluorescence intensity measured in the nucleus. Normalized E93 nuclear fluorescence intensity was determined as a ratio of nuclear E93 fluorescence intensity in a clone NB to nuclear E93 fluorescence intensity in a control NB in the same z-plane. All data is represented as mean ± standard error of the mean and statistical significance was determined using unpaired two-tailed Student’s t-tests or ANOVAs in Prism 9.

RNA-Seq Data Analysis: Differential Gene Expression (DGE) analysis was performed using the publicly available dataset published in Liu *et al.*, 2015 (GSE71103). We aligned FASTQ data to a reference Dm genome using STAR (version 2.7.2b) (Dobin et al., 2013). Read count tables were generated using the htseq-count package from bioconda, and DGE was performed using DESeq2 (Love et al., 2014). We made contrasts between 24h ALH and 84h ALH antennal lobe NBs in order to identify differentially expressed genes of interest (adjPvalue <0.1 and log Fold Change >1). The list of 1861 differentially expressed genes was compared to the list of downstream genetic interactions of Notch pathway from FlyBase (https://flybase.org/reports/FBgn0004647) to obtain the list of 59 differentially expressed genes of interest. Finally, VST normalized counts of the 59 genes were used to generate the heatmaps

using heatmap.2 from the ggplot2 package (https://ggplot2.tidyverse.org). To obtain common target genes between Notch and Imp, we compared our list of 59 genes to the top 1000 Imp target genes obtained via RIP-seq by Samuels *et al*., 2020. We performed similar comparison on the 274 Syp target genes obtained via RIP-seq by McDermott *et al.,* 2014 and our list of 59 genes to obtain common Notch and Syp target genes.

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Supplemental Table 1: *Drosophila* genotypes per figure panel

Figure	Genotype	
Fig. 1B	*worGAL4/+ (OregonR) ; pcnaGFP/+ (OregonR)*	control
Fig. 1C	*worGAL4/+; pcnaGFP/UAS-NotchRNAi*	
Fig. 1F	*hsflp, tubgal80, FRT19A/Notch55e11 FRT19A ; tubGal4, UAS-mCD8GFP/+*	
Fig. 1G,I	*worGAL4/+; pcnaGFP/UAS-KuzRNAi*	
Fig. 1H	*worGAL4/+; pcnaGFP/UAS-Su(H)RNAi*	
Fig. 2A,B,C	*NP0577GAL4/+; UAS-mCD8RFP/+; DeltaGFP/+*	
Fig. 2D,E,F	*repoGAL4, UAS-mCD8RFP/SerrateGFP*	
Fig. 2G	*worGAL4/+; E(spl)mγGFP/+*	control
Fig. 2H	*worGAL4/+; E(spl)mγGFP/UAS-NotchRNAi*	
Fig. 2I	*worGAL4/+; E(spl)mγGFP/UAS-DeltaRNAi*	
Fig. 2J	*repoGAL4/+; E(spl)mγGFP/+*	control
Fig. 2K	*repoGAL4/+; E(spl)mγGFP/UAS-DeltaRNAi*	
Fig. 2L	*repoGAL4/+; E(spl)mγGFP/UAS-SerrateRNAi*	
Fig. 3A	*worGAL4/+; pcnaGFP/UAS-DeltaRNAi*	
Fig. 3B	*worGAL4/+; pcnaGFP/UAS-DeltaRNAi*	
Fig. 3E	*NP0577GAL4/+; pcnaGFP/UAS-DeltaRNAi*	
Fig. 4A	*worGAL4,tubGAL80(ts)/+; pcnaGFP/UAS-NotchRNAi* (left panel)	
Fig. 4A	*worGAL4,tubGAL80(ts)/+; pcnaGFP/UAS-DeltaRNAi* (right panel)	
Fig. 4B	*worGAL4,tubGAL80(ts)/+; pcnaGFP/UAS-NotchRNAi* (left panel)	
Fig. 4B	*worGAL4,tubGAL80(ts)/+; pcnaGFP/UAS-DeltaRNAi* (right panel)	
Fig. 5C	*worGAL4/+ (OregonR)*	control
Fig. 5D	*worGAL4/+; UAS-NotchRNAi/+*	
Fig. 5E	*worGAL4/+; UAS-DeltaRNAi/+*	
Fig. 5F	*ImpGFP/+ (OregonR); worGAL4/+ (OregonR)*	control
Fig. 5G	*ImpGFP/+; worGAL4/+; UAS-NotchRNAi/+*	
Fig. 5H	*ImpGFP/+; worGAL4/+; UAS-DeltaRNAi/+*	
Fig. 5J	*hsFlp; repoGAL80; UAS-DeltaRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-RFP*	
Fig. 6A	*hsflp, tubgal80, FRT19A/Notch55e11 FRT19A ; tubGal4, UAS-mCD8GFP/+*	
Fig. 6B	*worGAL4/+; UAS-DeltaRNAi/+*	
Fig. 6C	*worGAL4/+; pcnaGFP/UAS-ImpRNAi*	
Fig. 6D	*pcnaGFP; worGAL4,tubGAL80(ts)/+; UAS-DeltaRNAi/UAS-ImpRNAi*	
Fig. 6E	*worGAL4/+; pcnaGFP/UAS-Eip93F WT*	
Fig. 6F	*pcnaGFP; worGAL4,tubGAL80(ts)/+; UAS-DeltaRNAi/UAS-ImpRNAi*	
Fig. 7D	*DeltaGFP/+*	control
Fig. 7E	*worGAL4/+; DeltaGFP/UAS-ImpRNAi*	
Fig. 7F	*worGAL4/UAS-Syp-RB-HA; DeltaGFP/+*	
Fig. 7G	*worGAL4/+; DeltaGFP/UAS-Eip93F WT*	
Supplementary Figures		

