A Notch-dependent transcriptional mechanism controls expression of temporal patterning factors in *Drosophila* medulla

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Abstract

Temporal patterning is an important mechanism for generating a great diversity of neuron subtypes from a seemingly homogenous progenitor pool in both vertebrates and invertebrates. *Drosophila* neuroblasts have been shown to be temporally patterned by sequentially expressed Temporal Transcription Factors (TTFs). These TTFs are proposed to form a transcriptional cascade based on mutant phenotypes, although direct transcriptional regulation between TTFs has not been verified in most cases. Furthermore, it is not known how the temporal transitions are coupled with generation of the appropriate number of neurons at each stage. We use neuroblasts of the *Drosophila* optic lobe medulla to address these questions, and show that the expression of TTFs Sloppy-paired 1/ 2 (Slp1/2) is regulated at transcriptional level directly by two other TTFs and the cell-cycle dependent Notch signaling through two cis-regulatory elements. We also show that supplying transcriptional active Notch can rescue the delayed transition into the Slp stage in cell cycle arrested neuroblasts. Our findings reveal how an interplay between temporal patterning, the neuroblast cell cycle and key signaling pathways

such as Notch achieves precise regulation of patterning transcription factor gene expression that is characteristic of these programs.

Introduction

Generation of a great diversity of neurons from a small pool of neural progenitors is critical for constructing functional nervous systems. This is achieved in part by integration of temporal patterning and spatial patterning of neural progenitors [reviewed in [1-4]]. Temporal patterning of neural progenitors refers to the generation of differently fated progeny in a birth-order dependent manner, and this is observed in both invertebrates and vertebrates [reviewed in [5-7]]. The central nervous system in *Drosophila melanogaster* has been an excellent model to study temporal patterning. Neural progenitors called neuroblasts (NBs) have been shown to sequentially express series of temporal transcription factors (TTFs), which are each required to specify subsets of neuron fates [reviewed in [8,9]]. For example, neuroblasts in the embryonic ventral nerve cord (vnc) are temporally patterned by a TTF cascade Hunchback (Hb), Kruppel (Kr), Nubbin/Pdm2 (Pdm), Castor (Cas) and Grainy head (Grh) [10-13], while *Drosophila* optic lobe medulla neuroblasts utilize a different TTF cascade composed of Homothorax (Hth), SoxNeuro (SoxN), Doublesex-Mab related 99B (Dmrt99B), Odd paired (Opa), Eyeless (Ey), Earmuff (Erm), Homeobrain (Hbn), Sloppy-paired (Slp1,Slp2), Scarecrow (Scro), Dichaete (D), BarH1/2, Tailless (Tll), Glial cell missing (Gcm), and Nerfin-1 [14-19]. In these TTF cascades, cross-regulations were identified among TTF genes based on loss and gain of function phenotypes, and they were proposed to form a transcriptional cascade [11-13,19,20] that can in theory self-propagate [21]. However, with a few exceptions [22], the cis-regulatory elements of these TTF genes haven't been characterized, and thus it is not known whether these cross-regulations are direct

transcriptional regulation. Moreover, in the embryonic TTF cascade, although mis-expression of one TTF is sufficient to activate the next TTF and repress the “next plus one” TTF, loss of one TTF often does not block temporal progression, but only causes the corresponding fates to be skipped [11]. In the medulla TTF cascade, one TTF is required but not sufficient to promote the TTF cascade progression [19]. These studies suggest that additional mechanisms in addition to cross-regulations among TTFs might function to regulate TTF progression.

As the neural progenitors go through the TTF cascade, they need to generate a certain number of postmitotic progeny as each temporal stage. Therefore, it has been questioned whether cell cycle progression is required for the TTF cascade progression. In the embryonic VNC TTF cascade, cytokinesis is required for the first transition (Hb to Kr) by promoting the nuclear export of seven-up (*svp)* mRNA encoding a switching factor [23], but all later temporal transitions progress normally in G2-arrested neuroblasts [24]. In larval VNC NBs, timely transition from the Imp /Castor/Chinmo to Syncrip/Broad stage also requires cell cycle progression [25]. These results suggest that the requirement of cell-cycle progression varies depending on the specific temporal transition. In vertebrate cortical progenitors, the temporal transition from generating deep layer neurons to generating upper layer neurons is not affected by blocking cell cycle progression when constitutively active Notch signaling is provided [26]. However, it is still possible that Notch signaling might be promoting the temporal transition in the absence of cell cycle progression.

Notch signaling plays pleiotropic roles in neurogenesis and neural development in both vertebrates and invertebrates [reviewed in [27]]. Upon binding to a ligand from a neighboring cell, the Notch receptor is cleaved to release the Notch intracellular domain (NICD), which then enters the nucleus and associates with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1). The NICD-CSL then recruits transcriptional coactivator Mastermind (MAM) to activate transcription of Notch target genes, mainly the bHLH transcriptional repressors of the HES (hairy and enhancer of split) /HEY families [reviewed in [28-30]]. Drosophila NBs divide asymmetrically multiple times and at each division generate a self-renewed neuroblast and a more differentiated progeny, that is either an intermediate progenitor [Ganglion Mother Cell (GMC) for type I NBs, Intermediate Neural Progenitor (INP) for type II NBs], or a postmitotic progeny (type 0 NBs) [reviewed in [31]]. During the asymmetric division of Drosophila neuroblasts, Numb, a negative regulator of Notch signaling, is asymmetrically localized to the intermediate progenitor, resulting in unidirectional Delta-Notch signaling from the intermediate progenitor to the neuroblast [32-35]. Ectopic activation of Notch signaling in the progeny causes them to revert back into neuroblasts and over-proliferate, while inhibition of Notch signaling in type II NBs eliminated the whole lineage [36-39]. In contrast, in most type I NB lineages, loss of N signaling does not affect neuroblast maintenance, and it was shown that E(spl) complex proteins and Deadpan act redundantly to maintain the un-differentiated state (stemness) of neuroblasts by repressing differentiation genes [39-41]. Notch signaling is also involved in controlling the daughter cell proliferation modes specifically the type I to type 0 switch in embryonic VNC NBs [42,43]. Work in vertebrate central nervous system suggest that during the asymmetric division of neural progenitors, the more differentiated progeny expresses Delta and

activates Notch signaling in the sister cell to maintain the neural progenitor fate [reviewed in [27]]. In addition, Notch signaling show cell cycle dependent activation and the expression of Notch target Hes genes oscillates during the cell cycle in neural progenitors [reviewed in [44]]. Although Notch signaling has been shown to play many important roles in neural lineage development, whether Notch signaling also has a role in temporal transitions between TTFs hasn't been investigated.

Here, we show that the expression of Slp1/2 in the *Drosophila* medulla TTF cascade is regulated directly by two other TTFs and the Notch signaling pathway. We show that two TTFs, Ey and Scro, and Su(H)/NICD regulate the transcription of Slp through binding to two cis-regulatory elements in the *slp* locus. Slp1/2 expression is delayed when N signaling is lost or when cell cycle progression is blocked which also causes loss of N signaling. Furthermore, we show that supplying transcriptionally active Notch can rescue Slp1/2 expression in cell-cycle arrested neuroblasts. Thus, our work demonstrates that Notch signaling cooperates with TTFs to promote the progression of the TTF transcriptional cascade.

Results

Expression of Slp1 and Slp2 in medulla neuroblasts is regulated at the level of transcription by coordinated function of at least two enhancers.

Observed distribution of temporal patterning transcription factors may be regulated at either the transcriptional or post-transcriptional level, and the protein expression pattern is not necessarily the same as that of the mRNA transcripts. A case in point is the expression pattern

of the SoxN protein recently reported to function alongside Dmrt99B and Hth to specify identities of early-born neurons in the medulla; while mRNA transcripts of soxN gene are widely distributed in neuroblasts of ages, SoxN protein is only expressed in the youngest neuroblasts, suggesting that post-transcriptional mechanisms are at work to confine the actual domain of SoxN protein expression[17]. To examine whether the expression of Slp1/2 in medulla neuroblasts is regulated at transcription we observed the distributions of *slp1* and *slp2* mRNA transcripts by fluorescence *in situ*-hybridization (Fig. 1C-C'''). The observed patterns of slp1 and slp2 mRNA expression closely parallel the expression of Slp1 and Slp2 proteins (Fig. 1B-B'''), and are also corroborated by scRNA-seq data[17], indicating that the expression of *slp1* and *slp2* is directly regulated at transcription.

We then sought to identify transcriptional enhancers that enable expression of the Slp genes in the characteristic pattern observed in medulla neuroblasts. From our examination of expression patterns of GFP in third instar larval brains driven by Gal4 lines under the regulation of putative enhancer elements [45-47] (available from the Janelia FlyLight image database), we identified the GMR35H02 Gal4 line as containing a candidate enhancer driving Slp1/2 expression in medulla neuroblasts. We then verified the expression of UAS-GFP driven by GMR35H02-Gal4 and found that indeed the GFP expression in medulla neuroblasts was initiated at the same time as endogenous Slp1 and Slp2 (Supplementary Fig. S1A-B'). Subsequently, we cloned the 3.75 kbp element corresponding to the GMR35H02 Gal4 regulatory sequence upstream of a GFP reporter gene in the vector pJR12[48] and constructed transgenic lines. In these lines GFP was expressed in the same pattern as the GMR35H02Gal4 driving UASGFP. Through successive iterations of enhancer-bashing experiments whereby we cloned progressively smaller segments

of the initial 3.75 kbp element in the above described vector and observed GFP reporter expression in transgenic flies, we identified a 220 bp fragment within the larger GMR35H02 regulatory DNA which preserved the expression pattern of the GFP reporter (Fig. 1E-E’’’) as that of UAS-GFP driven by the GMR35H02 -Gal4.This we reasoned is a minimal enhancer driving expression of Slp genes in medulla neuroblasts.

Deletion of this element by CRISPR-Cas9 however failed to completely eliminate the expression of Slp1 and Slp2 in neuroblasts (Supplementary Fig. S1C-C’’’). Given the likelihood of developmentally regulated gene expression being controlled by multiple enhancers [49,50] and the importance of enhancer redundance to achieving robust developmental outcomes [49,51,52], this hinted that other enhancers also influenced Slp1/2 expression in medulla neuroblasts. We identified other potential enhancers of Slp1/2 expression from the REDfly database[53,54] and tested their roles in driving Slp1/2 expression in the context of medulla neuroblasts. We screened available transgenic lines expressing GFP or lacZ reporters driven by putative enhancer elements (kind gifts from Dr. Miki Fujioka and Dr. James B. Jaynes) and cloned other candidate regulatory segments in the pJR12 GFP-reporter vector to compare GFP transgene expression to endogenous Slp1/2 expression. We then identified a second regulatory segment corresponding to the REDfly enhancer d5778 (Fig. 1D) that can drive GFP expression in medulla neuroblasts in a pattern that partially overlaps that of endogenous Slp1 and Slp2. Subsequently we found that an 850 bp segment within the d5778 enhancer is sufficient to initiate GFP expression in this pattern (Fig. 1F-F’’’). Reporter expression driven by this second enhancer (henceforth referred to as d5778 850bp enhancer) is initiated at a slightly later time compared to the first identified enhancer, in older Slp expressing neuroblasts and also in glial cells at the

junction of the medulla and the central brain. Our analysis of the REDfly enhancers that supposedly regulate expression of Slp1/2 also revealed that the earlier identified 220 bp enhancer segment is contained within another REDfly enhancer named u8772 (Fig. 1D). Thus, we refer to the first identified enhancer henceforth as u8772 220bp. As in case of the u8772 220bp enhancer deletion, sole deletion of d5778 enhancer by CRISPR-Cas9 again did not abolish Slp1/2 expression(Supplementary Fig. S1D-D'''), suggesting that these enhancers function redundantly and may make additive contributions to attaining the observed levels of Slp1 and Slp2 expression.

