Role of *enok* in epigenetic gene regulation in neural stem cell development in *Drosophila melanogaster*

A dissertation submitted to the faculty of biology at the Johannes Gutenberg University Mainz, in partial fulfilment of the requirements for the degree of

Doctor rerum naturalium (Dr. rer. nat.)

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FACHBEREICH BIOLOGIE DER JOHANNES GUTENBERG-UNIVERSITÄT MAINZ Nr. / 20

Zusammenfassung der Dissertation von: Apurv Kulkarni

Thema: Role of *enok* in epigenetic gene regulation in neural stem cell development in *Drosophila melanogaster*

In humans, the acetyltransferase MOZ (a.k.a MYST3 and KAT6A) and its paralog MORF (a.k.a MYST4 and KAT6B) are known to function in a complex containing BRPF1 as a scaffold protein. Both MOZ/MORF and BRPF1 are known to play essential roles in embryonic CNS development, stem cell maintenance and adult neurogenesis in vertebrates. However, the molecular mechanism for these functions and the CNS specific acetylation targets of this complex remain unknown. Here, I show, that the fly homolog of *MOZ/MORF*, *enok* (enoki mushroom) is necessary for maintenance of a specific subpopulation of neuroblasts, termed the type II neuroblasts in the larval central brain. We show that conversion of type II neuroblasts to type I neuroblast fate accounts for this loss and this function of enok is dependent on the activity of the HAT complex. Interestingly, overexpression of enok leads to type I neuroblast converting to type II neuroblast fate. overexpression of enok also results in supernumerary Dpn+ neuroblast like cells in both type I and type II neuroblast lineages suggesting that progeny of enok overexpressing NB fail to differentiate. By performing ChIP-Seq and qRT-PCR analysis we show that enok binds to pnt and btd (two genes previously known to be necessary in type II neuroblast maintenance) loci and positively regulates their expression. Overexpression of pnt in enok knockdown background rescued the loss of type II neuroblasts suggesting that enok maintains type II neuroblasts by positively regulating its targets.

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Abbreviations

ALH.....After larval hatching

Ase.....Asense

Br140.....Bromodomain-containing protein, 140kD

BRPF1.....Bromodomain- and PHD finger-containing protein 1

Btd.....Buttonhead

CB.....Central brain

ChIP-Seq.....Chromatin immunoprecipitation - sequencing

CNS.....Central nervous system

DL(1).....Driver line 1

DL(2).....Driver line 2

Dpn.....Deadpan

Enok.....Enoki mushroom

Erm.....Earmuff

GMC.....Ganglion mother cell

H.....Histone

HAT.....Histone acetyltransferase

HDAC.....Histone deacetylase

HDM.....Histone demethylase

HMT.....Histone methyltransferase

imINP.....Immature intermediate neural precursor

K.....Lysine

L3.....Third instar larval stage

MBNB.....Mushroom body neuroblast

mINP.....Mature intermediate neural precursor

MORF.....MOZ related factor

MOZ.....Monocytic leukemia zinc finger protein

MYST.....MOZ Ybf2 Sas3 Tip60

NB.....Neuroblast

NICD.....Notch intracellular domain

NSC.....Neural stem cell

Pnt.....Pointed

Pros.....Prospero

PTM.....Post translational modification

RNAi.....RNA interference

S2 cells.....Schneider 2 cells

UAS.....Upstream activating sequence

VNC.....Ventral nerve cord

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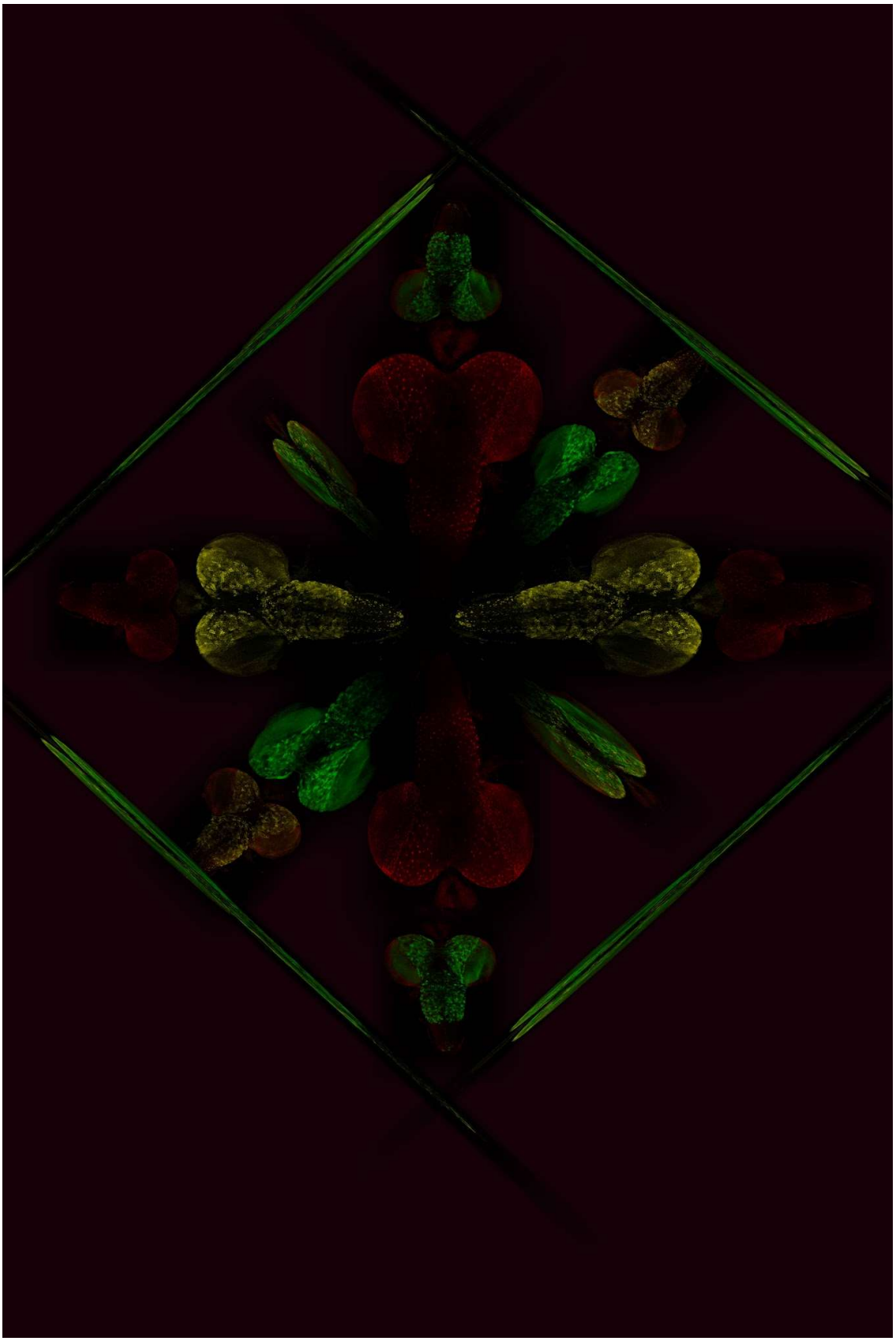
To the future, and to the role of biological sciences in it



1. Introduction

1.1 Epigenetic gene regulation in neural stem cell development

Conrad Waddington coined the term ‘epigenetics’ in 1942 to describe, interplay between genes and the environment, which together make the expressed characteristic traits of an organism, called the phenotype (Waddington, 1942). Today, there are several definitions of the term epigenetics, but it is generally accepted that the term refers to changes in gene activity independent of changes in primary DNA sequence. These changes, however, can be inherited by the progeny of the cells or the individuals. The processes and entities that bring about the epigenetic change can be divided into three groups: (i) DNA methylation, (ii) histone post translational modifications (iii) non coding RNAs (Podobinska et al., 2017).

Chromatin, the polymer of DNA and histones, is made up of fundamental units called nucleosomes. A typical nucleosome consists 146 basepairs of DNA wrapped around a histone octamer. The histone octamer core is made up of two copies each of H2A, H2B, H3, and H4. Two adjacent nucleosomes are connected by 10-50 base pairs long linker DNA and H1 (Luger et al., 1997). Histones have a globular C-terminal end and an N-terminal tail. The N-terminal tails have numerous sites for various post translational modifications, like, acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, etc. Post translational modifications of histone N-terminal tails regulate the structure of nucleosomes by altering their interaction with DNA and other parts of the histones. For instance, in the process of histone acetylation, where acetyl groups are added to ε-amino group of lysine residues of the histone N-termini of core histones, H3 and H4. This changes the charge and decreases the interaction between positively charged histones and negatively charged DNA. It also reduces interaction between core histones and linker histone, H1(Ridsdale et al., 1990). Collectively, this leads to chromatin decondensation. This relaxation of chromatin makes it more accessible for transcription machinery and transcription factor binding. Histone acetylation is generally associated with open chromatin and is considered to be an transcription activating mark. Histone

methylation on the other hand can be either activating or repressing depending upon which residue gets methylated. Opposing actions of two types of enzymes, histone acetyltransferases, and histone deacetylases govern the process of acetylation. Similarly the process of histone methylation in governed by opposing actions of histone methyltransferases and histone demethylases (Lilja et al., 2012).

The histone tails are decorated with various PTMs, and the resultant of presence or absence of various histone modification creates a layer of information, called "the histone code" on top of the information stored in the primary DNA sequence. The histone code is used by the effector molecules like the transcription factors to make downstream decisions such as scilnensing or expressing a gene. (Jenuwein and Allis, 2001).