Fig. S1A,B	*worGAL4,tubGAL80(ts)/+; E(spl)mγGFP/UAS-DeltaRNAi*	
Fig. S2A	*ImpGFP/+ (OregonR); worGAL4/+ (OregonR)*	control
Fig. S2B	*ImpGFP/+; worGAL4/+; UAS-NotchRNAi/+*	
Fig. S2C	*ImpGFP/+; worGAL4/+; UAS-DeltaRNAi/+*	
Fig. S2E	control: *worGAL/+ (Oregon R)* *worGAL4/+; UAS-DeltaRNAi/+*	
Fig. S2F	*prosGAL4/+; UAS-DeltaRNAi/+*	
Fig. S2G	*worGAL4,UAS-dp110/+; UAS-DeltaRNAi/+*	
Fig. S3A	*worGAL4/+; pcnaGFP/UAS-ImpRNAi*	

Supplemental Table 2: Key Resources

Reagent	Source	Identifier
ANTIBODIES		
rat ant-Dpn (1:100)	Abcam	ab195173
chicken anti-GFP (1:500)	Abcam	ab13970
rabbit anti-dsRed (1:1000)	Clontech	632496
rabbit anti-PH3 (1:1000)	Millipore	06-570
mouse anti-Repo (1:5)	Developmental Studies Hybridoma Bank	8D12
mouse anti-Prospero (1:1000)	Developmental Studies Hybridoma Bank	MR1A
mouse anti-Svp (1:10)	Developmental Studies Hybridoma Bank	5B11
mouse anti-Dlg (1:40)	Developmental Studies Hybridoma Bank	4F3
rabbit anti-Scribble (1:500)	Gift from Chris Q. Doe	
rabbit anti-Cas (1:500)	Gift from Chris Q. Doe	
rabbit anti-Syp (1:250)	Gift from Chris Q. Doe	
guinea pig anti-E93 (1:250)	Gift from Chris Q. Doe	
rabbit anti-Imp (1:250)	Gift from Paul MacDonald	
rat anti-Imp (1:250)	Gift from Claude Desplan	
guinea pig anti-Dpn (1:1000)	Gift from Claude Desplan	
goat anti-chicken Alexa 488 (1:300)	Thermo Fisher Scientific	A32931
goat anti-rat Alexa 555 (1:300)	Thermo Fisher Scientific	A48263
goat anti-rat Alexa 647 (1:300)	Thermo Fisher Scientific	A48265
goat anti-rabbit Alexa 405 (1:300)	Thermo Fisher Scientific	A48254
goat anti-rabbit Alexa 555 (1:300)	Thermo Fisher Scientific	A21428
goat anti-rabbit Alexa 633 (1:300)	Thermo Fisher Scientific	A21071
goat anti-mouse Alexa 405 (1:300)	Thermo Fisher Scientific	A48255
goat anti-mouse Alexa 488 (1:300)	Thermo Fisher Scientific	A11001
goat anti-mouse Alexa 555 (1:300)	Thermo Fisher Scientific	A32727
goat anti-guinea pig Alexa 488 (1:300)	Thermo Fisher Scientific	A11073
goat anti-guinea pig Alexa 555 (1:300)	Thermo Fisher Scientific	A21435

CHEMICALS		
SlowFade™ Diamond antifade reagent	Invitrogen	Catalog # S36963
SlowFade™ Gold antifade reagent	Invitrogen	Catalog # S36937

Normal Goat Serum	Thermo Fisher Scientific	Catalog # 31873
Paraformaldehyde 16% solution EM grade	Electron Microscopy Sciences	Catalog # 15710
Schneider's Drosophila media	Gibco	Catalog # 21720-024
Triton X-100	Sigma	Catalog # T9284

SOFTWARE		
ImageJ/Fiji	Fiji	http://fiji.sc/
LAS AF	Leica Microsystems	https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/
Prism 9	Graphpad	https://www.graphpad.com/scientific-software/prism/
Photoshop 2022	Adobe	https://www.adobe.com/products/photoshop.html
Illustrator 2022	Adobe	https://www.adobe.com/products/illustrator.html
R-studio	R-studio	https://www.rstudio.com/

EXPERIMENTAL MODEL: *Drosophila melanogastor*		
Oregon R	Bloomington Drosophila Stock Center	5
wor-Gal4	(Albertson and Doe, 2003)	
tubulin-Gal80(ts)	Bloomington Drosophila Stock Center	7108
repo-Gal4	Bloomington Drosophila Stock Center	7415
NP0577-Gal4	Kyoto Stock Center	112228
repo-Gal80	(Awasaki et al., 2008)	
UAS-Notch RNAi (HMS00001)	Bloomington Drosophila Stock Center	33611
UAS-Kuzbanian RNAi (HMS05424)	Bloomington Drosophila Stock Center	66958
UAS-Su(H)RNAi (HMS05748)	Bloomington Drosophila Stock Center	67928
UAS-Delta RNAi (HMS01309)	Bloomington Drosophila Stock Center	34322
UAS-Serrate RNAi (HMS01179)	Bloomington Drosophila Stock Center	34700

UAS-dp110	Bloomington Drosophila Stock Center	25914
Delta-GFP	Bloomington Drosophila Stock Center	59819
Serrate-GFP	Bloomington Drosophila Stock Center	59824
pcna-GFP	(Thacker et al., 2003)	
E(spl)mγ-GFP	(Almeida and Bray, 2005)	
Imp-GFP	Bloomington Drosophila Stock Center	60237
UAS-Imp RNAi (HMS01168)	Bloomington Drosophila Stock Center	34977
UAS-Syp-RB-HA	gift from Tzumin Lee	
UAS-Eip93F WT	Zurich FlyORF	F000587
hsFlp (on X)	gift from Iswar Hariharan	
Act5c-FRT-CD2-FRT-Gal4, UAS-RFP	Bloomington Drosophila Stock Center	30558
Act5c-FRT-CD2-FRT-Gal4, UAS-GFP	gift from Iswar Hariharan	
UAS-mCD8-mRFP	Bloomington Drosophila Stock Center	27399
UAS-mCD8-mGFP	Bloomington Drosophila Stock Center	5137
Notch55e11 FRT19A	Bloomington Drosophila Stock Center	28813
hsflp, tubgal80, FRT19A; tubGal4, UASmCD8GFP	gift from Ben Ohlstein	