Bioinformatic analyses of the sequences of our identified enhancers (see Methods) identified binding sites for the previous temporal transcription factor Ey, the CSL transcription factor Su(H), Slp1 itself, and a Nk2-class homeodomain transcription factor Ventralized (vnd) (Supplementary Table 1). The previous TTF Ey has been shown to be required but not sufficient for activating Slp genes' expression in medulla neuroblasts, but it was not known whether this regulation is direct. Vnd is not expressed in medulla neuroblasts according to our single-cell RNA-sequencing data[17] but a related homologous NK-2 homeobox transcription factor Scarecrow[55] (Scro) is expressed around the same time as Slp1 [16,17]. The CSL transcription factor Su(H) is the Drosophila homolog of RBP-J[56,57], and the key DNA-binding component of the active Notch transcriptional complex. The Notch signaling pathway plays an overarching role as a master regulator of development and differentiation in neuroblasts, but it hasn't been shown to regulate the expression of temporal patterning genes in medulla neuroblasts. Therefore, we next tested whether Slp1/2 expression is regulated by Notch signaling.

<u>The Notch pathway regulates Slp1 and Slp2 expression in medulla neuroblasts</u>

We observed expression of Slp1 and Slp2 in RNAi knockdown clones of key Notch pathway components induced by *ayGal4* (*actin>FRT-y$^+$-STOP-FRT-Gal4*, in which *actin* promoter drives Gal4 expression only after a STOP cassette is excised by the action of heat shock activated Flippase)[58]. Since Slp1 and Slp2 are two homologous genes located in proximity [59] and expressed in patterns very similar to one another with the timing of Slp1 expression slightly preceding that of Slp2 expression (Fig. 1B-B'''), in all subsequent figures we have shown data for Slp2 only with the assumption that Slp1 expression reflects the same pattern. On inducing RNAi knockdowns of the critical Notch pathway components Notch and Su(H) – which are part of the tripartite core of the active Notch transcriptional complex [60], it is seen that Slp2 expression is delayed within the RNAi knockdown clones marked by GFP expression (Fig. 2A-A''', 2B-B''' respectively) compared to wild type neuroblasts of the same age (i.e., in the same vertical 'stripe'). A similar effect is seen when dominant negative variant of the third core component of the Notch transcriptional complex mastermind (mam) is expressed under the control ayGal4 (Fig. 2C-C'''). These data suggest that timing of Slp1/2 expression in medulla neuroblasts is regulated by the Notch pathway.

To identify the cognate ligand of Notch that functions in activating the Notch signaling in medulla neuroblasts, we examined expression of known Notch ligands- Delta [61], Serrate[62,63] and Weary[64] in medulla neuroblasts (data not shown). Of these only UAS-GFP driven by Delta-Gal4 was seen to be expressed in medulla neuroblasts and in their progeny GMCs. We then examined whether knockdown of Dl recapitulates the effects we observe on Slp1/2 expression with loss of function of core Notch transcription complex components, if indeed Dl is required for activating Notch signaling in these cells. RNAi knockdown of Dl resulted in a similar delay in

initiating Slp2 expression as with loss of function of other key actors in the Notch pathway (Fig. 2D-D’’’) confirming this expectation.

Notch pathway, Ey and Scro regulate the activity of slp enhancers.

Since Su(H), Ey and Scro are all required for Slp expression, we next tested whether these regulations are mediated through the identified enhancers. We tested whether knockdown of each factor would affect GFP reporter expression driven by these enhancers. We reasoned that if any of the factors we had identified binding sites for bound to the enhancers and enabled them to initiate Slp1/2 transcription, GFP reporter expression driven by these enhancers would be lost in loss of function clones of these transcription factors. We induced expressions of RNAi knockdowns for Ey, Su(H) and Scro using ayGal4 in transgenic flies expressing wild type GFP reporters driven by these two enhancers and compared the expression of GFP in wild type neuroblasts and in neuroblasts where each of these TFs had been individually knocked down.

For both enhancers, GFP reporter expression was lost in eyRNAi clones (Fig 3A-A’ and Fig B-B’) confirming an earlier observation that Ey was required for initiating Slp1/2 expression. It also strengthened the evidence that these two were indeed bona-fide enhancers activating Slp1/2 expression. Similarly, GFP reporter expression was lost or delayed in Su(H) RNAi clones for both enhancers (Fig 3C-C’ and Fig 3D-D’). We also tested the effect of loss of Slp1/2 function on the expression of our GFP reporters. To this end we induced Slp1/2 loss of function (LOF) mutant clones by MARCM in UbiRFPFRT40A; GFP reporter expressing transgenic flies. In Fig.3D-D’ and Fig. 3E-E’ Slp LOF clones are visible as dark regions whereas wild type neuroblasts are marked by RFP expression from the Ubiquitin promoter. Reporter expression driven by both enhancers

within Slp LOF neuroblasts is normal as in wild type neuroblasts. Though this suggests that Slp1/2 are not required to initiate their own expression, it does not preclude a possible role of Slp1/2 in sustaining their own expression in older Slp1/2 stage neuroblasts or progeny, but the perdurance of the GFP reporter prevents us from testing this possible role. In summary, our genetic experiments confirmed that identified enhancers of *slp1/2* respond to activation by Ey, Su(H) and Scro.

Mutations of potential binding sites for Ey, Su(H) and Scro in slp1/2 enhancers impair expression of the reporters

To further test whether the regulators we identified indeed regulate Slp1/2 expression directly through binding to the enhancers, we set out to mutate all possible binding sites for each of our regulators- Ey, Su(H), Scro and Slp1 on each enhancer. We identified all potential matches to consensus binding motifs for these factors using the DNA Pattern Recognition Tool within the Sequence Manipulation Suite[65]. Custom-designed gene blocks containing the mutated enhancer sequences were cloned upstream of a GFP reporter in pJR12 vector as before, and transgenic flies expressing these reporter constructs were then made (Supplementary Table1). We next compared the expression patterns and intensities of GFP reporters with mutated TF binding sites against GFP reporter expression driven by the 'wild type' version of that same enhancer(Fig. 4A-A''', Fig. 5A-A'''). To compare GFP reporter intensities between wild type and mutated variants of an enhancer, identical microscope illumination and imaging settings were used for all variants of the same enhancer.

Mutation of all potential Ey binding sites nearly eliminated GFP expression for both enhancers (Fig. 4B-B’’’ and 5C-C’’’), as did the mutation of all possible Su(H) binding sites (Fig. 4C-C’’’ and 5D-D’’’). For the d5778 850 bp enhancer, the expression in glial cells are still present when Su(H) binding sites are mutated. Mutation of scro sites in the u8772 220bp enhancer also led to a loss of GFP expression (Fig. 4D-D’’’), whereas GFP expression was considerably weakened in the d5778 850bp scro mutant enhancer (Fig. 5B-B’’’). In contrast, mutation of Slp1 binding sites did not significantly affect the reporter expression (shown for u8772 220bp enhancer in 4E-E’’’). In summary, our results provided strong evidence that Ey, Su(H) and Scro directly regulate Slp1/2 expression through binding to its two enhancers.

DamID-seq confirms binding of Ey and Su(H) to identified enhancers of Slp1/2.

Genome-wide binding patterns of Ey and Su(H) in medulla neuroblasts was inferred by DamID-seq[66,67] by driving the expression of UAS-DamSu(H) or UAS-DamEy fusion proteins in medulla neuroblasts of transgenic flies using tubG80ts; SoxN-Gal4 induced at 29 °C for 72 hours. For each transcription factor two biological replicates were sequenced. Peaks of Ey and Su(H) binding were observed in both replicates in the genomic region corresponding to the identified enhancers of Slp1/2 transcription (Fig. 6A). Additionally, we also observed peaks of Ey and Su(H) binding in a region mapping to an intron within the *scro* gene (Supplementary Fig. S2A). It has been reported that Scro expression is initiated in medulla neuroblasts at about the same time as Slp1/2 expression is initiated. This also raises the possibility that Scro expression may be initiated by either Ey, Su(H) or both. In another temporal gene *homeobrain* (*hbn*) that is turned on in the Ey stage [16,18], Ey and Su(H) binding peaks were also observed (Supplementary Fig. S2B). Genome-wide about 66% of all Su(H) bound genes and 71% of all Ey bound genes were

bound by both Ey and Su(H) (Fig. 6B). Enriched GO terms for these co-bound genes include: neuroblast fate determination; neuroblast development, regulation of stem cell division, regulation of G1/S transition of mitotic cell cycle and Notch signaling pathway (data not shown). Taken together these data support evidence from genetics studies we have conducted that suggest Ey and Su(H)/N pathway activate Slp1/2 transcription by direct binding to Slp1/2 enhancers.

Notch signaling is dependent on cell-cycle progression.

Although it has been well-established that GMC generated by the asymmetric division of neuroblasts signal to their sister neuroblast to provide the Notch signaling[32,33], it hasn't been demonstrated whether Notch signaling is lost in cell-cycle arrested medulla neuroblasts since they fail to generate GMCs. We used a regional Gal4, *vsxGal4* that is expressed in the center domain of the medulla crescent starting in the neuroepithelium[68], to drive UAS-DCR2 and a RNAi line against Proliferating Cell Nuclear Antigen (PCNA),which is essential for DNA replication and S phase progression[69]. Blocking cell cycle progression caused premature transformation of neuroepithelial cells to neuroblasts, as has been previously reported [70]. These neuroblasts express the normal neuroblast marker Deadpan (Dpn) (Fig. 7A-A''), but produce few progeny, and some neuroblasts are present in the deep layers which would normally be occupied by neuronal progeny. A large fraction of these neuroblasts in the central domain don't express E(spl)mγGFP, a Notch signaling reporter [61], while all neuroblasts in the control regions express E(spl)mγGFP (Fig. 7A-A''). Thus, Notch signaling is largely lost in cell-cycle arrested neuroblasts.

The role of cell cycle progression in the medulla TTF cascade.

Next we tested whether blocking cell-cycle progression also affected the medulla TTF cascade progression. In *vsxGal4>PCNA RNAi* brains, neuroblasts in the affected region do not express either Ey or Slp1/2, but keep expressing SoxN (Fig. 7B-C''). This suggests that an earlier temporal transition step is blocked which is required for Ey expression, and this prevents us from examining whether the Ey to Slp transition also requires cell cycle progression. To circumvent this problem, we used the *ay*Gal4 to drive *UAS-DCR2* and PCNA RNAi. By adjusting heat shock timing to induce clones, we were able to obtain a condition where Ey expression is minimally affected, and at this condition we observed that Slp2 expression is still severely delayed (Fig. 7E-E'''). In addition to the timing of clone induction, the *ayGal4* is also likely to be weaker than VsxGal4 at inducing RNAi, and the cell cycle is likely only slowed down rather than arrested when using *ayGal4*, because there still are some progeny produced in such clones. Furthermore, Slp1/2 expression is also delayed when we knock down String /Cdc25 required for the G2 to M phase transition[71], or overexpress Dacapo (Dap, a Cyclin-dependent kinase inhibitor in the CIP/KIP family)[72] using *ayGal4* (Supplementary Figure S3A-B''). Thus, cell cycle progression is also required for the precise timing of Ey to Slp1/2 transition.

Notch signaling can rescue Slp expression delay caused by cell cycle defects.

Finally, we tested if supplying Notch signaling to cell-cycle arrested or delayed neuroblasts can rescue the timing of Slp expression initiation. We supplied active Notch in neuroblasts using a *dpn>FRT-stop-FRT3-FRT-NICD* (drives the expression of NICD from the *dpn* enhancer in presence of heat shock activated Flippase; see Methods) while simultaneously inducing

knockdown of PCNA using ayGal4 under the same heat shock condition that leaves Ey expression largely unaffected. Supplying NICD was sufficient to rescue Slp2 expression in PCNA RNAi clones. This suggests that for the Ey to Slp transition, the presence of active Notch signaling can substitute for the requirement for the cell-cycle progression.