There are several examples which show the importance of epigenetic modification in neural stem cell development (reviewed in Murao et al., 2016). I would like to discuss one representative example. In vertebrates, neurogenin 1(*NEUROG1*) plays crucial roles in neurogenesis in NSCs. Whereas signal transducer and activator of transcription 3 (*STAT3*) is a key regulator of astrogenesis. In embryonic CNS development, neurogenesis takes place during mid gestation and precedes astrogenesis, which takes place in late gestation. In NSCs, NEUROG1 and STAT3 compete for CREB binding protein (CBP) binding, a HAT. CBP acts as a coactivator for the downstream genes of both, *NEUROG1* and *STAT3* . In mid gestation, when NEUROG1 levels are high, NEUROG1sequasters CBP. This activates downstream genes of *NEUROG1* and leads to neurogenesis. Only in late gestation, when NEUROG1 levels drop, CBP becomes available for STAT3 binding and activation of its downstream genes, resulting in astrogenesis. During mid gestation, *WNT/TCF,* signaling activity is responsible for inducing and maintaining the expression of *NEUROG1* in the NSCs. However, during late gestation, *NEUROG1* is no longer responsive to *WNT/TCF* activity and is downregulated, even though,*WNT/TCF* signalling is still active. Enhancer of zeste 2 (*Ezh2*), a polycomb repressive complex 2 subunit, is responsible for depositing H3K27me3 repressive marks, and is highly expressed in NSCs during astrogenesis. In the course of transition from neurogenesis to astrogenesis, the promoter of *NEUROG1* acquires the H3K27me3 mark. The loss of competence of

NEUROG1 locus to respond to *WNT* signalling is attributed to Ezh2 mediated H3K27me3. Indeed NSC specific loss of *Ezh2,* before the onset of astrogenesis, leads to prolonged neurogenesis and reduced astrogenesis. Furthermore, inhibiting HDACs, in adult hippocampal NSCs, using inhibitors, such as valproic acid (VPA), leads to increased neurogenesis and reduced gliogenesis. VPA-mediated neurogenesis is accompanied by acetylation of *NEUROG1* promoter and upregulation of *NEUROG1* expression. Thus in this case histone PTMs, methylation and acetylation, together regulate neurogenesis and gliogenesis, by regulating *NEUROG1* expression and also by regulating the expression of its downstream targets in both embryonic and adult NSCs (Sun et al., 2001; Hibayashi et al., 2009; Yu et al., 2009; Pereira et al., 2010)

1.2 *MOZ, MORF*, and *enok*

The genomes of eukaryotes code of several HATs. Based on the sequence similarities, HATs are categorized in different families. Gcn5/PCAF family of HATs, CBP/P300 family of HATs and MYST family of HATs are three of the highly conserved and most studied families of HATs. HATs of the MYST family are conserved from yeast to humans. The MYST acronym derives from the four earliest discovered member of this family: human MOZ (monocytic leukemia zinc finger protein), yeast Ybf2 (also known as, Sas3 (something about silencing 3)), yeast Sas2, and mammalian TIP60 (HIV Tatinteracting 60 kDa protein) (Yang, 2004). MOZ and MORF (MOZ-related factor) are two related proteins of the MYST family. In various studies, using exome sequencing, it was found out that *MORF* was mutated in individuals with Noonan syndrome-like disorder (Kraft et al., 2011), Ohdo syndrome (Clayton-Smith, et al., 2011; Szakszon et al., 2013), genitopatellar syndrome (Campeau et al., 2012; Simpson et al., 2012) and blepharophimosis–ptosis–epicanthus inversus syndrome (Yu et al., 214). Retarded growth, faciocranial deformities, intellectual disability are the common features of these disorders. Similarly *MOZ* has been found to be mutated in previously unrecognized syndromes, where individuals with *MOZ* mutation have microcephaly, intellectual disability, and global delay in development (Arboleda et al., 2015; Tham et al., 2015). Suggesting that *MORF* and *MOZ* play important roles in human development. In mice

MOZ is required for fetal hematopoietic stem cell maintenance. *MOZ* homozygous mutant mice are bloodless, have small liver, and die around embryonic day 14 (Katsumoto et al., 2006). The mouse homolog of *MORF* is called Querkopf (*Qkf*), named after the square shape of the head in mice mutant for the gene. *Qkf* is expressed in the adult neural stem cells of the subventricular zone. Querkopf mutant adult neural stem cells have reduced self-renewal ability, resulting in impaired adult neurogenesis in the olfactory bulb (Merson et al., 2006). Apart from the normal development, *MOZ* and *MORF* have also been implicated in tumor formation. Chromosome translocations leading to fusion of *MOZ/MORF* have been identified in various cancers. MOZ-CBP, MOZ-P300, MOZ-TIF2, MORF-CBP fusions have been found play important roles in acute myeloid leukemia and other hematologic malignancies. Suggesting that miss-targeted acetylation caused by these chimeric proteins is oncogenic (Liang et al., 1998; Chaffanet et al., 2000; Panagopoulos et al., 2001; Kojima et al., 2003). MOZ, MORF, and their fly homolog Enok (enoki mushroom) , along with all the other members of MYST family, share a ~370 amino acid residue long MYST domain. MYST domains of MOZ and MORF contain C2HC fingers, which are essential for their catalytic HAT activity. MOZ, MORF and Enok have two more domains that are common to all three, namely the NEMM (N-terminal region in Enok, MOZ and MORF) and the PHD (plant homeodomain-like) Domain. In addition to the above mentioned domains, Enok contains an uncharacterized, neurofilament protein-like, domain which is not present in both MOZ and MORF. In vertebrates, MOZ and MORF are known to function as a part of a multisubunit protein complex. The complex consists of either MOZ or MORF and three other proteins, BRPF1 (bromodomain and PHD finger containing 1), ING5 (inhibitor of growth 5), EAF6 (homolog of yeast Esa1-associated factor 6). In the complex the C-terminal motif of BRPF1 binds to ING5 and EAF6 whereas the N-terminal motif binds either MOZ or MORF, indicating that BRPF1 acts as a scaffold protein for the complex. BRPF1 contains two PHD fingers, which flank a C2HC zinc knuckle. Together this region is called a PZP (PHD-zinc knuckle-PHD) module. The PZP module has been shown to bind to DNA. The PZP module also binds to unmodified N-terminus of H3. This binding is sensitive to modifications at lysine 4. Suggesting that the complex might also have the ability to 'read' other histone modifications (Yang, 2015).

MOZ/MORF form a tetrameric complex in vertebrates. All members of this complex have homologs in *Drosophila*, *enok*, *br140* (Bromodomain-containing protein, 140kD), *ING5* (Inhibitor of growth family member 5), *Eaf6* (Esa1-associated factor 6) are homologs of vertebrate HAT complex subunits, *MOZ/MORF*, *BRPF1*, *ING5* and *Eaf6* respectively. It was shown using MudPIT (multidimensional protein identification technology) of S2 cell nuclear extracts, that flag affinity purification using one component protein of the complex as bait, pulled down rest of the three component proteins of the complex, irrespective of which component protein was used as bait. Suggesting that the complex is conserved in flies . To find out which histone residue is acetylated by Enok, western blot analysis using antibodies against 10 different histone acetyl modifications, was done in S2 cells treated with dsRNA against *enok*. Out of the 10 modifications checked only H3K23ac was reduced after *enok* knockdown. It was also shown that knockdown of any of the other components of the HAT comlex using dsRNA in S2 cells also resulted in reduced H2K23ac, but did not affect any other modification. Suggesting that Enok specifically acetylates H3K23 residues, and the entire complex is needed for the process (Huang et al., 2014; Huang et al., 2016).

The mushroom bodies are a pair of structures in the insect brain involved in olfactory learning and memory (Strausfeld et al., 1998). $Enok^{1}$ is a nonsense mutation which truncates the protein at end of the C2HC finger motif and it renders the protein functionally null. $Enok^{1}$ homozygous larvae show global developmental delay and compared to the heterozygotes take twice as much time to reach wandering larval stage. Very few larvae reach the pupa stage, and none reach adult stage. It has been shown that *enok* is necessary for development of mushroom bodies. MARCM analysis (mosaic analysis with a repressible cell marker) with a null mutation for *enok*, $enok^{1}$, coupled with BrdU pulse-chase experiment it was shown that *enok* mutant mushroom body NBs stop producing neurons prematurely resulting in small mushroom bodies. The name *enok* refers to a type of mushroom with thin stalk and small cap (Scott et al., 2001)

Enok has been shown to be necessary for maintaining germline stem cells (GSCs), both cell autonomously, and non-cell autonomously. Loss of *enok* results in premature

differentiation of GSCs. The cell autonomous effect of *enok* is mediated via a pro-differentiation factor bruno. Loss of *enok* results in de-repression of bruno, a pro-differentiation factor, in GSCs, which leads to their premature differentiation. Loss of *enok* in cap cells, which form the niche for the GSCs leads to decrease in cap cell number leading to differentiation of GSCs (Xin et al., 2013).

During early embryogenesis *enok* has been shown to play a role in germ plasm development. Knocking down *enok* maternally using the ovoD system led to defects in posterior localization of oskar mRNA, which in turn leads to defects in germ plasm development. It was shown that Enok acetylates the H3K23 residues in the promoter regions of spire and maelstrom loci and positively regulates their transcription. Spire and maelstrom are necessary for posterior localization of oskar mRNA. Hence it was shown that *enok* regulates germ plasm development via positively regulating the expression of its target genes spire and maelstrom (Huang et al., 2014).