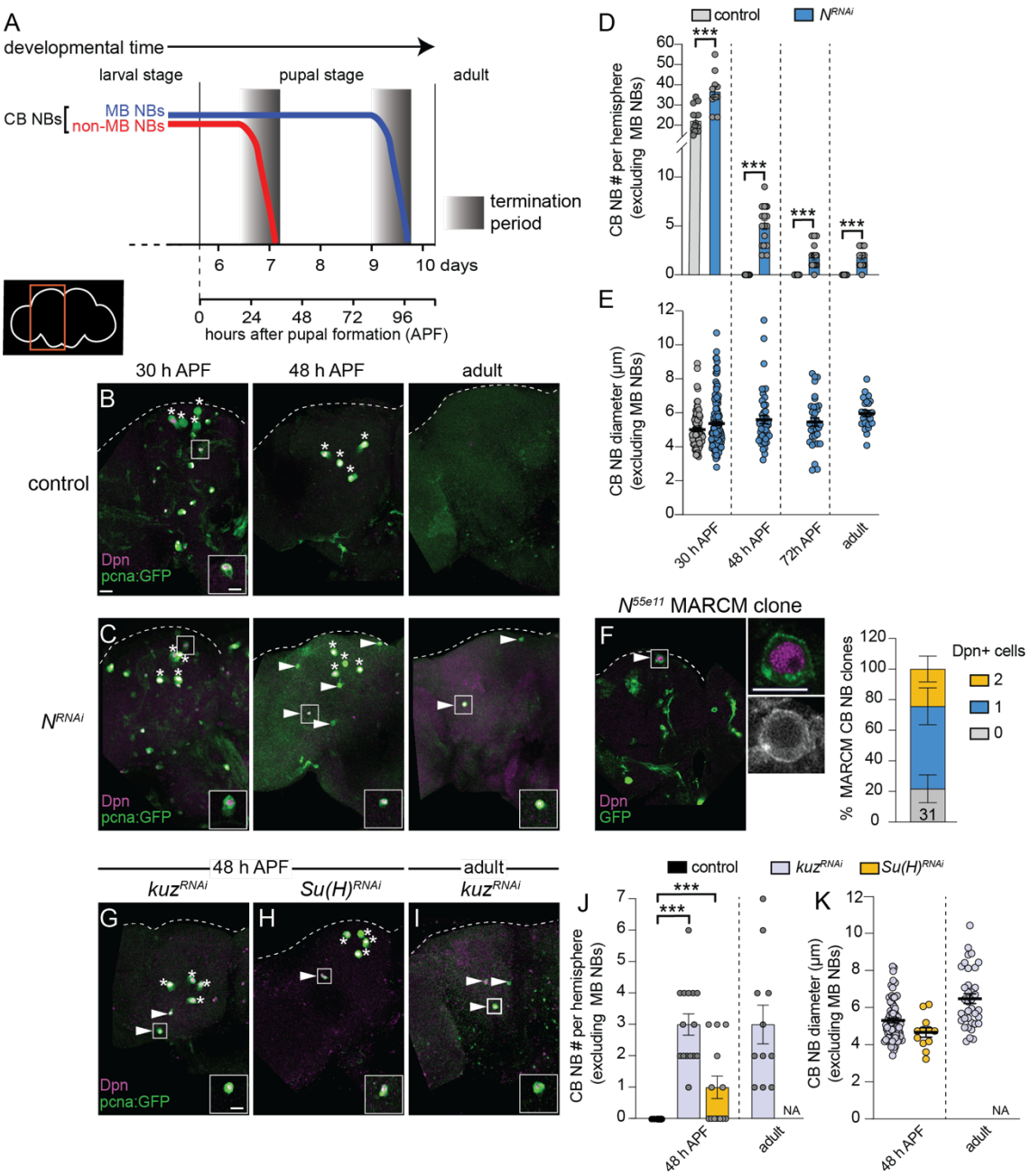

Figure 1: Notch signaling regulates timing of CB NB elimination and neurogenesis termination.

(A) Schematic showing developmental timeframe of CB NB elimination with timeline below used for developmental staging. (B-C, G-I) Maximum intensity projections of single brain hemispheres from indicated genotypes, times, with markers listed in bottom left. Asterisks indicate the four

MB NBs and arrowheads indicate some of the ectopically proliferating CB NBs (non-MB NBs). One ectopic CB NB (white box) shown at higher magnification in bottom right. (D,J) Quantification of CB NB number (excluding MB NBs) per brain hemisphere at indicated times and genotypes. Each data point represents one brain hemisphere, mean ± SEM, ***p-value≤0.001 (unpaired two-tailed Student's *t*-test). (E,K) Quantification of average CB NB diameter, used as a proxy for NB size, at indicated times and genotypes. Each data point equals one CB NB (n≥4 animals per genotype), mean±SEM. (F) Single optical section of a brain hemisphere from indicated genotype at 48 hours APF with markers listed in panels with high magnification panel to right of ectopic CB NB in white box. Distribution of *Notch*^{55e11} MARCM CB NB clones containing Dpn positive NBs. Scale bar equals 20 µms (panels) or 10 µms (insets) in this and all subsequent figures. Panel genotypes listed in Supplemental Table 1.