Discussion

Direct transcriptional activation in the TTF cascade.

Drosophila neuroblasts are temporally patterned by sequentially expressed TTFs. Although the expression pattern and mutant phenotypes suggest that TTFs form a transcriptional cascade, direct transcriptional regulation has not been demonstrated in most cases. In this work, we characterized two enhancers of the *slp* gene, in which binding sites for its previous TTF, Ey, were identified. The enhancer activity is lost when Ey is knocked down by RNAi in the brain, suggesting that this enhancer requires Ey protein to be activated. Furthermore, mutagenesis of Ey binding sites in each enhancer abolished the enhancer activity, and Dam-ID experiment confirmed that Ey indeed binds to the *slp* locus *in vivo*. Taken together, our study demonstrated for the first time that one TTF Ey directly activates the expression of the next TTF, Slp, by binding to its enhancers. In addition, another TTF Scro, which is activated at a similar time as Slp1/2, is required for Slp1/2 expression to reach the full level. Scro binding sites were also identified and mutagenesis of the binding site reduced the reporter expression level.

Notch signaling directly regulates the transcription of a TTF gene

Although the previous TTF Ey is necessary to activate Slp, over-expression of Ey is not sufficient to activate Slp expression right away. There is always a time delay after the start of Ey expression to the start of Slp expression to ensure the sufficient duration of the Ey window. This suggests that other mechanisms are at play in the regulation of Slp1/2 expression. Surprisingly, we identified multiple binding sites for Su(H) in both enhancers of *slp* gene locus. Then we showed that loss of N pathway components indeed caused a delay in Slp1/2 expression, and the activity of both enhancers also respond similarly to loss of N signaling. Consistent with a direct transcriptional regulation, mutagenesis of Su(H) binding sites attenuated the enhancer activity. Finally, Dam-ID experiment confirmed that Su(H) binds to the *slp* locus in vivo. Thus, our work provided strong evidence that N signaling, a general signaling pathway involved in the asymmetric division of neuroblasts, functions to speed up the activation of a TTF gene. This might be part of mechanisms to promote TTF cascade progression specifically in neuroblasts, where Notch signaling is active.

Cooperative regulation of target genes by the TTF Ey and Notch signaling

As observed in the enhancer sequences, as well as in the Dam-ID data, Ey binding sites and Su(H) binding sites are clustered together in the *slp* enhancers, suggesting that Ey and Notch signaling cooperate in the activation of the *slp1 and slp2* genes, and Ey might act to provide the specificity for the regulation. In addition to the *slp* genes, Dam-ID experiments of Ey and Su(H) showed that ~66% of all Su(H) bound genes and 71% of all Ey bound genes were bound by both Ey and Su(H), and these target genes are involved in neuroblast development. These data suggest that the TTF Ey and Notch signaling may bind to enhancers of target genes

cooperatively, providing a possible mechanism for activating target genes specifically in neuroblasts where Notch signaling is active. It has been shown in *Drosophila* embryos that tissue-specific transcription factors prime the enhancers which promotes synchronized and sustained activity in response to NICD [73]. It is possible that the previous TTF Ey may prime the enhancers of *slp1/2* and other target genes to facilitate its response to Notch signaling.

The role of cell cycle progression and Notch signaling in the medulla TTF cascade

Notch targeting genes and Dpn are transcriptional repressors that act partially redundantly to maintain neuroblast identity. In type II NBs, Dpn is dependent on Notch signaling, and loss of Dpn causes premature differentiation[37,74]. However, in type I NBs, Dpn is not lost when N signaling is lost, and N signaling seems dispensable for the self-renewing abilities of NBs[39-41]. In the medulla neuroblasts, we also observed that in *Su(H)* mutant clones, the clone size is not significantly affected, in contrast to the reduced clone size in cell cycle gene mutants. On the other hand, we observed that N signaling is dependent on cell-cycle progression, and N target gene is lost when cell cycle progression is blocked.

In the medulla, blocking cell cycle progression in neuroepithelial cells prematurely transform them into neuroblasts, and these neuroblasts seem to be arrested in the TTF cascade at the SoxN stage. The expression of Ey and Slp are never turned on in such neuroblasts. When we arrested or slowed down the cell cycle later in neuroblasts to preserve Ey expression, we observed Slp expression is still delayed. Therefore, cell cycle progression also has a role in the Ey to Slp transition. Further we showed that supplying Notch signaling is sufficient to rescue the delay in turning on of Slp expression caused by cell cycle defect. Taken together, these results

suggest that at the Ey to Slp transition, the cell cycle effect is mediated through the direct regulation of Slp transcription by Notch signaling. This can be part of a mechanism to coordinate the TTF temporal transition to the cell cycle progression to generate neural progeny.

Methods

Fly stocks and genetics

Flies were reared on yeast food at 25°C unless otherwise stated.

Enhancer identification- Flies carrying the *GMR35H02-Gal4* insertion (BDSC 49923) were crossed with transgenic flies expressing *UAS GFPnls* (BDSC 4776). GFP driven by the *GMR35H02-Gal4* was then compared to endogenous Slp1 and Slp2 expression. Procedure for making transgenic constructs and strains is described separately.

RNAi experiments- The following RNAi lines were used for RNAi knockdown experiments (In Fig 2); *UAS-ey-RNAi* (BDSC 32486), *UAS-scro*^{RNAi} (BDSC 33890), *UAS-N-RNAi* (BDSC 7078), *UAS-Su(H)-RNAi* (VDRC 103597), *UAS-Dl-RNAi* (VDRC 32788), *UAS-PCNA-RNAi* (VDRC 51253).

ayGal4 (*actin*>FRT-y^{+}-STOP-FRT-Gal4, in which *actin* promoter drives Gal4 expression after a STOP cassette is excised by the action of heat shock activated Flippase) was used to drive RNAi of *ey*, *scro* and *su(h)*. Flies of genotype *ywhsFlp; ayGal4>UAS GFP; UAS Dcr2/Tm6B* were crossed to the RNAi lines. Larvae were heat shocked at 37°C for 10 minutes 48 hours after egg laying and then raised at 29°C, until brains of third instar larvae were dissected and stained.

For observing effect of *ey RNAi* on the GFP reporter expression (Fig 3) flies of genotype *ywhsFlp; ayGal4> lacZ; eyRNAi* (made by combining *ayGal4>UAS lacZ* on chromosome II (BDSC 4410)

with *UAS-ey RNAi* on chromosome III) were crossed to flies with genotype *ywhsFlp; Sp/CyO; u8772 220bp>GFP* (enhancer1) and *ywhsFlp; Sp/CyO; d5778 870bp>GFP* (enhancer2).

For studying effects of *Su(H) RNAi* on GFP reporter expression, flies of genotype *ywhsFlp; ayGal4>lacZ; Tm2/Tm6B* were crossed to flies of genotype *Dcr2; Su(H) RNAi; u8772 220>GFP* (enhancer 1) or *Dcr2; Su(H) RNAi; d5778 870> GFP* (enhancer 2).

For studying effects of *scro RNAi* on GFP reporter expression, flies of genotype *ywhsFlp; ayGal4>lacZ; scro RNAi / Sm6-Tm6B* were crossed to flies of genotype *ywhsFlp; Sp/CyO; u8772 220bp>GFP* (enhancer1) and *ywhsFlp ;Sp/CyO; d5778 870bp>GFP* (enhancer2).

Heat shock protocols for RNAi experiments driven by *ayGal4* in presence of GFP reporters were the same as described above for *ayGal4* driven RNAi by themselves (i.e., without GFP reporters).

<u>Cell cycle arrest experiments</u>- For some experiments examining effect of cell cycle arrest on Slp1/2 expression and on expression of the Notch reporter E(spl)-mγ, *VsxGal4* was used to drive the expression of *UAS PCNA RNAi* and *UAS Dcr2* in neuroblasts. Flies of genotype *VsxG4; E(spl)-mγGFP;UAS Dcr2/Tm6B* were crossed to those with genotype *ywhsFlp; UAS-PCNA RNAi/CyO; T2/Tm6B*. Larvae were shifted to 29°C 48 hours after egg laying. Brains of third instar larvae were observed. For other experiments, flies of genotype *ywhsFlp; UAS-PCNA RNAi* were crossed to flies of genotype *ywhsFlp; ayGal4>UAS GFP; UAS Dcr2/Tm6B*. Larvae with genotype *ywhsFLP; ayGal4UASGFP /UAS-PCNARNAi; UASDCR2/+* were heat shocked for 8 minutes at 37°C 50 hours after egg laying (70 hours before they develop into climbing third instar larvae) and were then dissected. For rescuing Notch expression in cell cycle arrested neuroblasts flies of genotype

ywhsFlp; ayGal4>UAS GFP; UAS Dcr2/Tm6B were crossed to those with genotype *ywhsFlp; UAS PCNA RNAi; dpn>hsNICD*. Larvae of genotype *ywhsFLP; ayGal4UASGFP /UAS-PCNARNAi; UASDCR2/dpn>hsNICD* were then heat shocked for 8 minutes at 37°C 50 hours after egg laying (70 hours before the third instar stage) as before and then dissected.

Negative staining of Slp loss of function MARCM clones- *UbiFRT40ARFP/CyO* flies (BDSC 34500) were crossed with flies of genotype *ywhsFlp;Sp/CyO; u8772 220bp>GFP* (enhancer1) and *ywhsFlp ;Sp/CyO; d5778 870bp>GFP* (enhancer2) to create strains with genotypes *ywhsFlp; Ubi>RFPFRT40A; u8772 220bp>GFP* and *ywhsFlp; Ubi>RFPFRT40A; d5778 870bp>GFP* respectively. Flies of genotype *ywhsFlp; SlpS37A/Sm6-Tm6B* flies (a kind gift from Dr. Andrew Tomlinson) were then crossed to *ywhsFlp; Ubi>RFPFRT40A; u8772 220bp>GFP* and *ywhsFlp; Ubi>RFPFRT40A; d5778 870bp>GFP* and larvae were heat shocked for 45 minutes 48 hours after egg laying. Third instar larvae were then dissected. The *ywhsFlp; SlpS37A/Sm6-Tm6B* strain carries a deletion on chromosome II that spans both Slp1 and Slp2 genes. Clones carrying two copies of this deficiency are seen as dark regions amidst Ubi>RFP marked wild type neuroblasts.

Bioinformatic identification of TFBS

Initially, to identify a list of possible candidate TFs with binding sites within our enhancers we analyzed our enhancer sequences using the MEME suite tools[75] and TOMTOM[76] and the also by an analytic tool developed at EPFL (https://biss.epfl.ch) that uses FIMO[77] to identify motifs on a sequence (Michael Frochaux, Dr.Bart Deplancke personal communication) to complement our findings from TOMTOM. However mutating sites identified by this initial round of analysis did not always result in GFP reporter expression patterns that were consistent with our

observations from the genetic experiments (data not shown). We suspected this could be because these analyses may have failed to exhaustively identify all potential binding sites for our regulators. To this end we employed a slightly different strategy to identify all possible binding sites of Ey, Su(H), and Scro whose knockdown had affected the expression of GFP reporters in genetic experiments. We searched for consensus motifs of transcription factor binding sites for Ey, Su(H), Slp1 in JASPAR[78,79] and Fly Factor Survey[80] and then searched for potential matches to these consensus sequences in our enhancers using the DNA Pattern Recognition program in Sequence Manipulation Tool[65]. Since consensus motifs for Scro binding sites were not annotated in either JASPAR or Fly Factor Survey we instead picked 20 related transcription factors of the NK-domain TF family whose consensus binding motifs were annotated. We looked for potential matches to these motifs within our enhancers using the SMS Pattern Recognition Tool of the Sequence Manipulation Suite. Sequences that were most shared between the 20 NK domain containing TFs we examined were scored as the most likely motifs for d5778 870bp enhancer. Given the smaller size of the u8772 220bp enhancer, only motifs corresponding to vnd binding sites were mutated since Scro has been suggested to share the greatest homology to vnd of all other NK domain containing TFs.