In S2 cells it was shown that RNAi (ribonucleic acid interference) mediated knockdown of *enok* results in larger proportion of cells being present in G1 phase compared to the control. Cell cycle phase analysis revealed that this is due to both, faster M-G1 transition, and a slower G1-S transition. It was further shown that slower G1-S transition is mediated via *elg1* (enhanced level of genomic instability 1). Elg1 acts as an unloader of PCNA (Proliferating cell nuclear antigen) from DNA. Enok blocks the PCNA unloading activity and Elg1 and helps cell reach critical levels of DNA bound PCNA necessary for G1-S transition (Huang et al., 2016)

In another study it was shown that Enok binds to the components of PRC1 (Polycomb repressor complex 1). Using ChIP-Seq (chromatin immunoprecipitation followed by DNA sequencing) analysis, in whole embryos, it was shown that both the PRC1 and the Enok HAT complex bindings sites are enriched for developmental genes and both occupy nearly identical binding sites in the genome, suggesting that in early development the promoters of developmental genes may be decorated with bivalent marks, marking poised state (Kang et al., 2017)

1.3 Neuroblasts of *Drosophila* larval central nervous system

The *Drosophila* larval central nervous system (CNS) is made up of two brain lobes and a ventral nerve cord (VNC). Each lobe can further be divided into two regions, the central brain (CB), and the optic lobe (OL). The NSCs of the *Drosophila* larval CNS are called neuroblasts (NBs). Based on their lineage progression characteristics, the NBs can be divided in two categories, type I NBs and type II NBs. Type I NBs divide asymmetrically to produce one self renewed type I NB and a smaller ganglion mother cell (GMC). The GMC undergoes one terminal division to produce two differentiated neurons. Type II NBs also divide asymmetrically to produce one self-renewed type II NB and one smaller daughter cell. However, the smaller progeny of type II NBs, called intermediate neural progenitors (INPs) undergo limited rounds of asymmetric divisions to produce a self-renewed INP and a GMC. Similar to the GMC produced by type I NB, the GMC produced by the INP also divides only once to produce two terminally differentiated neurons. Before the INPs could start dividing, they undergo a process of maturation. The immature INPs (imINPs) are negative for Deadpan (Dpn), whereas the mature INPs are Dpn+. The imINP stage can be further divided into asense negative (Ase-) and Ase+ stages. Wherein, Ase- stage precedes the Ase+ stage. Ase continues to be expressed in the mature INP (mINP) stage. There are 8 type II NBs in each lobe of the larval CNS .The type II NBs are located dorsally in the the brain lobe close to the midline. The NBs that give rise to the mushroom bodies, a structure in the drosophila CNS required for olfactory learning and memory are called mushroom body NBs (MBNBs). MBNBs follow a type I mode of lineage progression. There are four MBNBs in each brain lobe. Apart from MBNBs, there are around 85 type I NBs in the CB region of each lobe (Homem et al., 2012).

All the NBs implement a common mechanism for achieving asymmetric cell division. When NBs delaminate from the epithelial cells of the neuroectoderm, they inherit the apical localization of proteins Par-3, Par-6 and aPKC (atypical protein kinase C). Apart from the Par-3-Par6-aPKC complex, another protein complex containing the proteins Gαi

(G protein α i subunit) and Pins (partner of inscuteable) is apically localized. Insc (inscuteable), the adapter protein, links these two complexes. Pins binds to a protein called Mud (mushroom body defect), which provides cortical attachment sites to astral microtubules and organizes the apico-basal orientation of microtubule spindle. These apical complexes also play a role in the basal localization of another set of proteins. Adaptor proteins Mira (miranda) and Pon (partner of numb) are basaly localized in late prometaphase. They facilitate basal localization of Numb and Brat (brain tumor). In type I NBs and in mINPs, Mira also facilitates the basal localization of prospero (Pros). The proteins that are localized apically during the NB division remain in the NBs, whereas basally localized proteins exclusively segregate into the GMC (or INPs in the case of type II NBs). Loss of the basal determinants leads to the GMC or INP reverting back to NB fate, which may lead to tumor formation (Wordaz et al., 2003; Knoblich, 2008).

1.4 Key regulators of NB development

Interplay between the seven genes, *dpn*, Notch, Enhancer of split mγ, helix-loop-helix (E(spl)mγ-HLH), pointed P1 (*pntP1*), earmuff(*erm*), *ase,* and *pros* plays a central role in development of NBs. I will briefly introduce the role each one of them plays in the following sections.

Pros is a homeodomain protein and is conserved from flies to humans. *Prox* (prospero related homeobox), a homolog of *pros*, is necessary in mice for neuronal differentiation (Torri et al., 1999). Using DamID (DNA adenine methyltransferase identification) (van Steensel et al., 2001) in embryonic CNS and in silico motif finding, it was shown that Pros binds to NB genes like *dpn, ase, insc* and to cell cycle genes E2F, cyclin E and string, GMC genes, like fushi tarazu and even skipped, and neuronal differentiation genes like Fasciclin 1 and Fasciclin 2. Using whole genome expression profiles, performed on dissected embryonic VNCs in wild type and *pros* mutant background, it was found that in *pros* mutants, NB genes, *dpn, ase, insc*, cell cycle genes, E2F, cyclin E and string are upregulated whereas neuronal differentiation genes Fasciclin 1 and Fasciclin 2 are downregulated. The authors conclude that *pros* plays a dual role in NB lineages, it

inhibits self renewal and facilitates cell cycle exit by down regulating NB and cell cycle genes and it promotes differentiation by positively regulating neuronal differentiation genes. They also show, that in *pros* mutant embryos the GMCs fail to differentiate or exit cell cycle and revert back to NB fate. Bello et al., 2006, show that in *brat* mutant clones, overexpression of *pros* is sufficient to rescue the tumor formation caused by failure to exit cell cycle. Collectively, above mentioned work suggests that transcriptional control by *pros* plays central role in NB proliferation control (Choksi et al., 2006).

Transcription factor dFezf/Earmuff (erm) plays a pivotal role in type II NB lineage development. Loss of *erm* results in ectopic type II NBs. The ectopic type II NBs in *erm* mutants arise from mature INPs reverting back to type II NB fate. Suggesting that *erm* is necessary to restrict the developmental potential of INPs (Weng et al., 2010). Using a specific antibody against Erm protein, Janssens et al., 2014, showed Erm is only present in type II lineages. In a type II lineage, *erm* is expressed exclusively in the Ase- and Ase+ imINPs. Further studies have shown that *erm* restricts the developmental potential of INPs by reducing their competence to respond to self renewal factors like *dpn*, klumpfuss and the Notch downstream effectors, enhancer of split genes. Furthermore, *erm* also upregulates expression of *pros*, a pro-differentiation factor. Moreover, it was shown that Erm physically binds to multiple components of brahma complex and HDAC3. And the dedifferentiation preventive function of *erm* is partly mediated by brahma complex and HDAC3 (Koe et al., 2014).

Pointed (*pnt*) is an Ets (E26 transformation specific) family transcription factor. Ets family transcription factors share conserved wing-helix-turn-helix DNA binding domain. In *Drosophila* two distinct isoforms of the pointed gene, pointed P1 and pointed P2 are expressed. Whereas *pntP2* is not expressed in any of the Dpn+ cells in the fly CNS, *pntP1* is specifically expressed in the type II lineages. In the type II lineage *pntP1* is expressed in the type II NB, Ase+ and Ase- imINPs. It is also expressed in the newly mature INP. But dramatically goes down as the mINP starts dividing. It was shown that overexpression of *pntP1* in type I NB lineages resulted in loss of *ase* expression in type I NBs and in production of INPs in type I lineages. Indicating that *pntP1* is sufficient to

convert type I NB fate into type II NB fate. Partial loss of *pntP1* function in type II NBs leads to three different phenotypes in the type II lineages. First, Type II NBs convert to type I NB fate and start expressing *ase* and stop producing INPs. Second, type II NBs stay Ase- but still stop producing INPs. Third, INPs in type II lineages revert back to produce several ectopic type II NBs. Studies from Yan lab (Zhu et al., 2012) and Zhu lab (Xie et al., 2014) shed light on why loss of *pntP1* results in such different and even contrasting phenotypes. According to the authors, the answer lies in the dual role of *pntP1* in the type II NB lineage development. *PntP1* acts as repressor of both *ase* and *pros*. It also acts as a positive regulator of *erm*. Derepression of *ase* and *pros* would, upon partial loss of *pntP1,* lead to type II NB converting to type I NB and loss on INPs. Derepression of *pros* alone leads to type II loss of INPs without the fate switch. Loss of *erm* without derepression of *ase* and *pros* would lead to INPs reverting back to type II NBs to produce ectopic type II NBs. It is still not clear what decides which of the above three possibilities will be favored over the others. Further, it has been shown that loss of buttonhead (*btd*), the *Drosophila* Sp8 transcription factor, in the background of partial loss of *pntP1* enhances the chances of the first possibility, where the type II NBs convert to type I NBs fate and stop producing INPs.