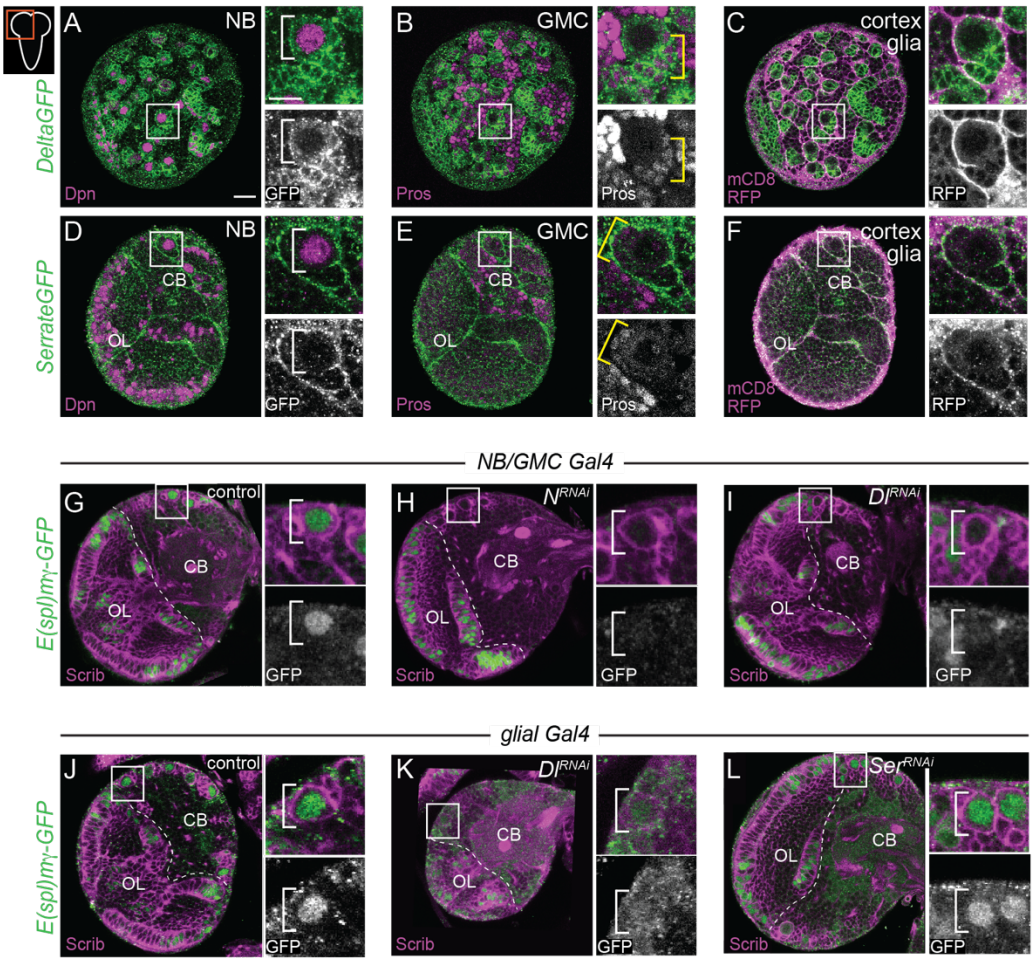

Figure 2: Delta expressed in CB NBs, GMCs, and cortex glia regulates CB NB Notch activity.

(A-F) Single optical section of a brain hemisphere from the indicated genotypes at wandering L3 stages. Higher magnification image of the CB NB highlighted by the white box is shown to the right of the colored overlays. Top panels are higher magnification colored overlay with single channel grayscale images below. White brackets indicate the CB NB and yellow brackets indicate newborn GMC progeny. (G-L) Single optical section of a brain hemisphere from the indicated genotypes at 72 hours ALH. Higher magnification image of the CB NB highlighted by the white box is shown to the right of the colored overlays. Scale bar equals 20 μms (panels) and 10 μms (insets). CB: central brain; OL: optic lobe. Panel genotypes listed in Supplemental Table 1.

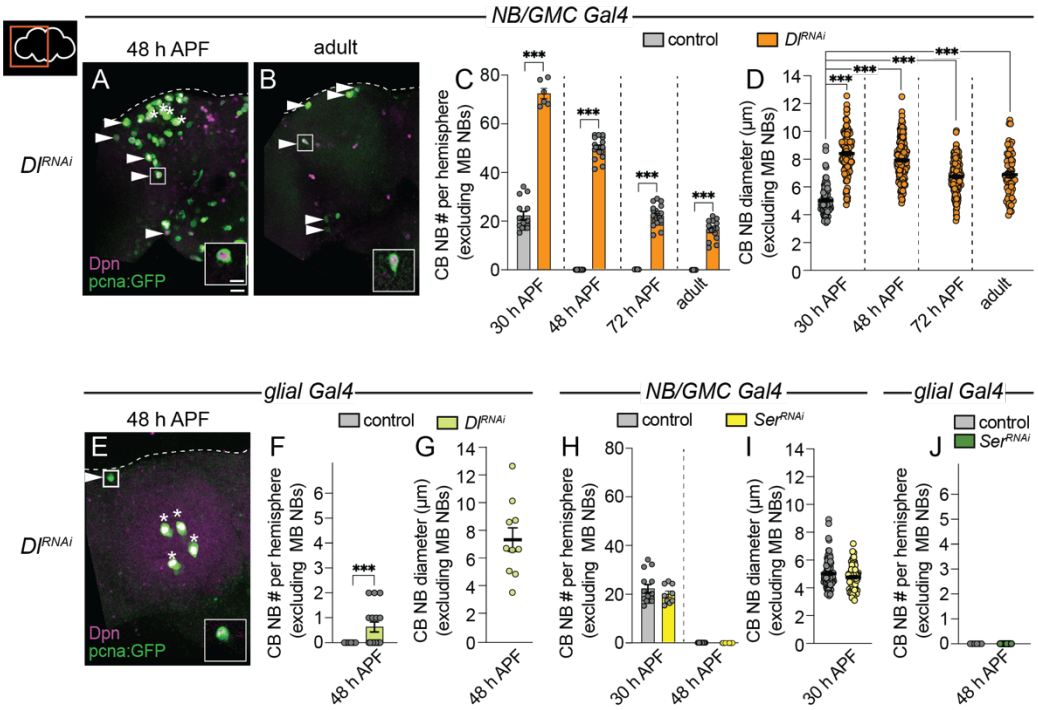

Figure 3: Delta is required to eliminate CB NBs and terminate neurogenesis.
(A,B,E) Maximum intensity projections of single brain hemispheres from indicated genotypes. Asterisks indicate the MB NBs and the white arrowheads indicate some of the ectopically proliferating CB NBs. Inset shows a higher magnification of the ectopically proliferating CB NB highlighted by the white box. (C,F,H,J) Quantification CB NB number (excluding the MB NBs). Each data point represents one brain hemisphere. Control data in (C) is the same as Figure 1D. Mean±SEM.***$P \leq 0.001$ (unpaired two-tailed Student's *t*-test). (D,G,I) Quantification of CB NB size (excluding the MB NBs) in the indicated genotypes and developmental times. Each data point represents one neuroblast (n≥4 animals per genotype). Control data in (D) is the same as Figure 1E. Mean±SEM.***$P \leq 0.001$, *$P \leq 0.033$ (Kruskal-Wallis test). Scale bar equals 20 µms (panels) and 10 µms (insets). Panel genotypes listed in Supplemental Table 1.