Plasmids constructs and making transgenic fly stocks

Primer sequences for all cloning are provided in a supplementary table (Supplementary Table 2).

For making all constructs DNA was amplified from template using Expand High-Fidelity Polymerase (Roche) unless stated otherwise and all constructs were verified by sequencing. All

fly embryo injections for making transgenic flies were carried out by Bestgene Inc, Chino Hills, CA.

Generating constructs for reporter assays and enhancer bashing and making transgenic reporter expressing stocks- Sequences corresponding to the fragments of regulatory DNA encoded in GMR35H02 and the d5778 REDfly enhancer were cloned from the BAC clone CH321-94O18[81] (BACPAC resources). Sequences were PCR amplified from this BAC and cloned into pJR12 vector (a kind gift from Dr. Jens Rister) between *AscI* and *NotI* sites. Transgenes were inserted at the landing site VK00027 on the third chromosome (BL9744) by ϕC31 integrase mediated transgenesis[82] and positive transformants were screened using the w+ marker originally present in the pJR12 plasmid. Mutated enhancers were custom synthesized as gene blocks (gBlocks,IDT DNA) and cloned as stated above. Split gBlocks were custom made for making the d5778 ey site mutant and the d5778 su(h) site mutants. These split gBlocks were then PCR spliced and cloned between NotI and AscI sites of the pJR12 vector. A custom-made gBlock was used for making the d5778 scro mutant reporter. All reporters were integrated at the same genomic site to ensure comparability across constructs and experiments.

Generation of CRISPR enhancer deletion constructs and transgenic stocks- CRISPR gRNAs were designed by entering sequences of genomic DNA of the target +/- 20 kbp into the CRISPR Optimal Target Finder web utility [83] (https://www.targtfinder.flycrispr.neuro.brown.edu). Four gRNAs-two upstream and two downstream of the target region to be deleted were then selected. All four gRNAs were then cloned into the vector pCFD5[84] (Addgene plasmid #73914 a kind gift from Dr. Simon Bullock) using NEB-Builder HiFi DNA Assembly master mix. Constructs were then injected into fly strain containing *M{nos-cas9}ZH-2A* (BL54591) along with a 120 bp

repair oligonucleotide that contained 60bp of wild type genome sequence flanking both ends of the expected double-stranded break. After injection, the *nos-cas9* source was eliminated in the subsequent generations by crossing individual G0 progeny flies to a double balancer of genotype *ywhsFlp; Sp/CyO; Tm2/Tm6B* and selecting male G1 progeny. Individual G1 males were then crossed to the same double balancer line to create stocks. The G1 males were then genotyped by PCR to identify whether their genome had been edited, using RedExtract-n-Amp tissue PCR kit (Sigma-Aldrich). G2 progeny of genome-edited males were then raised until homozygous stocks were established.

Generation of the heat shock inducible NICD construct: We modified the pJR12 vector used in enhancer bashing experiments to replace the GFP coding sequence with coding sequences of our interest. First, we generated a DNA fragment that matched the sequences of the pJR12 vector flanking the eGFP sequence but that did not contain the eGFP sequence itself by PCR splicing. We then cut-out the eGFP segment from the pJR12 vector and replaced it with our GFP deleted fragment using *NsiI* and *XhoI*. Next, an *FRT-stop-FRT3-FRT* segment was cloned from the CoinFlp plasmid[85] (Addgene plasmid # 52889, a kind gift from Dr. Iswar Hariharan) and PCR spliced with a fragment of Notch Intracellular Domain (NICD). Sequences corresponding to the Notch Intracellular Domain were PCR amplified from Notch cDNA LD34134 (DGRC). The *FRT-stop-FRT3-FRT-NICD* fragment was then cloned into the modified pJR12 vector between the *PmeI* and *AgeI* restriction sites. The deadpan enhancer sequence amplified from the BAC clone CH321-86A18 (BACPAC resources) was then cloned into the modified pJR12 vector to drive the heat shock inducible NICD expressing construct in presence of a heat shock activated Flippase or *hsFlp* (this construct is abbreviated as *dpn> hsNICD*). Construct was inserted at the VK00027

landing site (BL 9744) by ϕC31 integrase mediated transgenesis. Positive transformants were identified by the w+ eye color marker expression and stocks were created by crossing to a double balancer of genotype *ywhsFlp; Sp/CyO; Tm2/Tm6B*.

Generation of DamID-fusion constructs (*UAS Dam-Ey* and *UAS-Dam-Su(H)*) and transgene expressing stocks- Su(H) coding sequence was amplified from a cDNA clone GH10914 (DGRC) by PCR. The amplified Su(H) coding sequence was cloned into the pUAST-Dam-attB vector[67] (a kind gift from Dr. Andrea Brand) between the *NotI* and *XhoI* sites. A gBlock of Ey coding sequence was ordered from IDT and cloned into the pUAST-Dam-attB vector. Constructs were then incorporated at the VK00027 landing site (BL 9744) by ϕC31 mediated integration. Transformants were identified by expression of the w+ marker gene. Final stocks were established by crossing to a double balancer line of genotype *ywhsFlp; Sp/CyO; Tm2/Tm6B*.

Immunofluorescence staining- Antibody staining was carried out as described in [19] with a few modifications. The protocol is described briefly as follows: brains from climbing third instar larvae were dissected in 1XPBS and fixed in 4% Formaldehyde solution in 1X PBS for 30 minutes on ice. Brains were then incubated in primary antibody solution overnight at 4°C, washed three times for 30 minutes each at 4°C, then incubated in fluor conjugated- secondary antibody solution overnight at 4°C and then washed again thrice at room temperature each time for 30 minutes. Samples were mounted in Slowfade Gold antifade reagent (Invitrogen). Images are acquired using a Zeiss LSM500 Confocal Microscope.

Antibodies used in the study are as follows: rabbit anti-Slp1, guinea-pig anti-Slp2, rabbit anti-Ey (all used at 1:500) were kind gifts from Dr. Claude Desplan, guinea-pig anti-Dpn (1:500) (a kind

gift from Dr. Chris Doe). Commercially available antibodies include - sheep anti-GFP (1:500, AbD Serotec, 4745-1051), Chicken anti-beta-gal (1:500, Abcam ab9361), Rat anti-Deadpan [11D1BC7] (1:200, Abcam, ab195173) mouse anti-PCNA (1:50, Abcam ab29). These antibodies are provided by the Developmental Studies Hybridoma Bank (DSHB): mouse anti-eyeless (1:10), mouse anti-Pros (MR1A 1:20). Secondary antibodies are from Jackson or Invitrogen.

Fluorescence In situ hybridization (FISH)- FISH was carried out using custom made Stellaris probe sets as described in [86] except that incubation with sodium borohydride was skipped. Probes were generated using the slp1 mRNA and slp2 mRNA as templates. Concurrent FISH and immunofluorescence staining was carried out as described in the same publication.

DamID-Seq- Flies of genotype *ywhsFlp; Sp/CyO; UAS-Ey-Dam/ Tm6B* or *ywhsFlp; Sp/CyO; UAS-Su(H)-Dam /Tm6B* were crossed with *Dcr2; tubG80ts; SoxNGal4*. Mating crosses were incubated at 25 °C. Eggs laid within an 8-hour window were then shifted to 18°C for 3 days until they hatched. The expressions of the Dam-fusion proteins were then induced in larval brains by shifting the larvae to 29°C for 72 hours. Climbing third instar larvae were then dissected. Only the optic lobes were used for further sample preparation. For each sample around 100 brains were dissected.

Dam-ID libraries were made as described in [87]. Briefly, fly brains were dissected in 1X PBS and genomic DNA was isolated using QIAamp DNA Micro kit (Qiagen). Genomic DNA was then digested with DpnI and purified using spin column purification (Qiagen PCR-purification kit). To the DpnI digested DNA DamID-PCR adaptors were ligated. Subsequently the adaptor ligated DNA fragments were digested with DpnII. The DpnII digested genomic DNA fragments were

then amplified by PCR to enrich for bound GATC fragments. To make sequencing libraries the PCR enriched genomic DNA sample was sonicated using a Bioruptor bath sonicator (Diagenode), purified using AMPure XP beads, end-repaired and 3'-adenylated. Illumina sequencing indexes were then ligated to these fragments and the fragments. Index-labeled DNA fragments were then further enriched by PCR amplification, checked for quality and fragment size distribution on an Agilent Bioanalyzer and by qPCR (Quality control of all libraries was carried out at the Functional Genomics Unit of Roy J. Carver Biotechnology Center, UIUC). Libraries that were deemed acceptable were then sequenced on a single SP100 lane of an Illumina NovaSeq sequencer. Read lengths were 100bp. Two replicates each of *Ey-Dam*, *Su(H)-Dam* and *Dam* only samples were sequenced. Number of reads obtained were as follows: Dam replicate1: 53745480 reads, Dam replicate 2: 85637355 reads, Ey-Dam replicate 1: 81121107 reads, Ey-Dam replicate 2: 82085708 reads, SuH-Dam replicate 1: 77448608 reads, replicate 2: 80015207 reads. All samples exhibited good QC scores.

DamID-Seq data analysis- DamID-seq data was analyzed using the damidseq-pipeline [88]. Duplicate samples of Ey-Dam and Su(H)-Dam samples and Dam-only controls were aligned to the Drosophila reference genome UCSC dm6. Alignment rate was around 96% throughout for all samples. The main utility of the damid-seq pipeline was used to align reads to the genome using bowtie2, bin and count reads, normalize counts and generate log-2 ratio bedgraph files for each DamID-sample and its corresponding Dam-only control. The provided gatc.track file was used for running the script. Next, the findpeaks utility was used with an F.D.R. <0.01 to identify peaks. We then used the provided peaks2genes utility to assign peaks to genes. To assess the reproducibility of our data we also ran the findpeaks script using F.D.R <0.1 to

discover peaks with weaker statistical confidence and used this as input for the I.D.R. Python package (https://github.com/nboley/idr). 984 of 1810 (54.4.%) Ey-Dam and 972 of 1996 (48.7%) of Su(H)-Dam peaks passed an I.D.R. threshold of 0.05. Lists of genes bound by both replicates of Ey-Dam and by Su(H)-Dam were used in BioVenn[89] to visualize the overlap between these two lists and estimate overlap percentage. All replicate tracks for Ey and for Su(H) about genomic loci of interest were individually visualized in IGV [90].

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Competing interests.

The authors declare no competing or financial interests.

Materials & Correspondence. Publicly available fly lines (those from BDSC or VDRC) should be requested directly from the corresponding stock centers: https://bdsc.indiana.edu/ or

https://stockcenter.vdrc.at/control/main. Fly lines generated in this study can be requested without restriction. Correspondence and requests for materials should be addressed to Xin Li (lixin@illinois.edu).