Dpn, Enhancer of split mγ, helix-loop-helix (E(spl)mγ-HLH) and Notch act as NB self renewal factors. In larval NB lineages they are only expressed in the NBs and mature INPs. It has been shown that both type I and type II linges have dedicated mechanisms to prevent Notch from being segregated into the daughter cells (GMCS and imINPs) of NBs. Apart from its role in NBs specification during embryogenesis, Notch also plays important roles in larval NB development. Notch signalling is active in both type I and type II NBs. Loss of Notch completely removes type II lineages. However, loss of Notch, surprisingly, has no effect on the type I lineages. Both *dpn* and *E(spl)mγ-HLH* are bHLH-O transcription factors. E(spl)mγ-HLH is a direct target of canonical Notch pathway and requires Suppressor of Hairless as a mediator of Notch signalling. However, *dpn* expression in NBs is not dependent on Notch activity. Both *dpn* and *E(spl)mγ-HLH* are expressed in type I and type II NBs and mINPs. Single knockdowns of *dpn* and *E(spl)mγ-HLH* have only mild effect on type I NB lineages. Although type II NB are also

not affected in single knockdown, the type II NB linges are affected, that is, the single knockdowns of *dpn* and *E(spl)mγ-HLH* lead to approximately 50% reduction in INP number. Double knockdown of *dpn* and *E(spl)mγ-HLH* result in severe phenotypes in both type I and type II lineages. 75% reduction in type I NB number and a total loss of type II lineages in double mutants clones vs wild type clones. Suggesting that *dpn* and *E(spl)mγ-HLH* act completely redundantly in type I lineages whereas only partially redundantly in type II lineages. It has also been shown that Dpn and E(spl)mγ-HLH form both homo- and heterodimers. *Dpn* has been shown to be a negative regulator of *pros* expression. In type II NBs, Dpn and E(spl)mγ-HLH have been shown to bind to *erm* promoter and prevent it from being expressed in the type II NB (Zhakariodaki et al., 2012)

Ase is a bHLH transcription factor and a member of *achaete-scute* complex of proneural genes. *Ase* is expressed in type I NBs, all the GMCs, Ase+ imINPs and mature INPs. *Ase* is not expressed in type II NBs and Ase- imINPs. *Ase* has been shown to form a negative feedback loop with *pros*, wherein *ase* positively regulates *pros* expression in NBs and *pro*s, in the GMCs, where it becomes nuclearly localized, acts as a negative regulator of *ase* expression. Ectopic expression of *ase* in the type II NBs leads to *pros* expression in type II NBs (which are pros- in wt) and a type II to type I NB fate conversion, leading to loss of INP production (Yasugi et al., 2014).

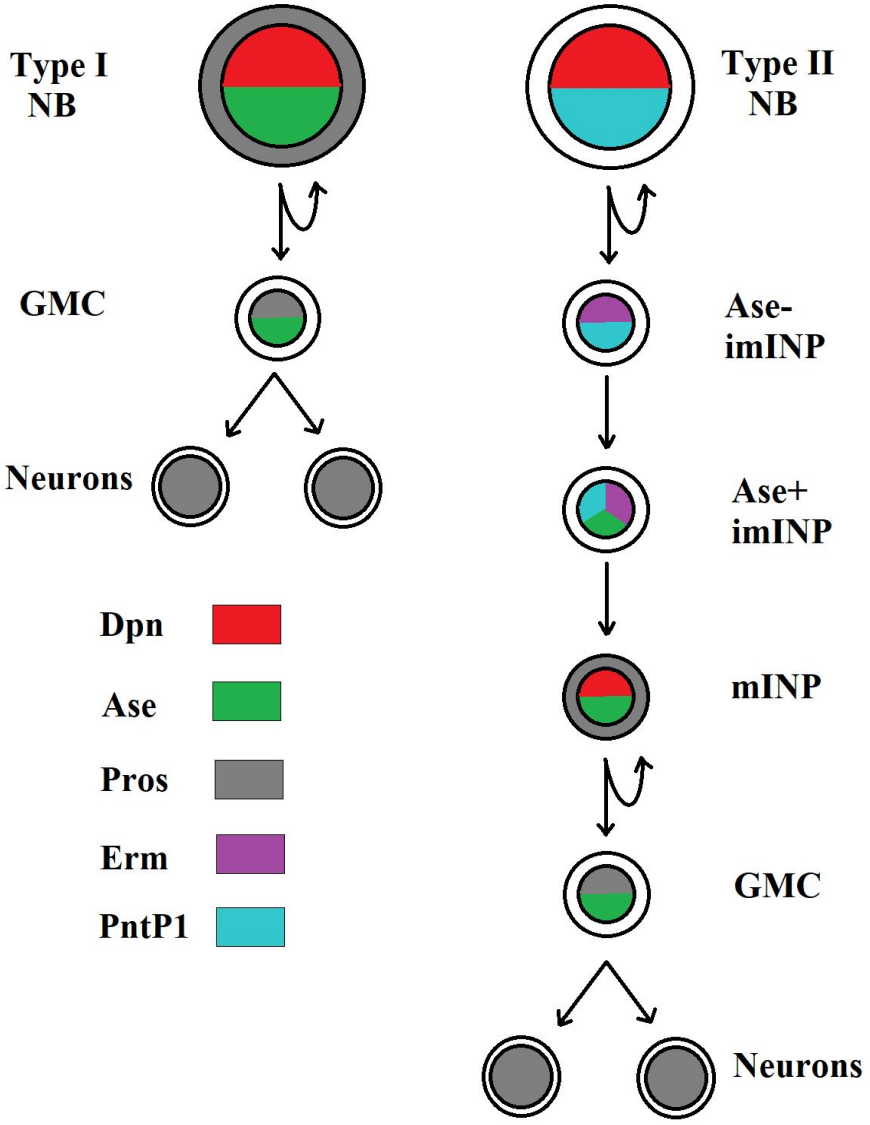


Figure 1. Expression of key regulators in type I and type II NB lineages

A schematic of type I and type II NB lineages. Expression of key regulators Dpn, Ase, Pros, Erm, and PntP1 in NBs, imINPs, mINPs, GMCs and newly born neurons in a NB lineage is depicted using different colors

2. Aims

The gene expression profiles in NSC lineages change dramatically, from the self-renewing NSCs, to the terminally differentiated neurons (Berger et al., 2012). Expression levels of key fate determinants are tightly regulated throughout the development of NSC lineage. Misregulation of key fate determinants in NSC lineages can lead to premature differentiation of NSCs resulting in reduced neurogenesis or failure to differentiate in NSCs leading to overproliferation or tumorigenesis (Homem et al., 2012). Gene regulation takes place at various levels. One such level of gene regulation is the so called 'epigenetic gene regulation'. Histone post-translational modifications play central role in epigenetic gene regulation. Histone acetylation and histone methylation are two of the most well studied histone modifications. Histone acetylation and histone methylation are governed by actions four types of histone modifiers: HATs, HDACs, HMTs, and HDMs (Lilja et al., 2013). The central aims of this work were to find the histone modifiers that have important functions in neural stem cell development and to shed some light on the mechanistic details of their function using a well characterized model system for stem cell biology, the *Drosophila melanogaster* larval NSCs, also known as the neuroblasts.

3. Results

3.1 RNAi mediated screen for histone modifiers

In order to find out the role of epigenetic modifiers belonging to one of the four following categories: (1) histone acetyltransferases (HATs), (2) histone deacetylases (HDACs), (3) histone methyltransferases(HMTs), and (4) histone demethylases (HDMs), in larval NB development, they were knocked down using RNAi via the UAS-Gal4 system (Brand et al., 1993). Of all the genes belonging to the above mentioned categories in the fly genome, candidates for the screen were selected only if they are expressed in the larval NBs (Table 1). For this, NB expression data published by Berger et al., 2012, was used.

For this screen I decided to use larvae from stage L3 of larval development. Stage L3 NBs provide a well established model system to study neural stem cell development (Homem et al., 2012). Staging and dissecting the stage L3 larvae is easier compared to other stages of post embryonic development.

Effect of knockdown of the candidate genes on type II NB lineages was the central focus of the screen. Compared to type I NBs, type II NBs have more complex lineages, and their mode of neurogenesis resembles that of the vertebrate neurogenesis. Type II NB have INPs, which dramatically increase their output in terms of neurons produced per NB. However, developmental potential of the INPs needs to be tightly regulated. Premature differentiation of INPs results in reduced number of neurons. Whereas, failure to limit the developmental potential of INPs results in INPs reverting back to NB fate, leading to exponential growth in type II NB number and tumorigenesis (Bello et al., 2006). Availability of specific markers, specific location in the central brain, peculiar size and shape of the lineages, and the fact that there are invariably only eight of them per lobe, make it easy to identify and count them unambiguously (Homem et al 2012).

Gene name	Catagory	Bloomington stock number
ash1	HMT	33705
CG4565	HMT	32893
chm	HAT	32484
E(z)	HMT	36068
egg	HMT	36797
Elp3	HAT	35488
enok	HAT	40917
G9a	HMT	36798
Gcn5	HAT	35601
gpp	HMT	34842
Hat1	HAT	34730
HDAC1	HDAC	36800
HDAC11	HDAC	32480
HDAC3	HDAC	64476
HDAC6	HDAC	34702
Kdm4A	HDM	34629
Kdm4B	HDM	35676
mof	HAT	36870
nej	HAT	31728
Sirt1	HDAC	32481
Sirt4	HDAC	36588
Sirt6	HDAC	36801
Su(var)3-3	HDM	36867
Su(var)3-9	HMT	43661
Taf1	HAT	35314
Tip60	HAT	18052

Table 1. List of candidate genes screened

Table shows the candidate genes screened for their role in larval NB development. The genes listed in column 1, belong to one of the four following categories given in column 2: (1) histone acetyltransferases, (2) histone deacetylases, (3) histone methyltransferases, and (4) histone demethylases. Column 3 shows the bloomington stock numbers of the RNAi lines used for the screen for respective genes in column one.