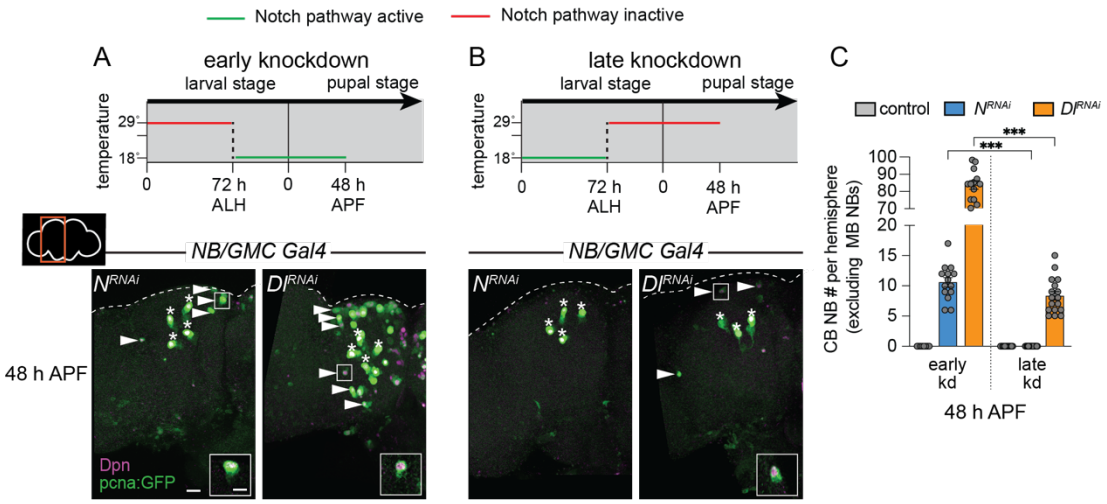

Figure 4: Notch is required early to eliminate CB NBs.

(A,B) Top, schematic depicting experimental setup to temporally control knockdown of Notch signaling. Bottom, maximum intensity projections of single brain hemispheres from indicated genotypes at 48 hours APF relative to 25ºC. Asterisks indicate the MB NBs and the white arrowheads indicate some of the ectopically proliferating CB NBs. Inset shows a higher magnification of an ectopically proliferating CB NB highlighted by the white box. (C) Quantification of CB NB number (excluding the MB NBs) in the indicated genotypes. Each data point represents one brain hemisphere. Mean±SEM. .***$P \leq 0.001$ (Welch's t test). Scale bar equals 20 µms (panels) and 10 µms (insets). Panel genotypes listed in Supplemental Table 1.