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Figure 1

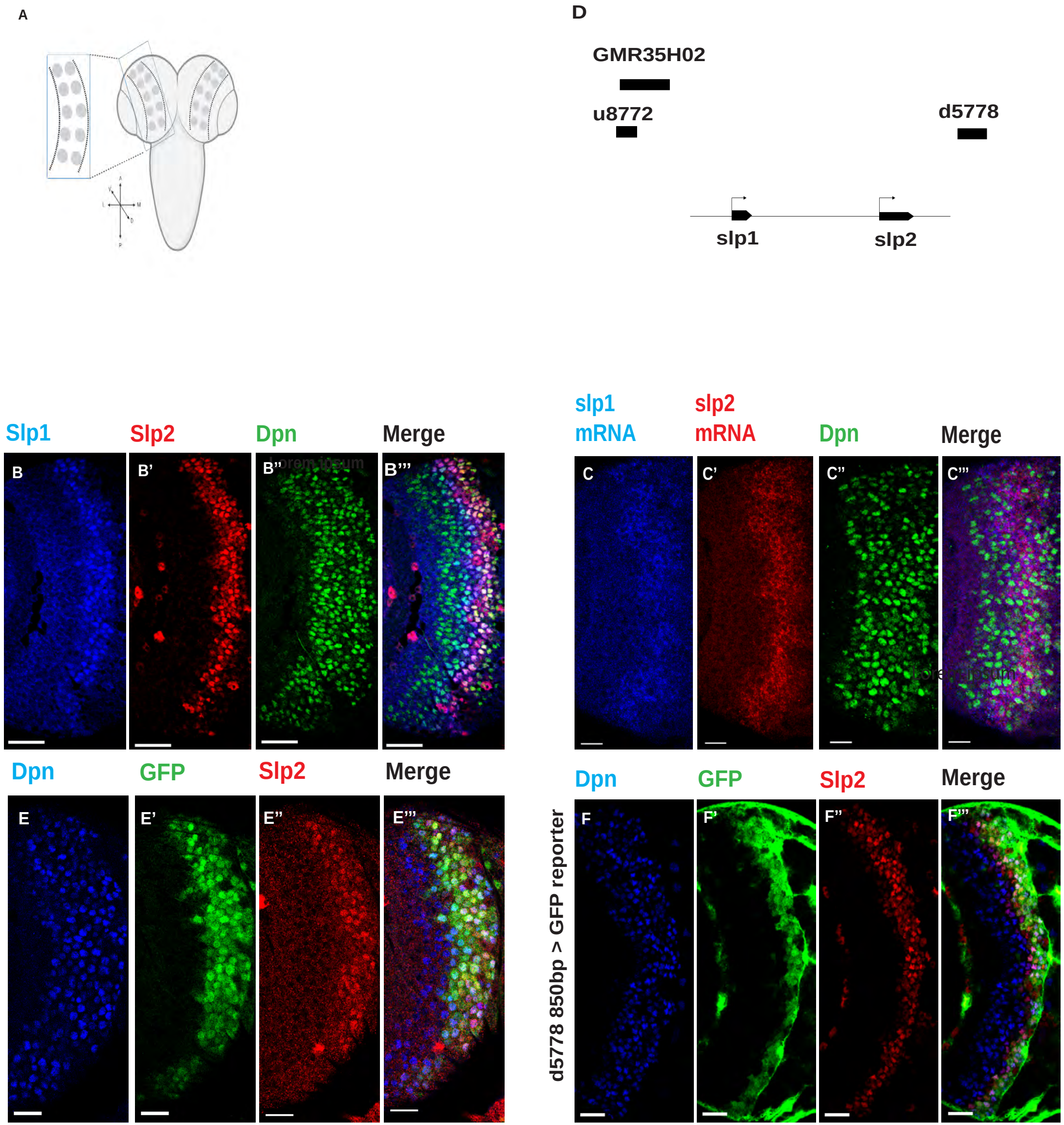

A
D
GMR35H02
u8772
d5778
slp1
slp2
Slp1
Slp2
Dpn
Merge
B
B'
B''
B'''
slp1 mRNA
slp2 mRNA
Dpn
Merge
C
C'
C''
C'''
Dpn
GFP
Slp2
Merge
u8772 220bp > GFP reporter
E
E'
E''
E'''
Dpn
GFP
Slp2
Merge
d5778 850bp > GFP reporter
F
F'
F''
F'''

Figure 1. Expression of Slp1 and Slp2 genes in medulla neuroblasts is controlled at transcription and achieved by coordinated action of at least two transcriptional enhancers. (A) Schematic of Drosophila brain at the third instar larval stage highlighting the location of the optic lobe medulla. Medulla neuroblasts are superficially located on the ventral surface of the optic lobe. (B) Expression patterns of Slp1 and Slp2 proteins in medulla neuroblasts identified by their expression of Deadpan (Dpn, a neuroblast marker). (C) Expression patterns of slp1 and slp2 mRNAs in Dpn expressing medulla neuroblast closely parallels the corresponding protein expression patterns suggesting regulation at transcription. (D) Schematic of the relative locations of slp1 and slp2 genes and transcriptional enhancers identified in regulating their expression. Positions of slp1 coding locus (2L:3,825,675..3,827,099 [+]), slp2 coding locus (2L:3,836,840..3,839,185 [+]), GMR35H02 (2L:3,817,265..3,821,014) and the REDfly enhancers u8772 (2L:3,816,967..3,818,532) and d5778 (2L:3,842,530..3,844,660) are shown relative to one another. A 220 bp enhancer located within the genomic segment covered by GMR35H02 and an 850 base pair enhancer element within the REDFly enhancer d5778 were identified as potential regulators of slp1 and slp2 genes. The distance between the start of GMR35H02 and the end of d5778 is around 27.394 kbp. (E) Expression patterns of a GFP reporter expressing GFP driven by the 220 bp enhancer closely approximates endogenous expressions of Slp1 and Slp2 in medulla neuroblasts. (F) Expression pattern of GFP reporter driven by the 850 bp enhancer shown alongside endogenous Slp1/2 expressions. This enhancer initiates GFP expression at a slightly later stage compared to the 220 bp enhancer and likely also regulates Slp2 expression in surface glia. Scale bar: 20 microns.

Figure 2

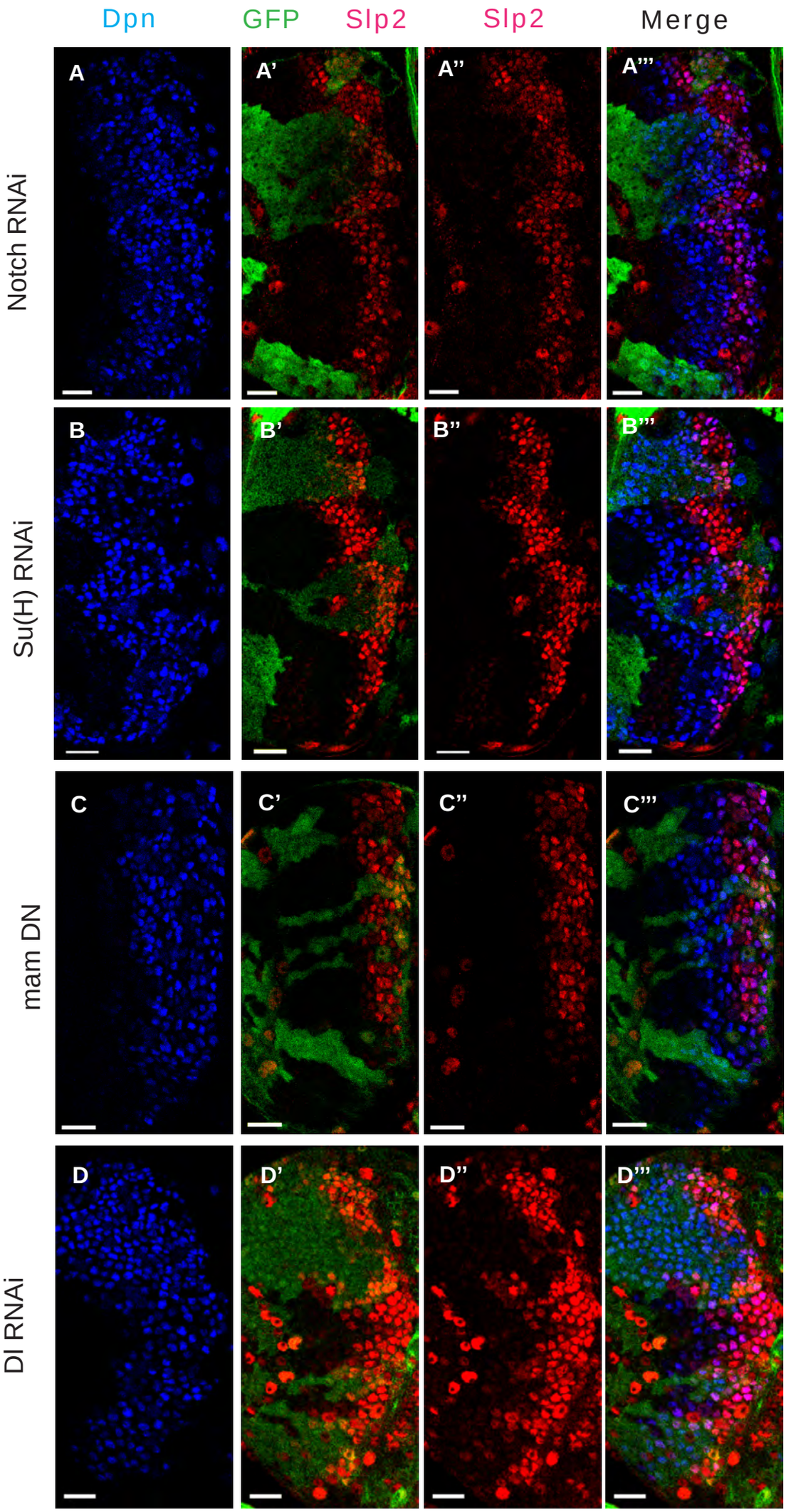

Dpn
GFP
Slp2
Slp2
Merge
Notch RNAi
Su(H) RNAi
mam DN
Dl RNAi
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
C'''
D
D'
D''
D'''

Figure 2. The Notch pathway regulates expression of Slp genes in medulla neuroblasts. (A) RNAi knockdown of Notch in GFP marked clones leads to a delay in expression of Slp genes compared to contemporaneous wild type neuroblasts outside the GFP marked clones. (B) Knockdown of su(h) shows a similar delay in Slp1/2 expression as for Notch-RNAi. (C) Expression of a dominant negative variant of mastermind, third component of the Notch transcription complex shows a similar phenotype as *NRNAi* and *Su(H) RNAi* and delays Slp1/2 expression. (D) Knockdown of Dl a Notch ligand has a similar delay on expression of Slp genes. Scale bar 20 microns.