As mentioned earlier, the screen was performed using the UAS-Gal4 system. The Gal4 driver line {UAS-*dicer-2*; *insc*-Gal4 UAS-*CD8::GFP*} (Neumüller et al., 2011) expresses Gal4 protein in the expression pattern of the gene inscuteable, which is expressed in the CNS. In the early larval stages, it is also expressed in tissues other than the CNS, such as the gut. Later in the larval development the expression becomes CNS specific. In the larval CNS this driver line is expressed in all NBs, including, MBNBs, type I NBs of both CB and VNC, type II NBs, and the optic lobe NBs. It is also expressed in the mINPs. Even though the promoter used in the driver line is active only in the NBs and mINPs, the expressed Gal4 protein and the dsRNA or any other expressed protein under the UAS control is inherited by the daughter cells of the NBs. As a result, in a NB lineage, we typically see the NB, INPs (in case of type II lineages), GMCs, and newly born neurons to be positive for expression of genes under UAS control, with the highest expression in the NBs and the lowest expression in the neurons. The driver line has UAS-*CD8::GFP,* as a result, in this driver line, the whole NB lineage (from NB to newly born neurons) gets marked by membrane bound GFP. From here on, I will refer to this Gal4 driver line as DL(1) (driver line 1). In cases where the expression of a candidate dsRNA resulted in early larval lethality or arrest in development, before stage L3, using DL(1), probably, due to the effects of the knockdown of the candidate gene in non-CNS tissues, resulting from non-CNS tissue expression of DL(1), a more specific Gal4 driver line {UAS-*dicer-2*; *wor*-Gal4 *ase*-Gal80} was used (Neumüller et al., 2011). I will refer to this second Gal4 driver line as DL(2) (driver line 2). DL(2) expresses Gal4 under the promoter of the gene *worniu* (*wor*). It also contains a Gal80 under the promoter of the gene *asense,* and UAS-*CD8::GFP*. *Wor* promoter is active in all NBs, INPs and GMCs. *Ase* promoter is active in all the cells whereas *wor* promoter is active except in type II NBs and Ase- imINPs. As a result the Gal4 is only active in type II NBs and Ase- imINPs. However, since any gene expressed under the UAS control will be inherited by the daughter cells of the cell that expressed it, we typically see genes expressed using DL(2) to be expressed in type II NBs, both, Ase+ and Ase- imINPs, late born mINPs and some of the newly born progeny of these late born mINPs. However type I lineages do not show any expression of genes under UAS control when driven by DL(2).

In this screen, the stage L3 larval CNSs were stained for Dpn, a pan neural stem cell marker. It marks the nuclei of MBNBs, type I NBs, type II NBs, optic lobe NBs, and mature INPs in type II NB lineages. At least five CNSs were imaged for every candidate gene.

Out of all the genes screened (Table 1), three genes showed a significant departure from the the wild type NB numbers upon RNAi mediated knockdown in larval stage L3. *HDAC1*, a histone deacetylase, when knocked down using DL(1) gives a developmental arrest in stage L1 of larval development. To circumvent this problem, DL(2) was used to drive UAS-*HDAC1*-RNAi. In control (DL(2)) brain lobes, 8 GFP+ type II NB lineages with one Dpn+ NB, and several Dpn+ mINPs are seen. Whereas, in *HDAC1* RNAi knockdowns only (3.6 ± 0.9) type II NBs were present. The surviving NBs have a fewer number of mINPs in each lineage((0.5 ± 0.8) compared to (23.9 ± 4)) in control (Figure 2). Since I have only used a type II NBs specific driver line to knockdown *HDAC1*, I cannot comment on the effect of its knockdown type I NBs.

The second gene whose knockdown affected the number type II NBs was *ash1*. *Ash1* is a histone methyltransferase. Knockdown of *ash1,* using DL(1), affects both type I and type II NB lineages. Upon *ash1* RNAi knockdown, all eight type II lineages are lost. It also reduces the number of type I NBs in the thoracic ventral nerve cord from (161.8 ± 3.5) in control VNCs (DL(1)) to (95 ± 9.5) (Figure 3 and 4).

The third gene whose knockdown affected the number type II NBs was *enok. Enok* is a histone acetyltransferase of MYST family. The vertebrate homologs of *enok* are called *MOZ* and *MORF*. Similar to *ash1*, *enok* knockdown also led to complete elimination of type II lineages. However, contrary to *ash1* knockdown, *enok* knockdown has no effect on the number of type I NBs in the thoracic VNC (Figure 3 and 4).

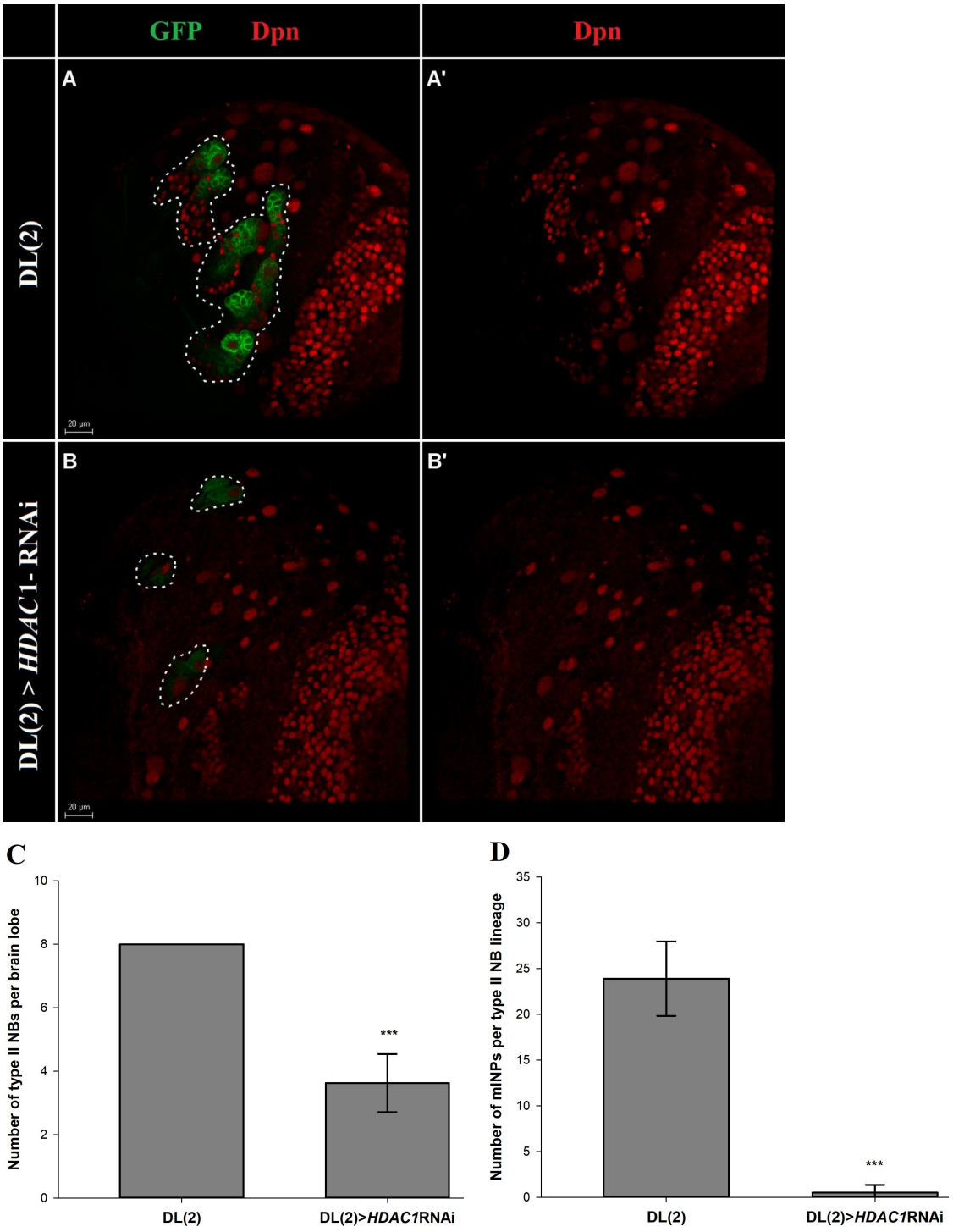
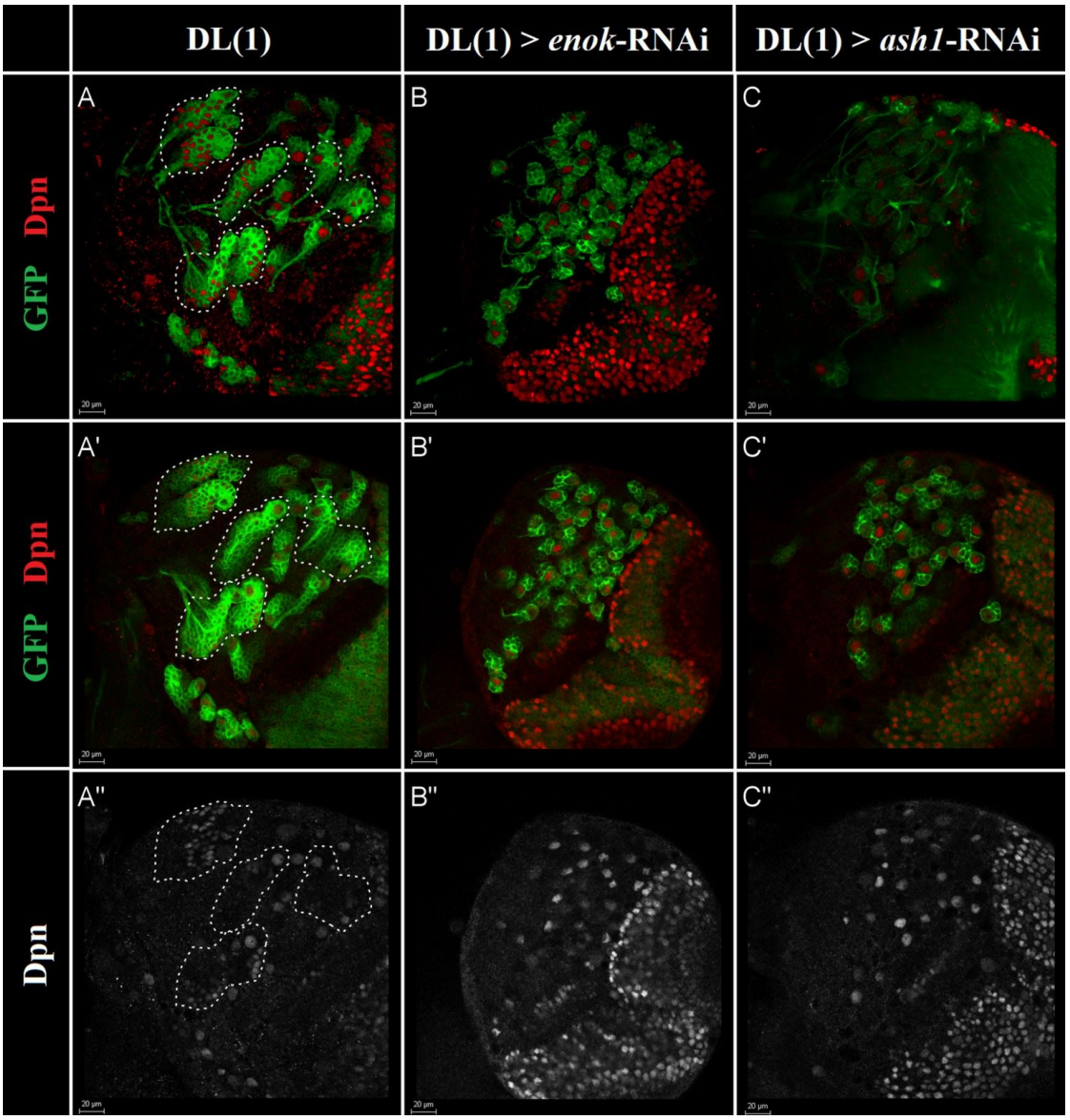