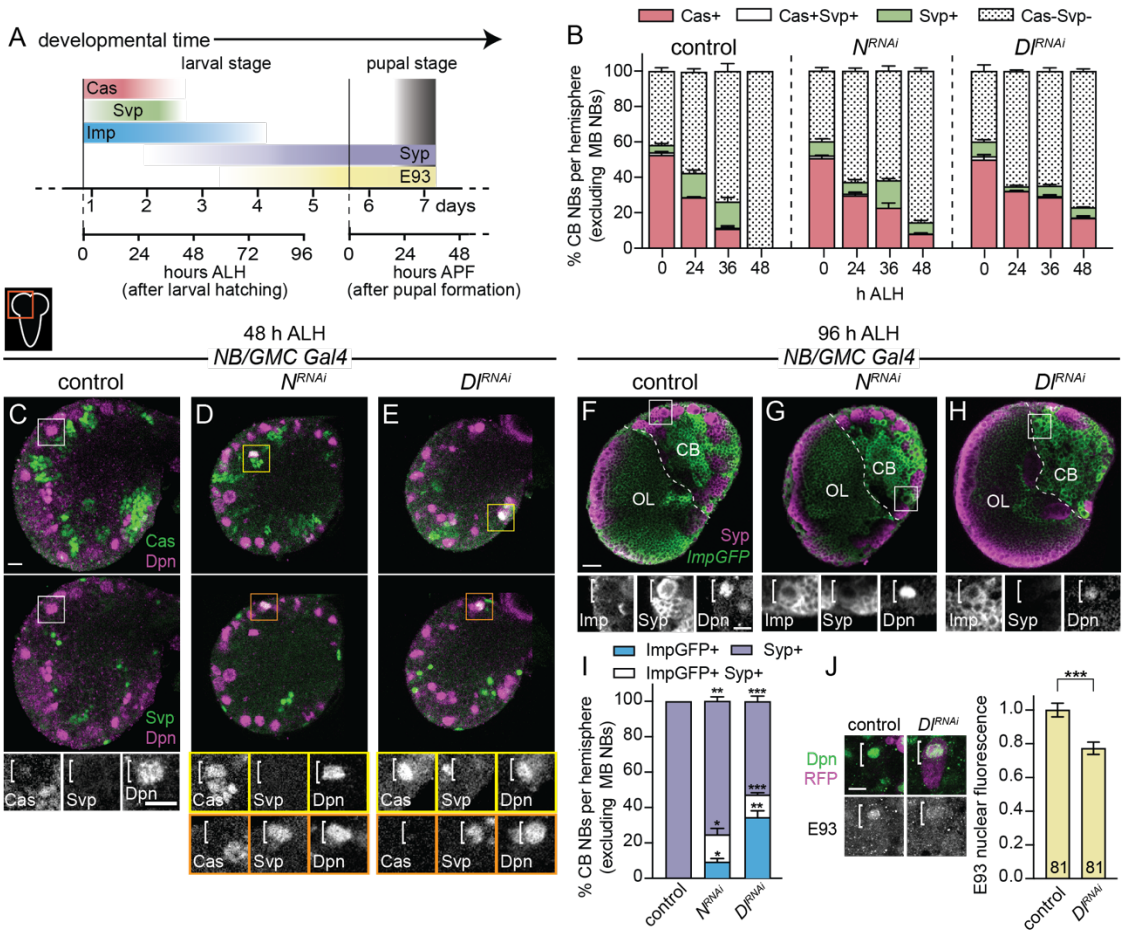

Figure 5: Notch signaling refines temporal factor expression boundaries.

(A) Schematic of temporal factor expression in CB NBs during larval and pupal development. Top timeline (days) refers to developmental timing with two timelines below used for developmental staging. Larva hatch 22 hours after egg lay. (B,I) Quantification of the percentage of CB NBs (excluding the MB NBs) in the indicated genotypes and developmental times expressing the indicated temporal factors. Mean±SEM. n≥3 animals, ***P≤0.001, **P≤0.002, *P≤0.033 (Two-way ANOVA). (C-H) Single optical section of a brain hemisphere from the indicated genotypes and developmental times. Higher magnification image of the CB NB highlighted by the white box is shown below the colored overlays. White brackets indicate the CB NB. (J) Single optical section of a CB NB, colored overlay with grayscale image below. White brackets indicate the CB NB with quantification of normalized nuclear E93 intensities. Column numbers indicate the number of CB NB clones (excluding the MB NBs) scored. Mean±SEM.***P≤0.001 (Mann-Whitney test). Scale bar equals 20 μms (panels) and 10 μms in

single CB NB panels. CB: central brain; OL: optic lobe. Panel genotypes listed in Supplemental Table 1.

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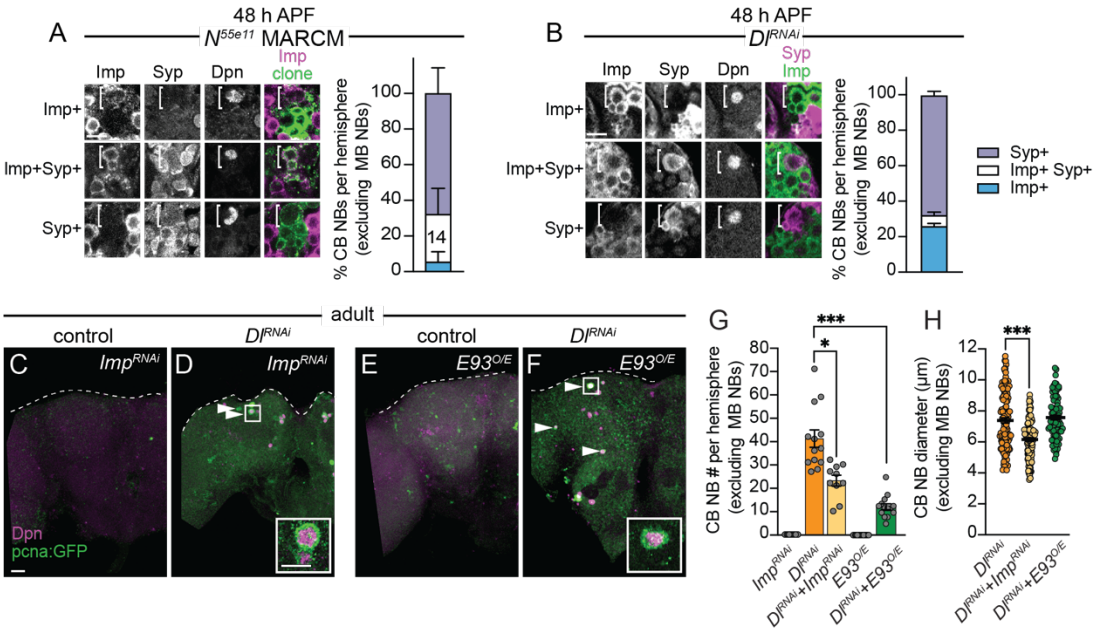

Figure 6: CB NBs ectopically persist due to prolonged early factor Imp expression and reduced late factor E93 expression.

(A,B) Single optical sections of CB NBs from indicated genotypes and developmental times. Single channel grayscale images with colored overlay to the right. White brackets indicate CB NBs. Right, percentage of CB NBs (excluding the MB NBs) in the indicated genotypes and developmental times expressing the indicated temporal factors. Column number (A) indicates number of clones scored. Mean±SEM. n≥3 animals (B). (C-F) Maximum intensity projections of single brain hemispheres from indicated genotypes in one-day old adults. White arrowheads indicate some ectopically proliferating CB NBs (MB NBs are absent). Inset shows a higher magnification of an ectopically proliferating CB NB highlighted by the white box. (G) Quantification of CB NB number (excluding MB NBs) in the indicated genotypes. Each data point represents one brain hemisphere. Mean±SEM. ***P≤0.001, *P≤0.033 (Kruskal-Wallis ANOVA). (H) Quantification of CB NB size (excluding the MB NBs) in the indicated genotypes. Each data point represents one CB NB (n≥4 animals per genotype). Mean±SEM. ***P≤0.001, *P≤0.033 (Kruskal-Wallis ANOVA). Scale bar equals 20 µms (panels) and 10 µms (insets). Panel genotypes listed in Supplemental Table 1.