Figure 3

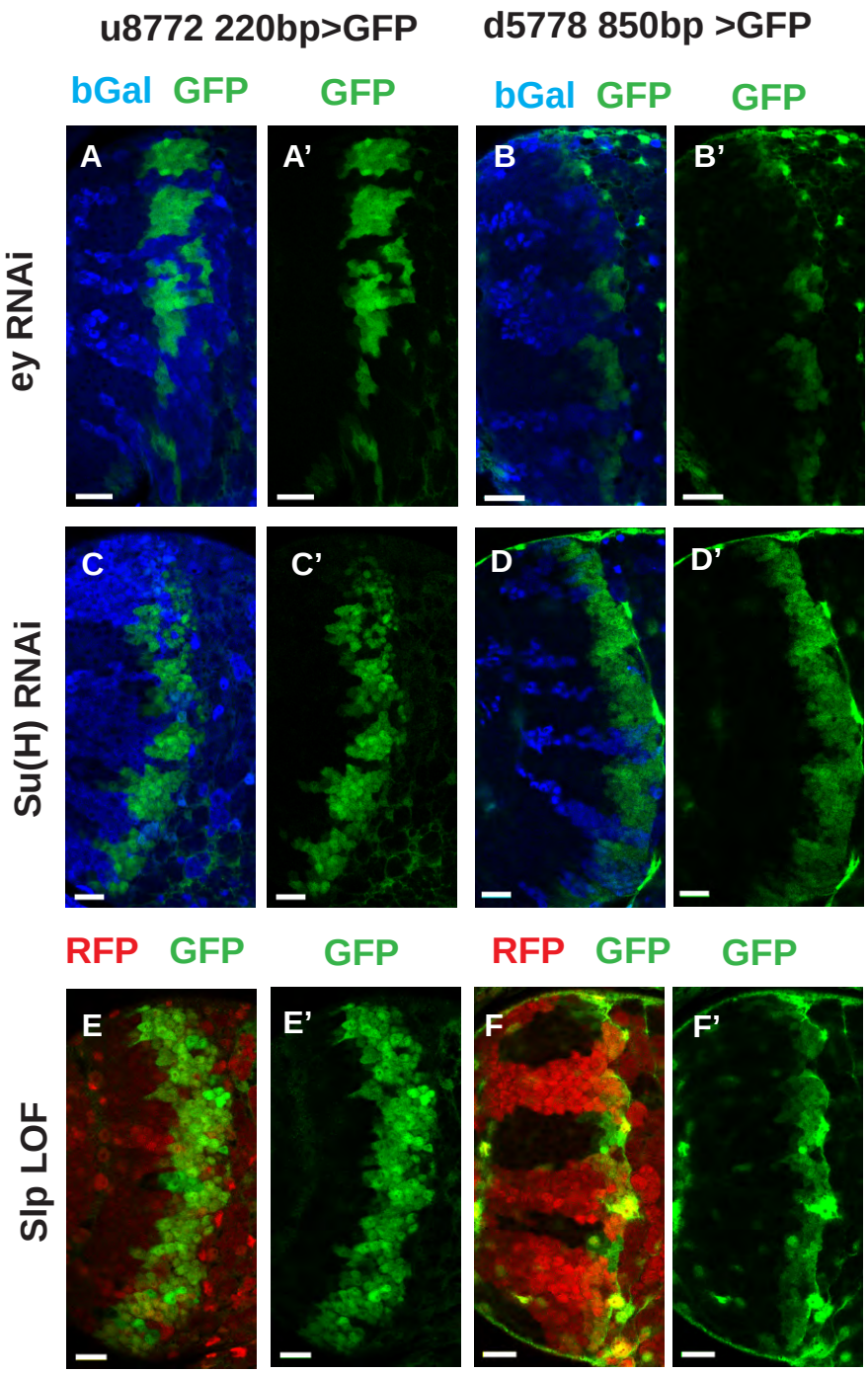

u8772 220bp>GFP
d5778 850bp >GFP
bGal GFP
GFP
bGal GFP
GFP
ey RNAi
A
A'
B
B'
Su(H) RNAi
C
C'
D
D'
RFP GFP
GFP
RFP GFP
GFP
Slp LOF
E
E'
F
F'

Figure 3. The Notch pathway and temporal patterning factors Eyeless and Scarecrow act through the identified 220 bp and 850 bp enhancers to regulate expression of Slp genes. (A,A') Observed expression of a GFP reporter encoding the 220 bp enhancer element in Ey knockdown clones. *Beta-galactosidase* (*bGal*) marks regions where ey is knocked down, regions unmarked by *bGal* are populated by wild type neuroblasts. (B,B') Observed expression of a GFP reporter encoding the 850 bp enhancer element in *ey RNAi* knockdown regions and in wild type neuroblasts. In case of both enhancers knockdown of Ey leads to a loss of GFP reporter expression, indicating GFP expression driven by these enhancers is sensitive to Ey. (C,C') 220bp enhancer GFP reporter expression in wild type and in Su(H) knockdown neuroblasts (as before, regions of *Su(H) RNAi* expression are marked by *bGal*). (D,D') 850bp enhancer GFP reporter expression in wild type and in Su(H) knockdown neuroblasts. In case of both enhancers expression of the GFP reporter is lost as far as the *bGal* marked *Su(H) RNAi* clone extends confirming that Su(H) is required for initiating GFP expression. (E, E') Expression of the 220 bp GFP reporter in Slp1 and Slp2 loss of function mitotic clones ('dark' regions) and in wild type neuroblasts (marked by RFP). (F,F') Expression of 850 bp enhancer GFP reporter in Slp LOF and wild type neuroblasts similar to (E,E'). Slp1 binding sites are identified in both the 220 bp and the 850 bp enhancer elements which may indicate the Slp genes may auto-regulate their expression. However, GFP reporter expression driven by these two enhancers remains preserved within Slp LOF neuroblasts as in wild type neuroblasts, suggesting that Slp1/2 genes are not required to initiate their own expression in neuroblasts.

Figure 4

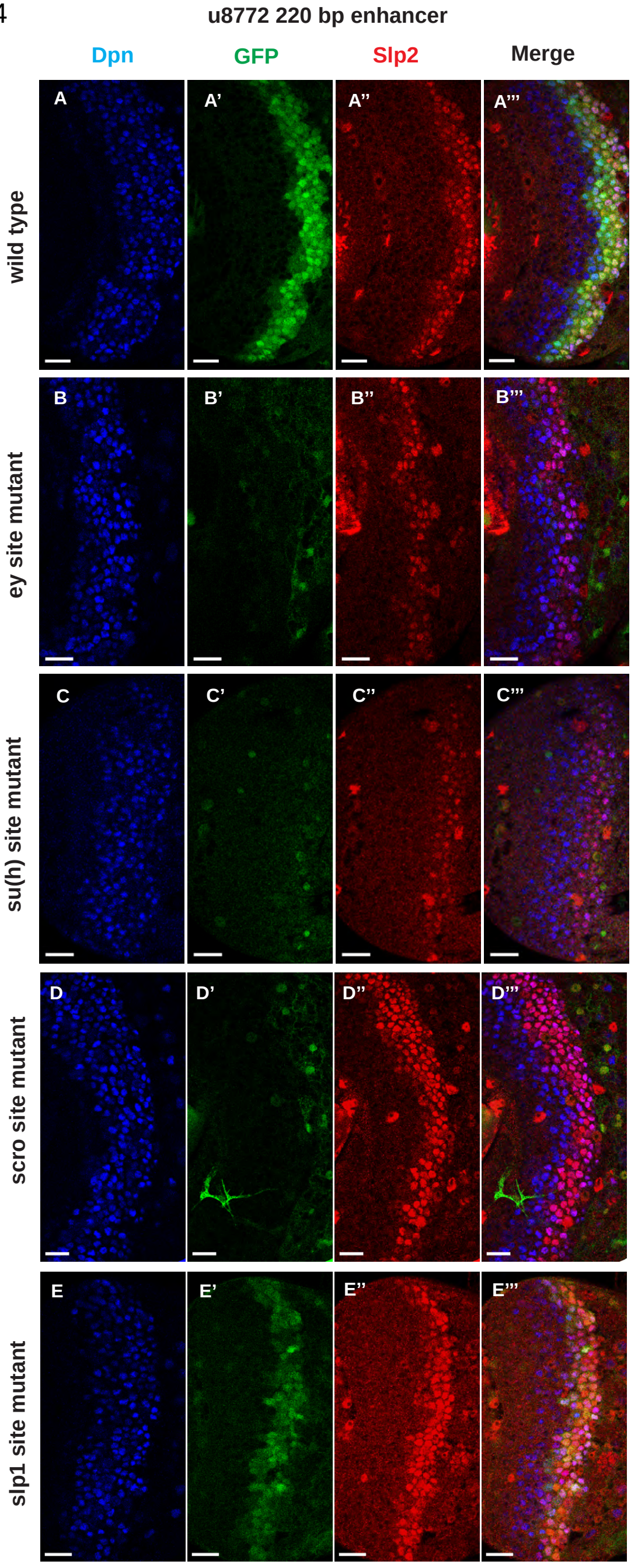

u8772 220 bp enhancer
Dpn
GFP
Slp2
Merge
wild type
ey site mutant
su(h) site mutant
scro site mutant
slp1 site mutant
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
C'''
D
D'
D''
D'''
E
E'
E''
E'''

Figure 4. Mutations of putative binding sites for Ey, Su(H) and Scro in u8772 220 bp enhancer confirm their requirement for activating expressions of Slp1/2 genes. Expression of GFP reporter driven by the whole (wild-type) 220 bp enhancer (A-A’’’) was compared against GFP reporters driven by mutated versions of the 220 bp enhancer in which predicted binding sites for specific transcription factors had been mutated: (B-B’’’) with predicted ey binding sites mutated, (C-C’’’) with predicted Su(H) binding sites mutated, (D-D’’) with predicted Scro binding sites mutated, (E-E’’’) Slp1 binding sites mutated. Loss of binding sites for Ey, Su(H) and Scro in the 220 bp enhancer led to a loss of GFP reporter expression.