Figure 2. *HDAC1* is necessary for type II NB and mINP maintenance

HDAC knockdown using RNAi with DL(2) leads to reduction in type II NB and mINP number. (A) & (B) 3D projection of dorsal, CB, from stage L3 larvae from DL(2) & UAS-*HDAC1*-RNAi driven by DL(2). Type II NB lineages are marked by white dotted lines. (A') & (B') Dpn channel from (A) & (B) respectively. (C) & (D) Quantification of phenotype shown in (A) & (B). (C) *HDAC1* knockdown leads to reduction in type II NB number from (8±0) in control to (3.6±0.9). $p < 0.001$. (D) *HDAC1* knockdown leads to reduction in number of mINPs from (23.9±4) to (0.5±0.8). $p < 0.001$.



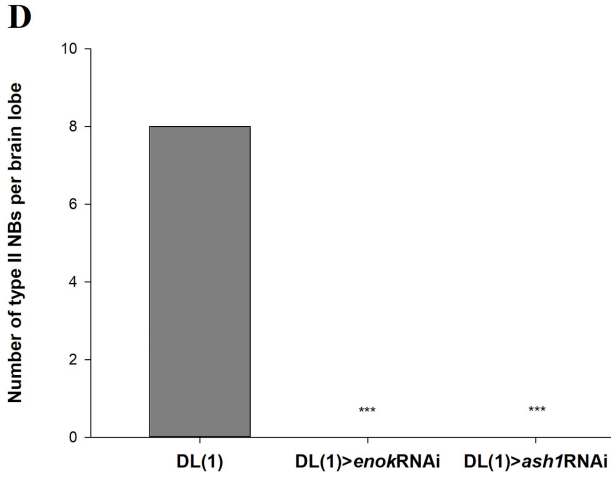


Figure 3. *Enok* and *ash1* are necessary for type II NB maintenance

Type II lineages are marked with dotted lines. Loss of *enok* or *ash1* resulted in loss of type II lineages. (A), (B), & (C) 3D projections of dorsal CB of DL(1)(control), DL(1) driving *enok*-RNAi, and DL(1) driving *ash1*-RNAi larvae, respectively, at L3 stage, stained for Dpn. (A'), (B'), and (C') single confocal slices from (A), (B), and (C) respectively. (A''), (B''), and (C'') Dpn channel from (A'),(B'), and (C'). (D) Quantification of type II neuroblasts. Each control brain lobe had 8 type II NBs (out of which 7 are visible here in (A)). RNAi knockdown of *enok* or *ash1* results in loss of all eight type II NBs with $p < 0.001$

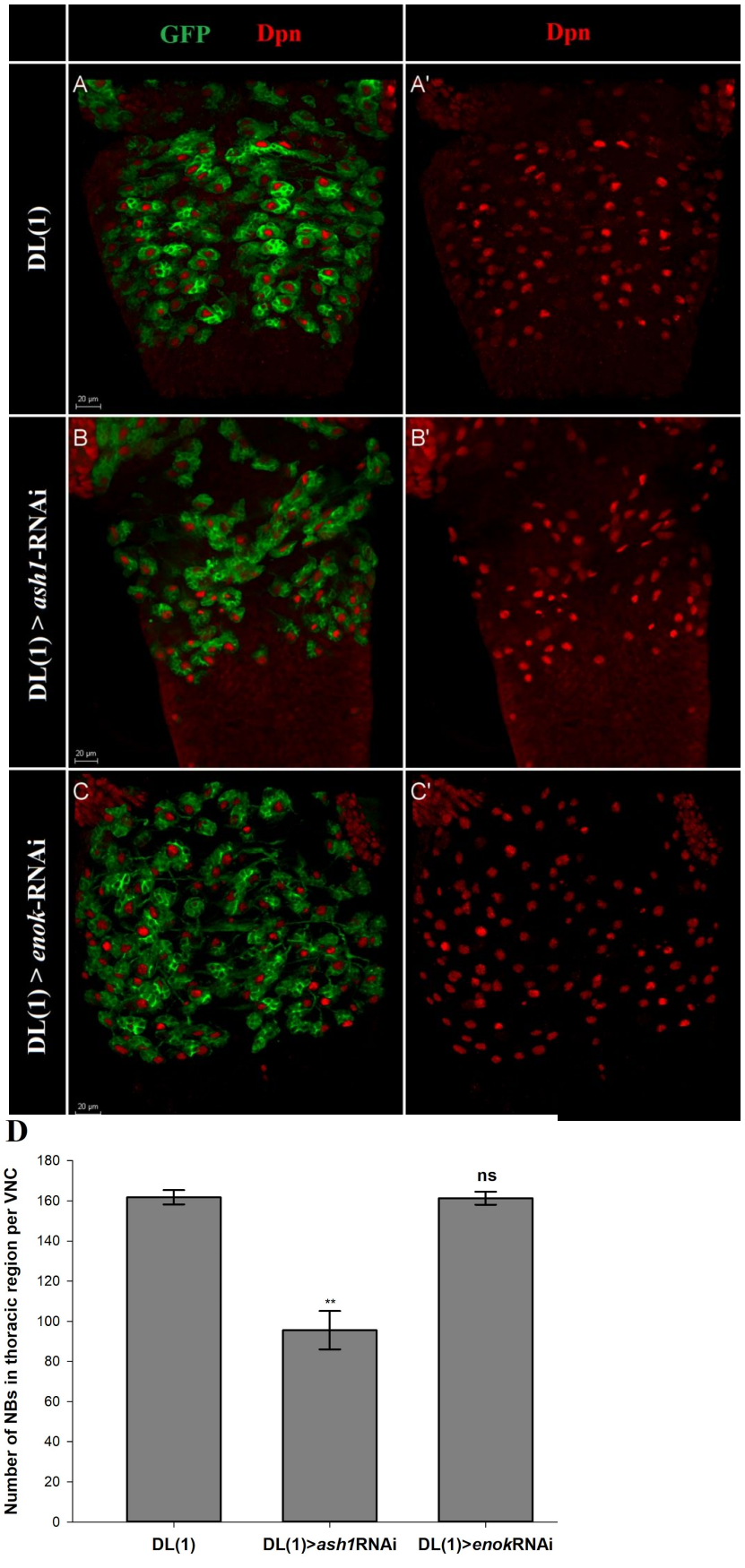


Figure 4. Loss of *ash1*, but not *enok*, affects number of type I NBs of the thoracic VNC

(A) to (C) 3D projection of the thoracic region of the VNC, from stage L3 larvae, stained with Dpn. RNAi knockdown of *ash1* using DL(1) results in reduced number of type I NBs, whereas *enok* knockdown does not have an effect on the number of type I NBs of the thoracic VNC. (A') to(C') the Dpn channel from (A) to (C) respectively. (D) Quantification of phenotypes depicted in (A) to (C). *Ash1* knockdown resulted in reduction in number of type I NBs of the VNC (95 ± 9.5) compared to control (161.8 ± 3.5), $p < 0.01$. However, in *enok* knockdowns the number of type I NBs of the thoracic VNC (161.3 ± 3.2) was not affected.