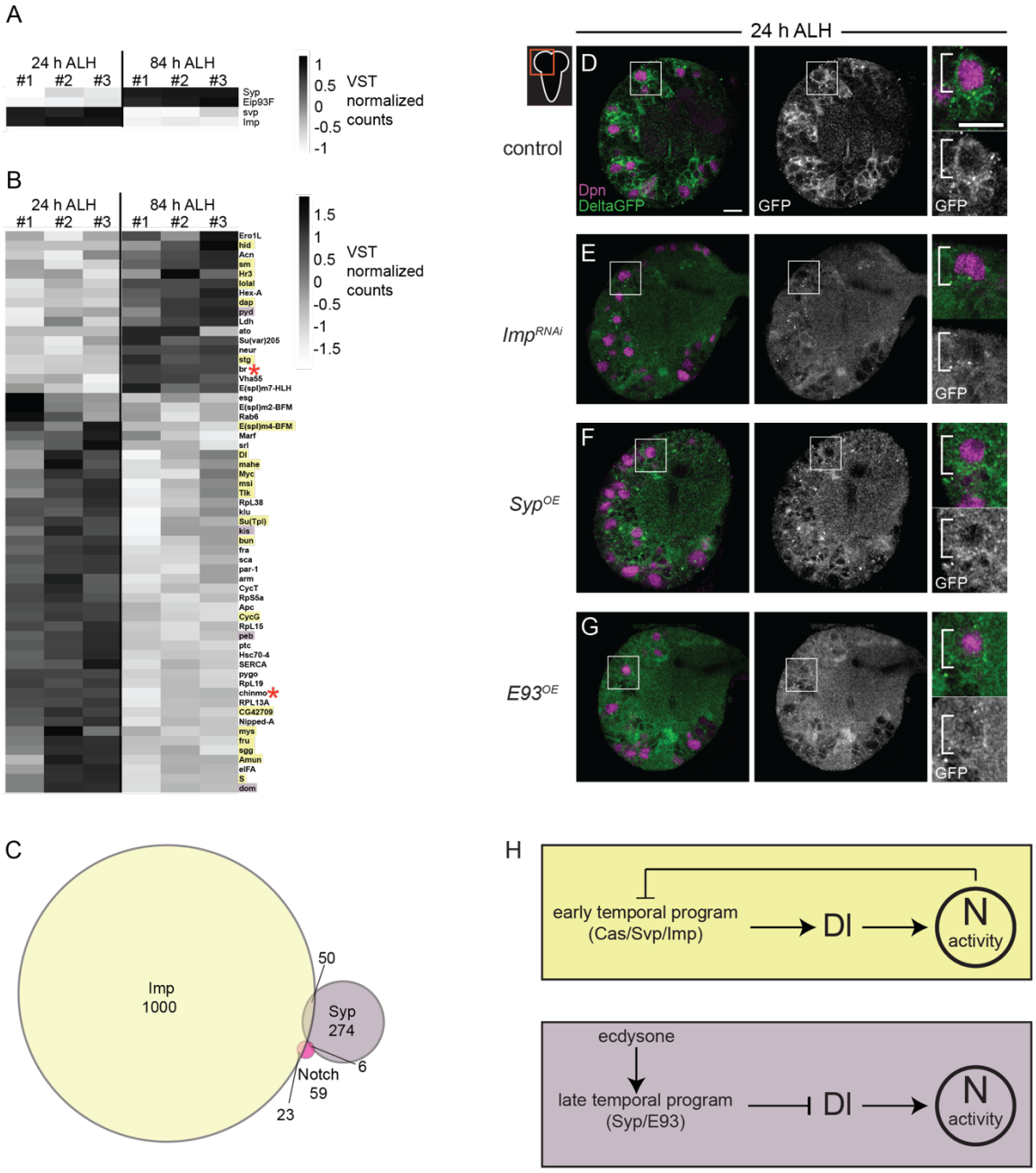

Figure 7: Delta is expressed at higher levels early and is positively regulated by the early temporal factor Imp.

(A-B) Heat map showing the list of genes that are differentially expressed in the AL NBs from 24 to 84 hours ALH. In (B) genes are color coded to show if they are also targets of either Imp (yellow) or Syp (purple) or both (red asterisk). Variance-stabilizing transformation (VST) normalized counts were used to plot the heat maps. (C) Venn diagram showing the number of

target genes analyzed that are common between Notch, Imp and Syp. (D-G) Single optical section of a brain hemisphere from the indicated genotypes at 24 hours ALH expressing *Delta-GFP*. Higher magnification image of the CB NB highlighted by the white box is shown to the right of the single channel grayscale images. Top panels are higher magnification colored overlay with single channel grayscale images below. White brackets indicate the NB. Scale bar equals 10 μms. Panel genotypes listed in Supplemental Table 1. (H) Model of Delta-dependent Notch activation in regulation of CB NB temporal patterning.