Figure 5

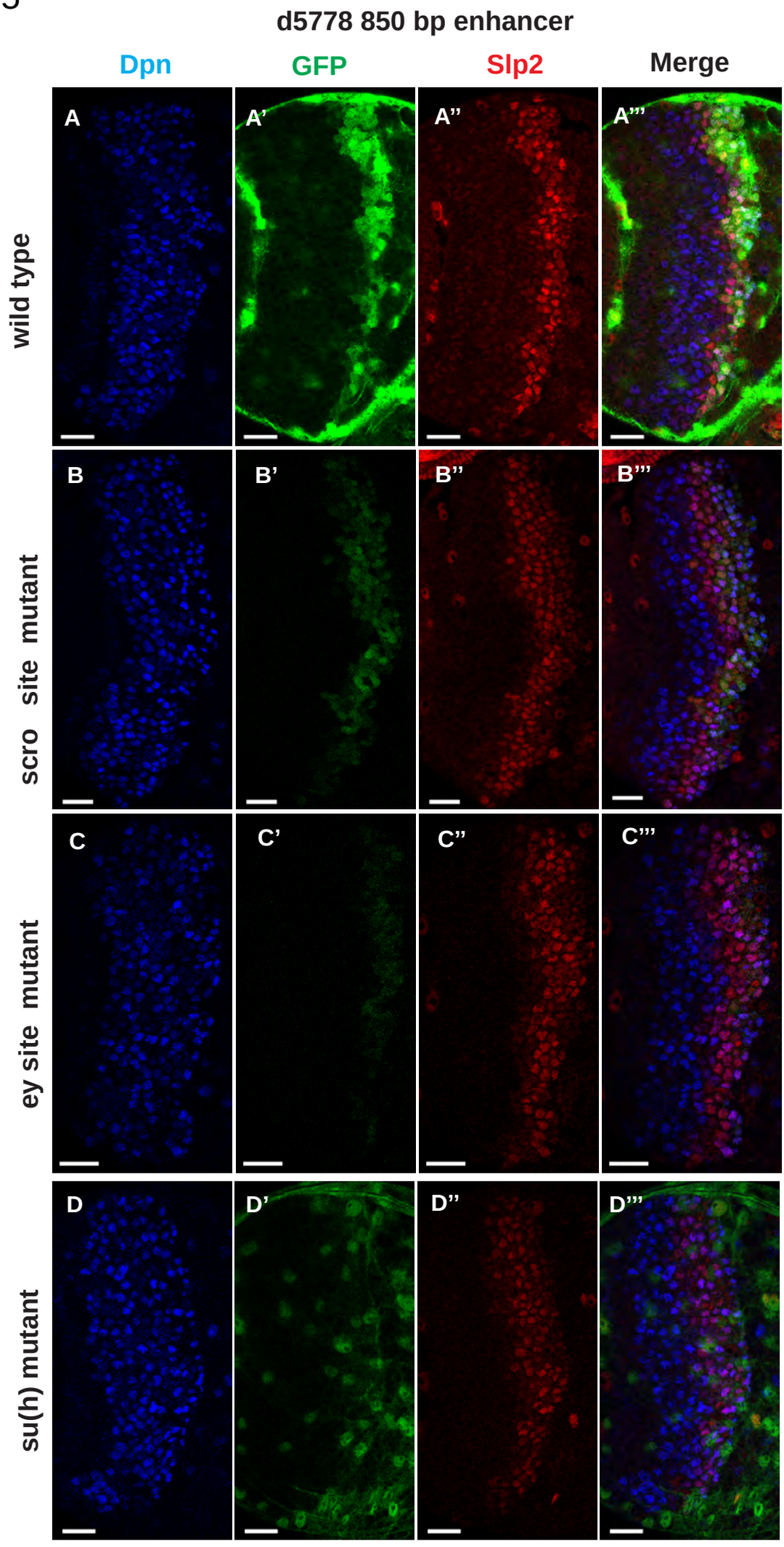
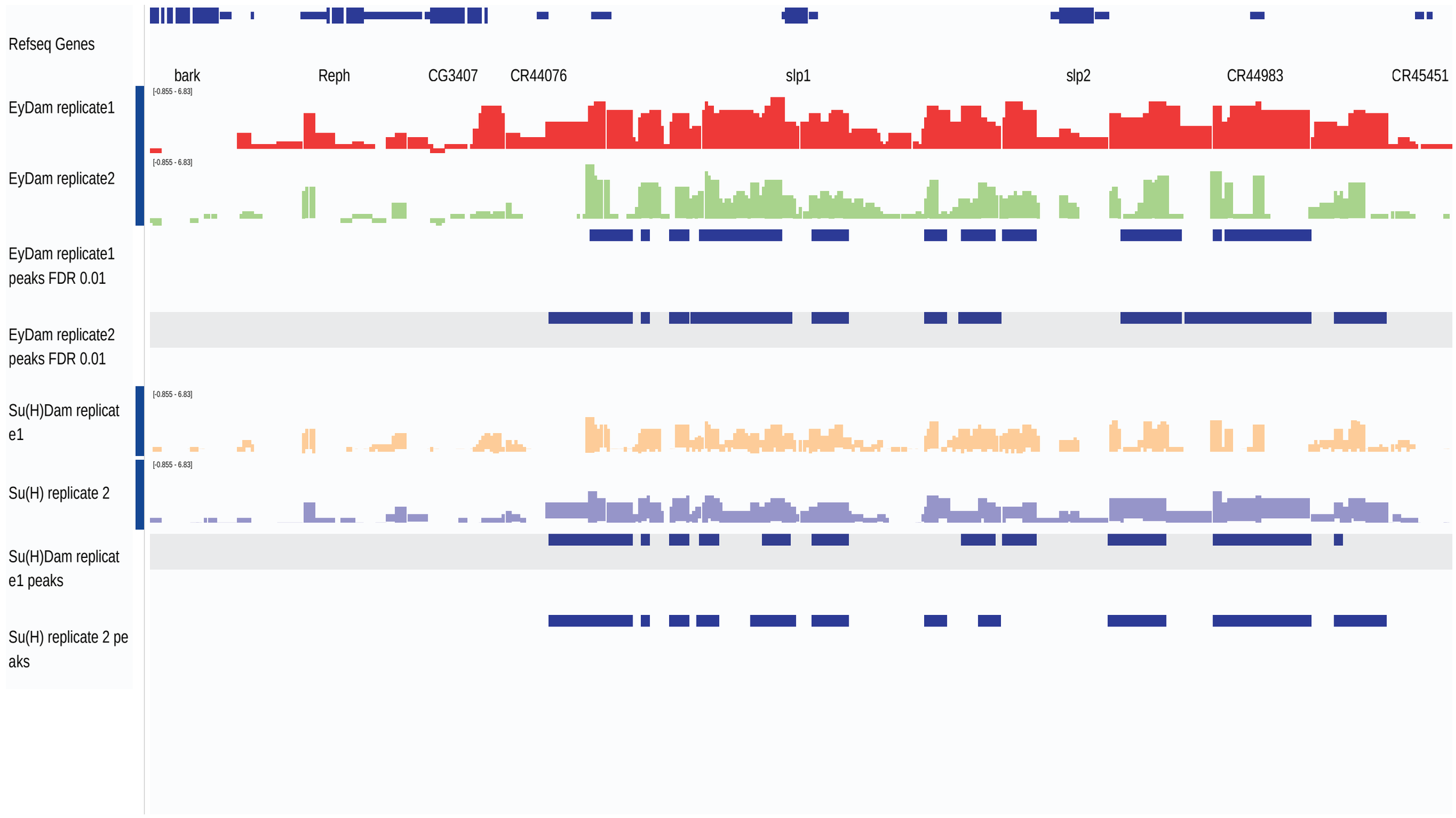

d5778 850 bp enhancer
Dpn
GFP
Slp2
Merge
wild type
scro site mutant
ey site mutant
su(h) mutant
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
C'''
D
D'
D''
D'''

Figure 5. Mutations of putative binding sites for Ey, Su(H) and Scro in d5778 850 bp enhancer confirm their requirement for activating expressions of Slp1.2 genes. A parallel comparison was made for GFP reporter expression driven by the wild type 850 bp enhancer (A-A’’’) to same GFP reporter expression driven by versions of the 850 bp enhancer with binding sites for specific transcription factors Scro, Ey and Su(H) mutated (B-B’’’, C-C’’’ and D-D’’’ respectively). Loss of binding sites for Ey nearly obliterated GFP reporter expression as did the loss of Su(H) binding sites. Additionally, in Su(H) site mutant expression of GFP reporter was up-regulated in surface glial cells. Loss of potential Scro binding sites also greatly reduced GFP reporter expression.

Figure 6

A


Refseq Genes
bark
Reph
CG3407
CR44076
slp1
slp2
CR44983
CR45451
EyDam replicate1
[-0.855 - 6.83]
EyDam replicate2
[-0.855 - 6.83]
EyDam replicate1 peaks FDR 0.01
EyDam replicate2 peaks FDR 0.01
Su(H)Dam replicate1
[-0.855 - 6.83]
Su(H) replicate 2
[-0.855 - 6.83]
Su(H)Dam replicate1 peaks
Su(H) replicate 2 peaks

B

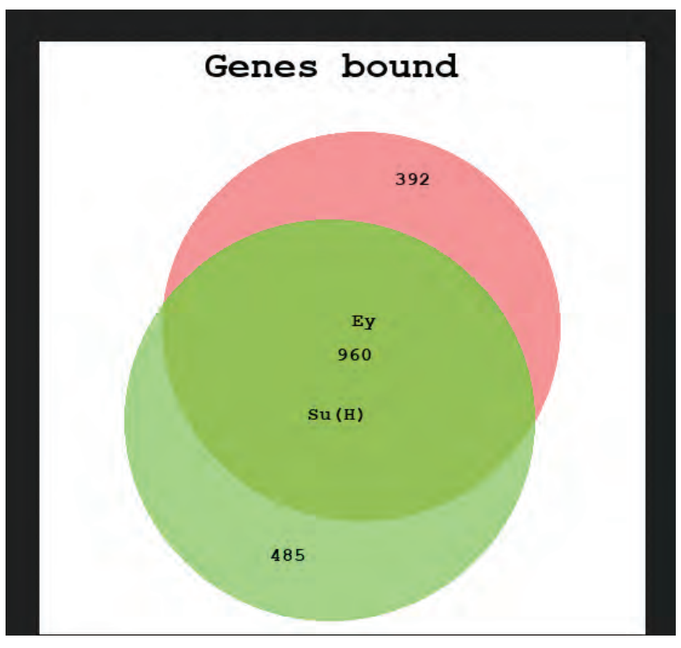

Genes bound
392
Ey
960
Su(H)
485

Figure 6. Genome-wide binding of Ey and Su(H) were observed by DamID-seq. (A) Prominent peaks of Ey and Su(H) binding are seen in neighborhood of the slp1 and slp2 gene loci including at the genomic locations of identified 220bp and 850 bp enhancers in both replicates of Ey-Dam and Su(H)-Dam experiments. (B) Output from BioVenn showing overlap between genes bound by EyDam and Su(H)Dam.

Figure 7

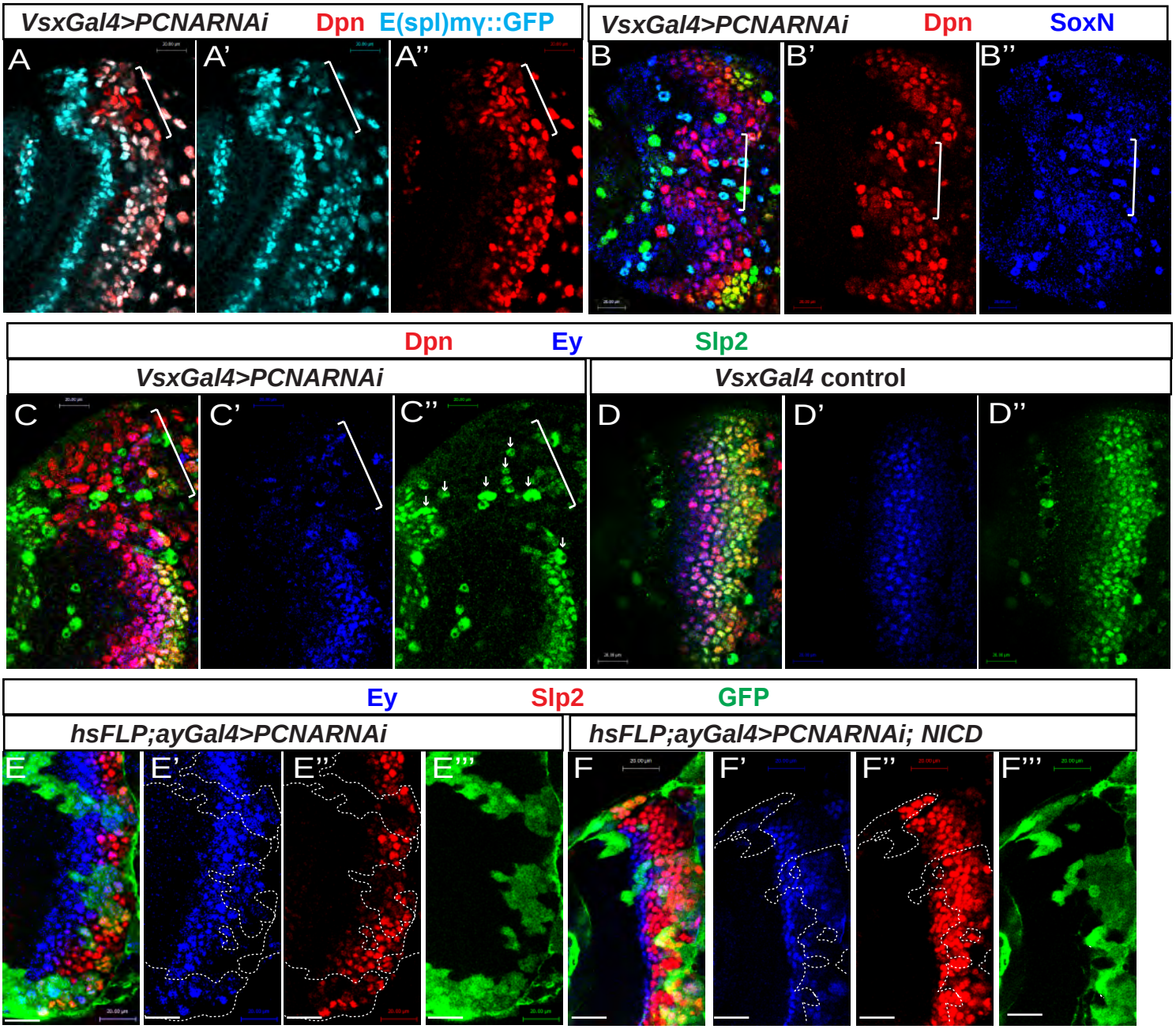
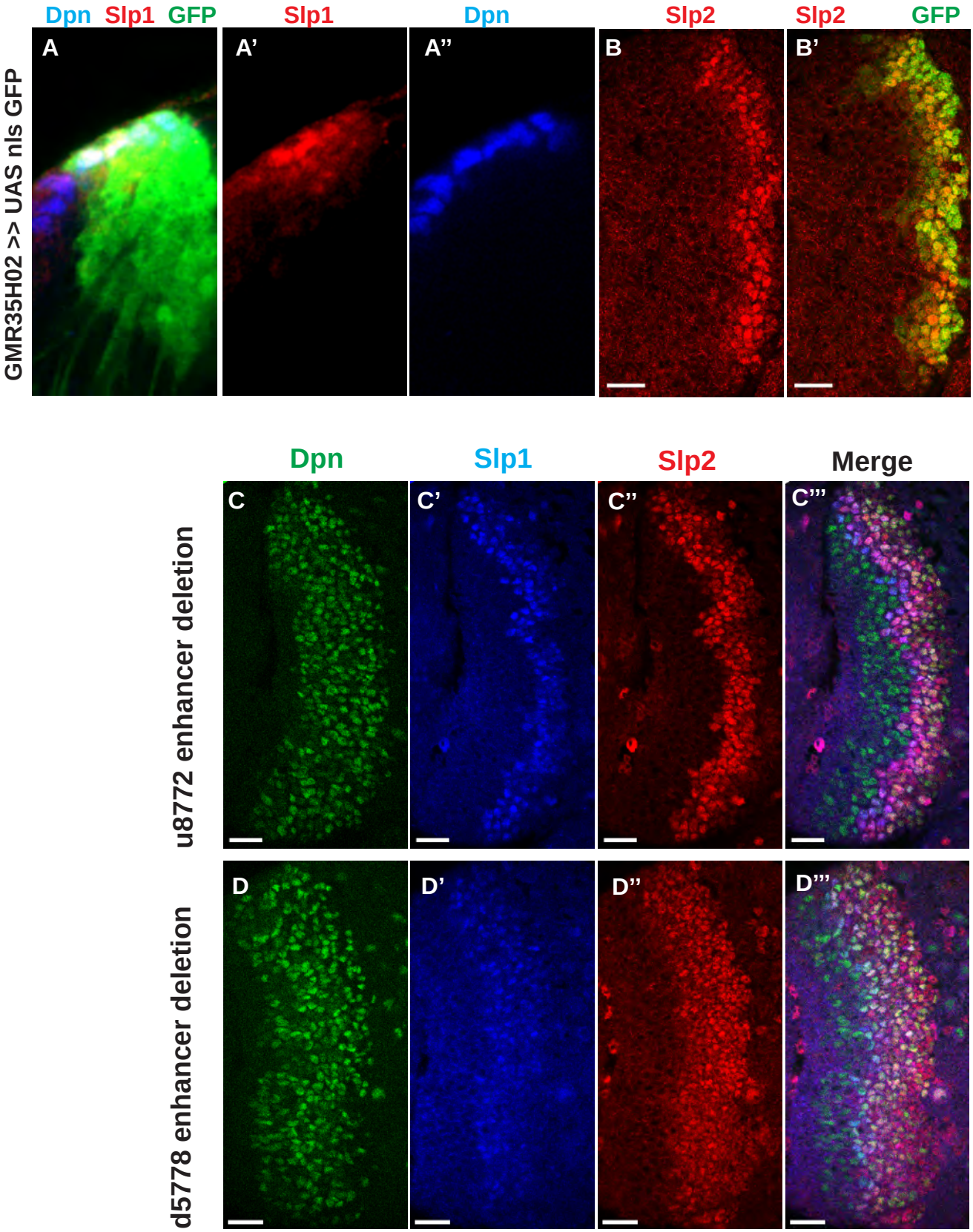