Enok, is conserved from yeast to humans (Yang et al., 2015). In flies, it has been reported to affect proliferation of MBNBs (Scott et al., 2001). Mushroom bodies are involved in olfactory learning and memory (Strausfeld et al., 1998). Loss of MOZ/MORF HAT complex activity leads to reduced size of olfactory bulb in mice (Marson et al., 2006). Individuals with mutations in *MOZ* or *MORF* have severe intellectual disability. Loss of *enok* leads to loss of type II NB lineages. Type II neuroblasts give rise to the central complex in *Drosophila*. Central complex is involved in memory and learning (Jiang et al., 2012). Intrigued by the apparent functional conservation of *enok* and the fact that it selectively affects type II NBs and not the type I NBs of the thoracic VNC, we decided to further investigate the role of *enok* in type II NB development.

3.2 Confirming the *enok* RNAi knockdown phenotype

In order to confirm the phenotype given by RNAi mediated knockdown of *enok* RNAi line #1 (Bloomington stock # 40917) (Figure 3 and 4), another RNAi line against *enok* was used (VDRC #108400). When driven by DL(2), the the second RNAi line also results in a complete loss of type II NB lineages (Figure 17). To further confirm the RNAi mediated *enok* loss of function phenotypes, I used, a previously described, null mutant for *enok*, named $enok^{1}$. $Enok^{1}$ homozygous mutants have a retarded growth phenotype and take twice as much time to reach wandering larval stages compared to the heterozygotes (Scott et al., 2001). Stained at this stage for Dpn, the homozygotes showed a lack of type II NBs, identified by Dpn staining, size and surrounding mINPs in the dorso-medial CB, suggesting that the mutants give phenotype similar to that of the RNAi knockdowns. Overexpression of full length *enok* rescues the loss of type II NBs in *enok* RNAi knockdown further confirming the specificity of RNAi lines used (Figure 5).

3.3 Expression pattern of Enok in the CB

From the transcriptome analysis it is known that *enok* transcript shows a 1.3 fold enrichment in NBs compared to differentiated neurons (Berger et al., 2012). I checked Enok protein expression using specific antibody raised against Enok protein, kindly

provided by Prof Michal Pankratz. The antibody has been previously tested and follows similar expression pattern as that of *enok* transcript (Zinke and Pankratz, unpublished). To analyze the expression of Enok in the CB, stage L3 larvae from DL(2) were stained with antibody against Enok, along with the antibody against the pan neural stem cell marker, Dpn. The anti-Enok antibody staining showed that Enok protein is present in the nuclei of all the cells of the central brain (Figure 6). However, it can be easily noted that it is expressed in higher levels in NBs compared to their progeny. In order to quantify the expression levels of Enok in different cells of NB lineages, nuclear pixel intensities of confocal images of NBs and their progeny were measured using ImageJ. For an 8-bit image, pixel intensities have numerical values between a minimum of 0 and a maximum of 255. The pixel intensities for type II NBs, type I NBs, mINPs, and GMCs or neurons (due to the similar size and lack of Dpn expression, I was unable to distinguish GMCs from neurons) were (123.3 ± 4.1), (120.6 ± 4.2), (110 ± 4.3), and (73.3 ± 3.3) respectively. The difference between pixel intensities of type II and type I NBs was not statistically significant ($p > 0.05$). However the difference between any other pair of cell types, was statistically significant ($p < 0.001$). This suggests that Enok is expressed in higher levels in NBs compared to their progeny. However both type I and type II NBs express similar levels of Enok.

3.4 Function of *enok* in type II NBs is HAT complex dependent

In flies, the Enok HAT complex is made up of four proteins, Enok, Br140, Eaf6, and ING5. In this complex Br140, a homolog of mammalian BRPF1, acts as a scaffold protein for the complex (Huang et al., 2016). In order to see if the *enok* knockdown phenotype comes from *enok*'s HAT complex dependent function or not, I decided to knockdown *br140* using RNAi. Similar to *enok* knockdown phenotype, RNAi mediated knockdown of *br140* driven by DL(1), resulted in a reduced type II NB number per brain lobe, from (8 ± 0) in control to (1.4 ± 0.54), ($p <0.001$) (Figure 7). Suggesting that the function of *enok* in maintenance of type II NB is dependent on the HAT complex.

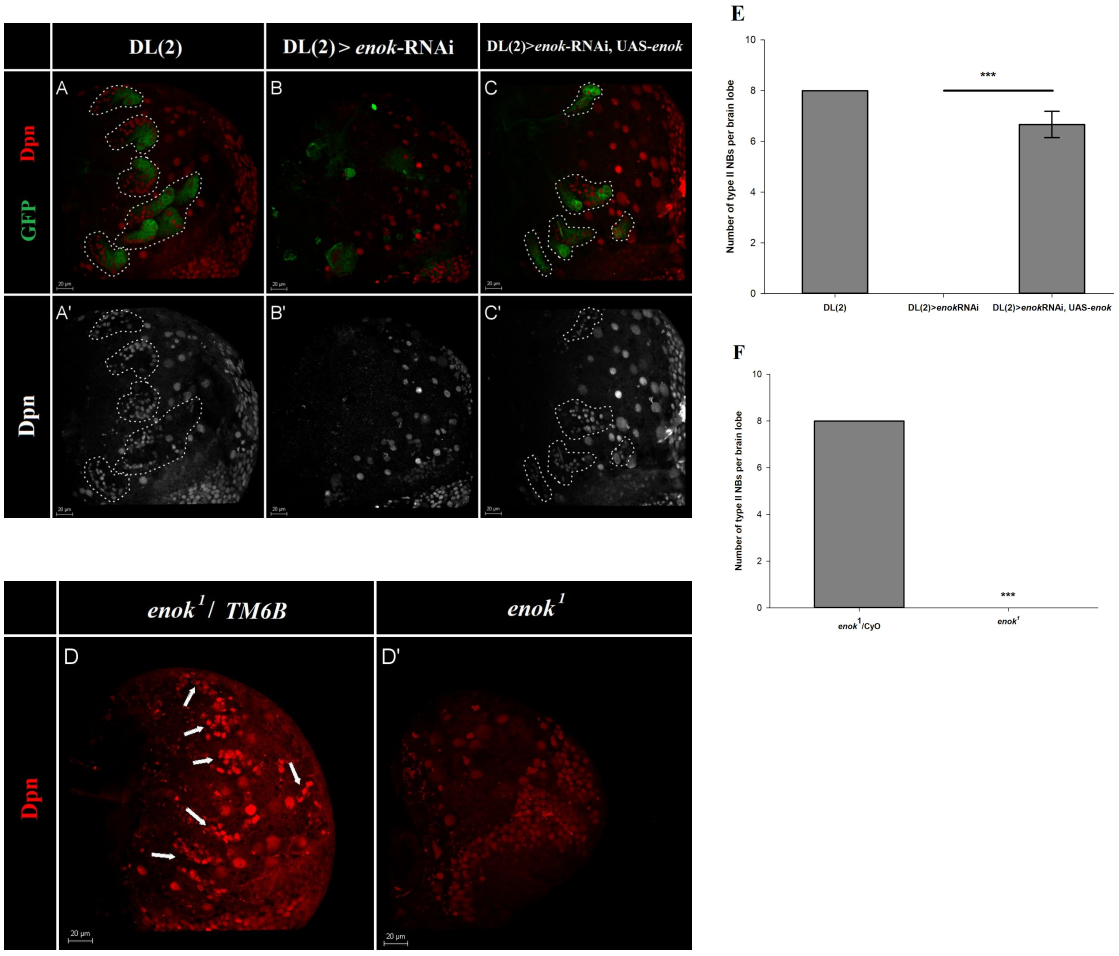
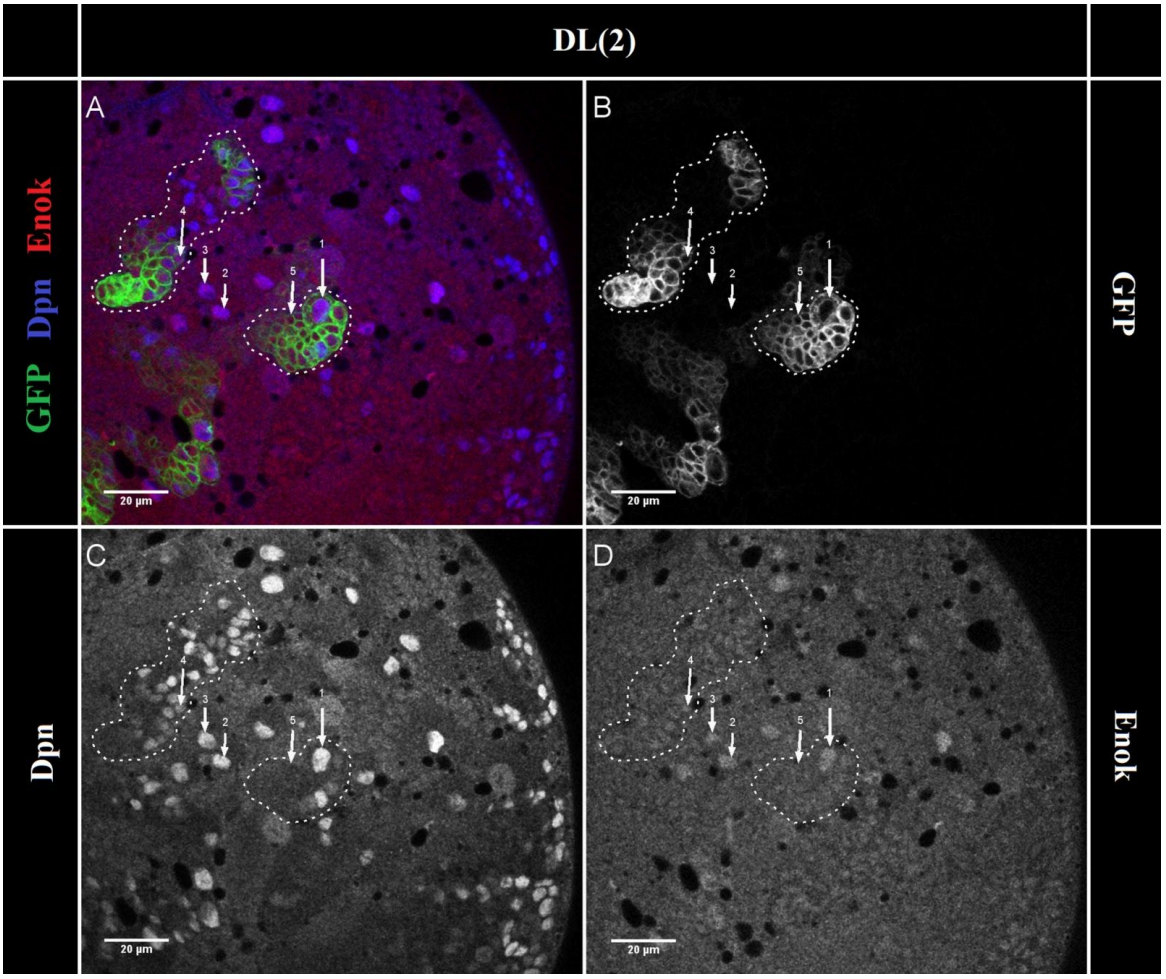