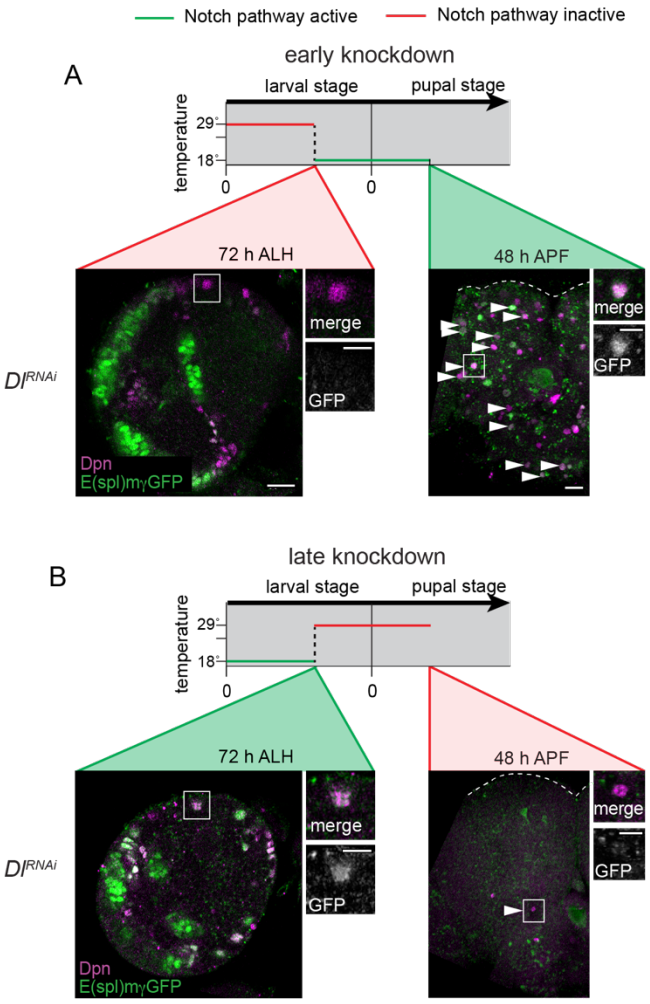

Figure S1: Temporal control of Notch activity in CB NBs. Related to Figure 4.
(A-B) Cartoon above depicts temperature shift regime. Below, single optical sections of brain hemispheres from indicated genotypes and developmental times, with markers listed in panels. Higher magnification image of the CB NB highlighted by the white box is shown to the right of the colored overlays. White arrow heads indicate some of the ectopically persisting CB NBs. Scale bar equals 20 µms (panels) and 10 µms (inset). Panel genotypes listed in Supplemental Table 1.

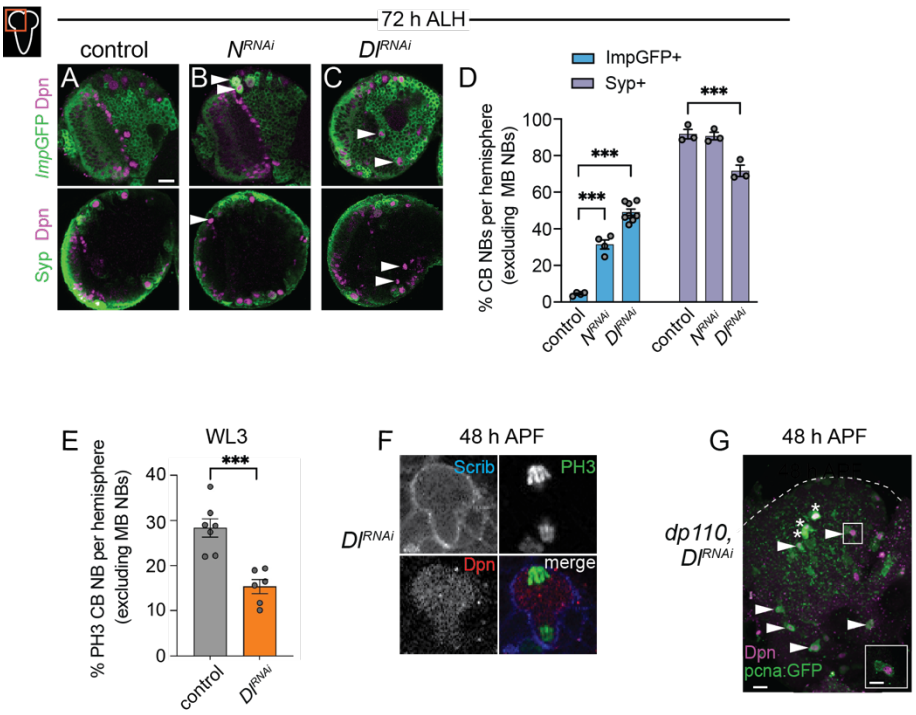

Figure S2: Imp expression is prolonged and Syp expression delayed in animals with reduced Notch pathway activity. Related to Figure 5.

(A-C) Single optical section of a brain hemisphere from the indicated genotypes at 72 hours ALH, with markers listed to left. White arrowheads indicate CB NBs with ectopic expression of either Imp-GFP or no Syp expression. (D) Quantification of the percentage of CB NBs (excluding the MB NBs) in the indicated genotypes at 72 hours ALH expressing the indicated temporal factors. Mean±SEM. n≥3 animals, ***P≤0.001 (Two-way ANOVA). (E) Quantification of the percentage of PH3 positive CB NBs (excluding the MB NBs) in the indicated genotypes at WL3 (wandering L3 stage). (F) Image of a mitotic non-MB NB at 48 hours ALH in Dl RNAi expressing animals. (G) Maximum intensity projection of a single brain hemisphere from indicated genotype and time with markers listed in bottom left. Asterisks indicate the MB NBs and arrowheads indicate some of the ectopically proliferating CB NBs (non-MB NBs). One ectopic CB NB (white box) shown at higher magnification in bottom right. Scale bar (A,G) equals 20 µms and inset (G) equals 10 µms. Panel genotypes listed in Supplemental Table 1.

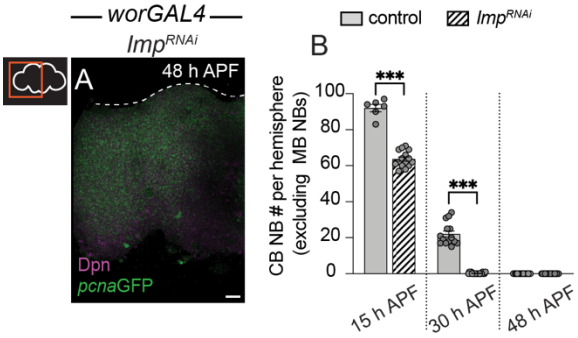

Figure S3: Defects in temporal patterning account for defects in timing of CB NB elimination and neurogenesis termination when Notch activity is reduced. Related to Figure 6.

(A) Maximum intensity projection of single brain hemisphere from indicated genotype at indicated time. (B) Quantification of CB NB number (excluding the MB NBs) in the indicated genotypes. Each data point represents one brain hemisphere. Mean±SEM. ***$P \leq 0.001$, **$P \leq 0.002$, (Kruskal-Wallis ANOVA). Scale bar equals 20 µms. Panel genotypes listed in Supplemental Table 1.