VsxGal4>PCNARNAi
Dpn
E(spl)mγ::GFP
A
A'
A''
VsxGal4>PCNARNAi
Dpn
SoxN
B
B'
B''
Dpn
Ey
Slp2
VsxGal4>PCNARNAi
VsxGal4 control
C
C'
C''
D
D'
D''
Ey
Slp2
GFP
hsFLP;ayGal4>PCNARNAi
hsFLP;ayGal4>PCNARNAi; NICD
E
E'
E''
E'''
F
F'
F''
F'''

Figure 7. Cell cycle progression is required for the precise timing of Slp expression and this is mediated in part through Notch signaling.

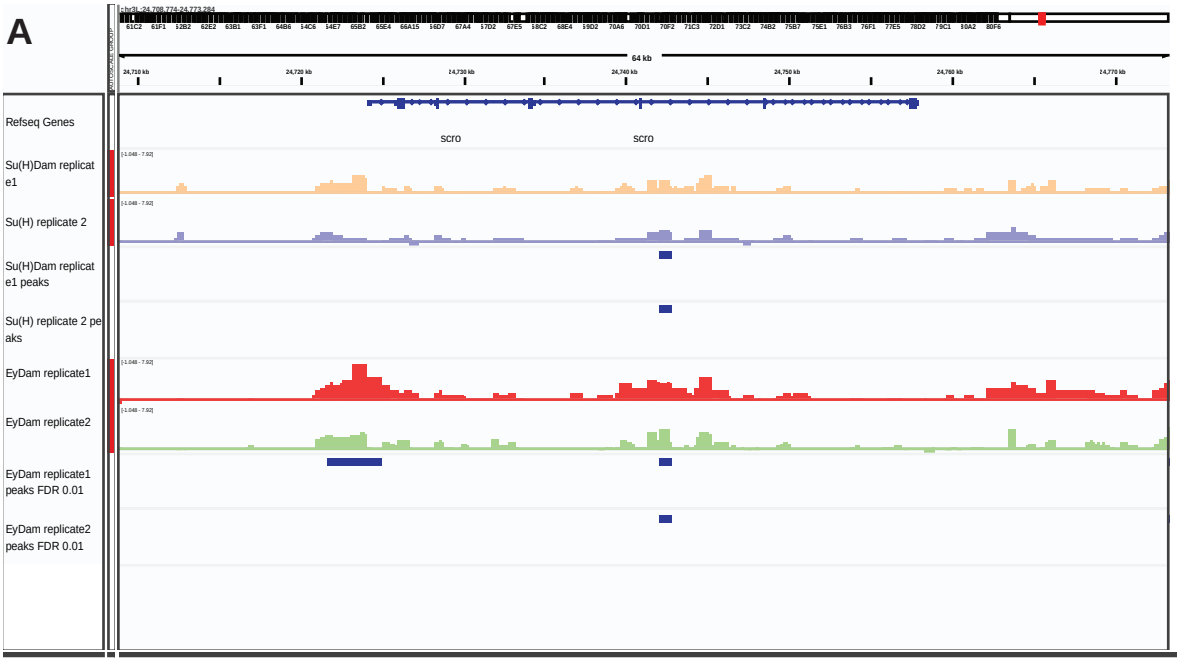
(A-A’’’) The expression of Dpn (red) and E(spl)mγGFP (cyan) in vsxGal4 driving UASDCR2 and UAS-PCNARNAi (VDRC51253) optic lobes. The affected domain is indicated by a bracket. (B-B’’’) The expression of Dpn (red) and SoxN (blue) in vsxGal4 driving UASDCR2 and UAS-PCNARNAi (VDRC51253) optic lobes. (C-C’’’) The expression of Dpn (red), Ey (blue) and Slp2 (green) in vsxGal4 driving UASDCR2 and UAS-PCNARNAi optic lobes. The affected domain is indicated by a bracket. Some glia express Slp2 but not Dpn are indicated by white arrows. (D-D’’’) The expression of Dpn (red), Ey (blue) and Slp2 (green) in vsxGal4; UASDCR2 control optic lobes. (E-E’’’) Larvae of genotype ywhsFLP; ayGal4UASGFP /UAS-PCNARNAi; UASDCR2/+ were heat shocked at 37C for 8min 70 hours before dissection at 3rd instar larval stage. Ey expression (blue) is minimally affected in clones marked by GFP (green), while Slp2 (red) expression is delayed. (F-F’’’) Larvae of genotype ywhsFLP; ayGal4UASGFP /UAS-PCNARNAi; UASDCR2/dpn>FRT-STOP-FRT-NICD were heat shocked at 37C for 8min 70 hours before dissection at 3rd instar larval stage. Ey expression (blue) is minimally affected in clones marked by GFP (green), while Slp2 (red) expression initiation is rescued. Scale bar 20 microns.

Supplementary Figure S1



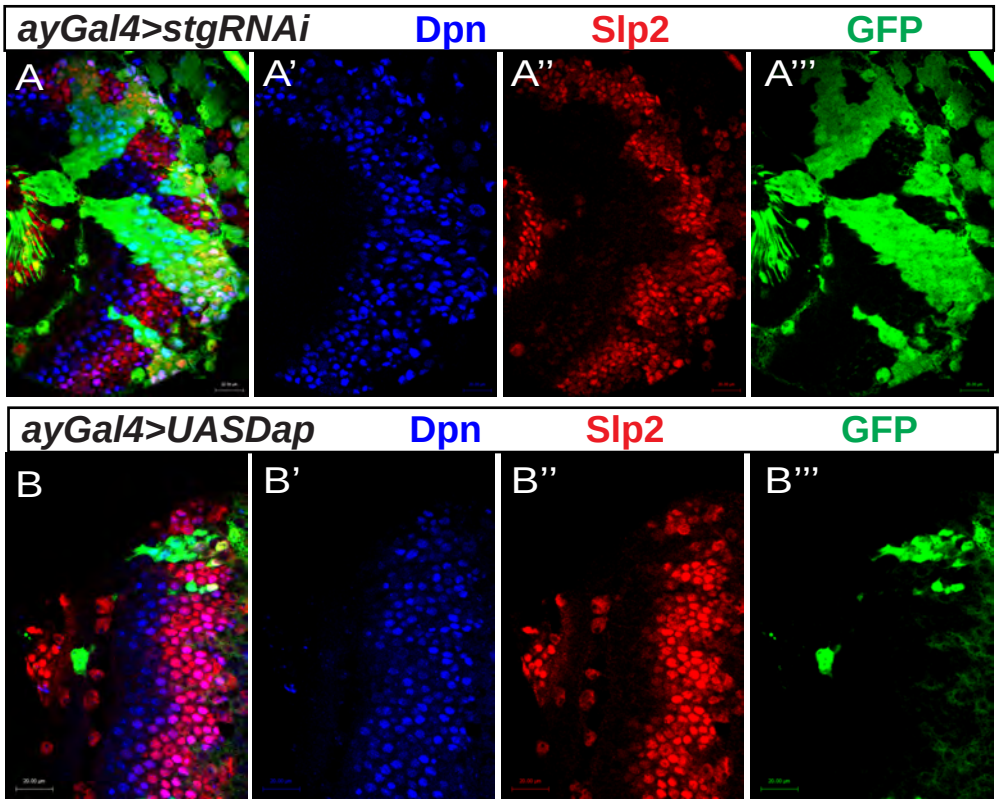
Supplementary Figure S1. Slp1/2 expression is regulated by at least two enhancers. (A-A’’’) UAS-GFP driven by GMR35H02-Gal4 is initiated at the same time as endogenous Slp1 in neuroblasts marked by Dpn. (B-B’) UAS-GFP driven by GMR35H02-Gal4 overlaps with Slp2 expression. (C-D’’’) CRISPR-Cas9 deletions of the u8772 enhancer (C-C’’’) and the d5778 enhancer (D-D’’’) individually do not abolish Slp1/2 expression suggesting possible redundance.

Supplementary Figure S2



Supplementary Figure S2. DamID-seq also confirms EyDam and Su(H)Dam binding to scro (A) and hbn (B) loci that have been suggested by other studies to function as TTFs in optic lobe medulla neuroblasts.

Supplementary Figure S3



Supplementary Figure S3. Cell cycle progression is required for the temporal cascade progression. (A-A''') Larvae of genotype ywhsFLP; ayGal4UASGFP /+; UASDCR2/UASstgRNAi (VDRC 17760) were heat shocked at 37C for 8min 70 hours before dissection at 3rd instar larval stage. Dpn expression (blue) is normal in clones marked by GFP (green), while Slp2 (red) expression is delayed. (B-B''')) Larvae of genotype ywhsFLP; ayGal4UASGFP /+; UASDCR2/UAS-Dap (BDSC 83338) were heat shocked at 37C for 8min 70 hours before dissection at 3rd instar larval stage. Dpn expression (blue) is normal in clones marked by GFP (green), while Slp2 (red) expression is delayed.