Figure 5. Confirming the *enok* RNAi knockdown phenotype

Overexpression of full length *enok,* in the *enok* RNAi knockdown background rescues the loss of type II NB phenotype. Similar to *enok* RNAi knockdown larvae, the larvae homozygous for *enok*[1], a null mutation, also show a loss of type II NB phenotype. (A) to (C) 3D projection of dorsal CB of stage L3 larvae from DL(2), DL(2) driving UAS-*enok*-RNAi, and DL(2) driving UAS-*enok*-RNAi together with full length *enok* under UAS control, respectively, stained for Dpn. (A') to (C') show Dpn channel form (A) to (C) respectively. (D) and (D') show 3D projection of dorsal CB of stage L3 larvae heterozygous and homozygous, respectively, for *enok*[1], stained with Dpn. (E) The quantification for phenotypes shown in (A) to (C). Simultaneous knockdown and overexpression of *enok* rescues the loss of type II NB phenotype from (0) to (6.6±05). $p < 0.001$. (F) The quantification for phenotypes shown in (D) and (D'). Larvae homozygous for *enok*[1] have (0) type II NBs compared to (8) type II NBs is heterozygotes. $p < 0.001$.



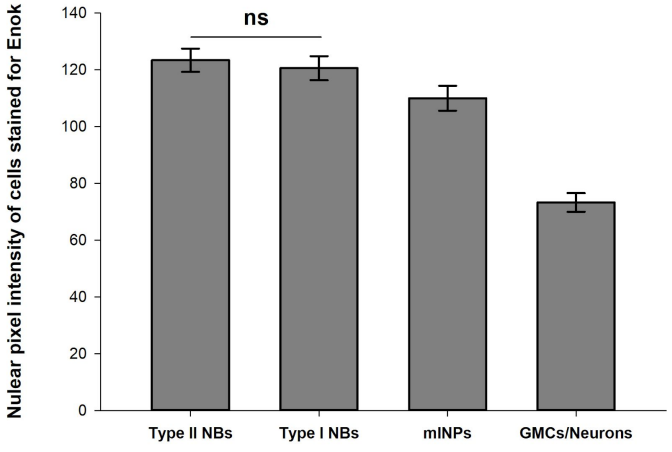
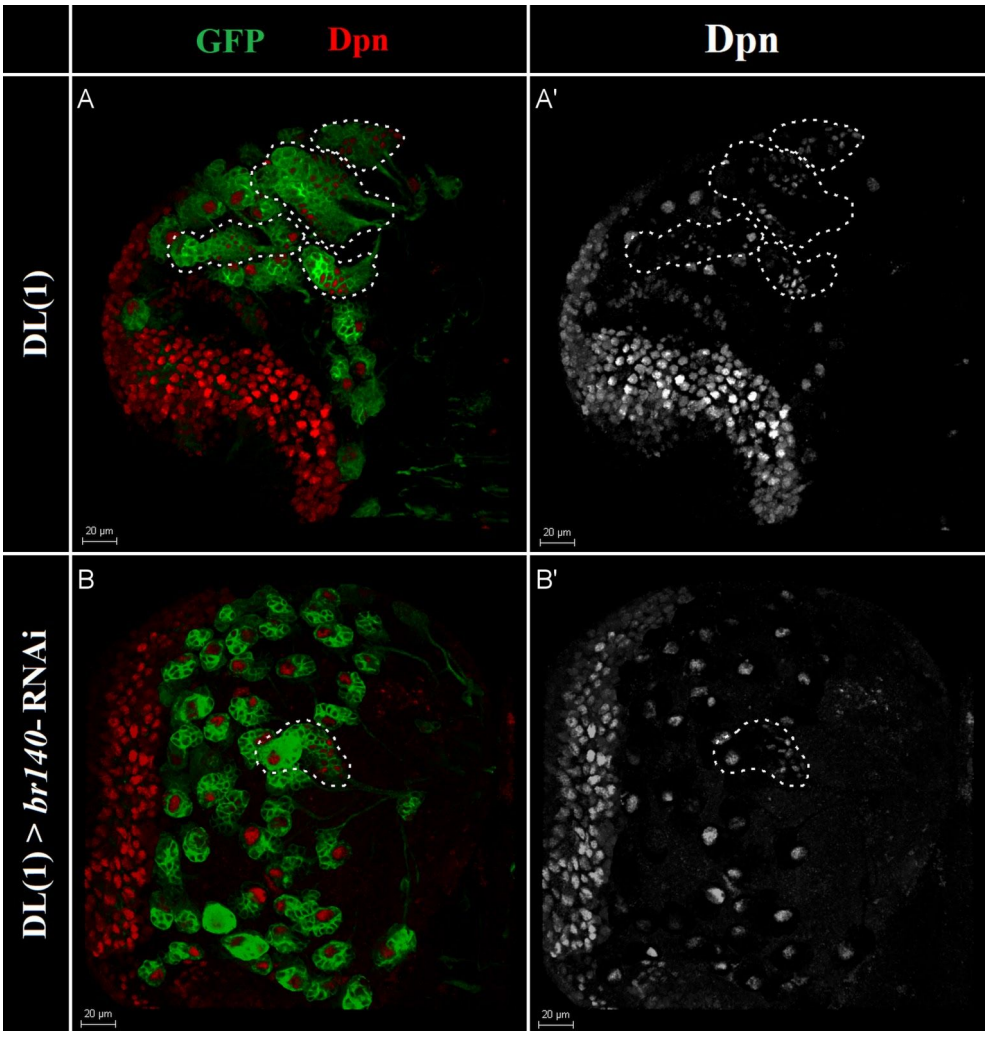


Figure 6. Expression of Enok in larval central brain

Enok protein is nuclearly localized. It is ubiquitously expressed in the larval CB. The expression level is higher in NBs compared to other cells of the lineage (A) Single confocal slice from dorsal, WT (DL(2)), third instar larval central brain, stained for Dpn and Enok. Arrow 1 to 5 mark, a type II NB, a couple of type I NBs, a mINP, and either a GMC or a neuron, respectively. (B) to (D) individual channels, GFP, Dpn, and Enok respectively, from (A). (E) Quantification of Enok expression in NB lineages using pixel intensity (0-255) as a readout for expression strength. There is no significant difference in Enok expression between type II (123.3 ± 4.1) and type I (120.6 ± 4.2) NBs. All other pairwise differences in pixel intensity means are statistically significant with $p < 0.001$. The pixel intensities for mINPs and GMCs or neurons are 110 ± 4.3 and 73.3 ± 3.3.



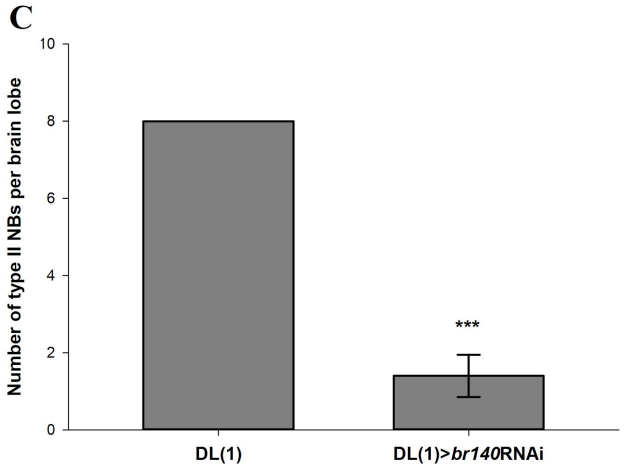
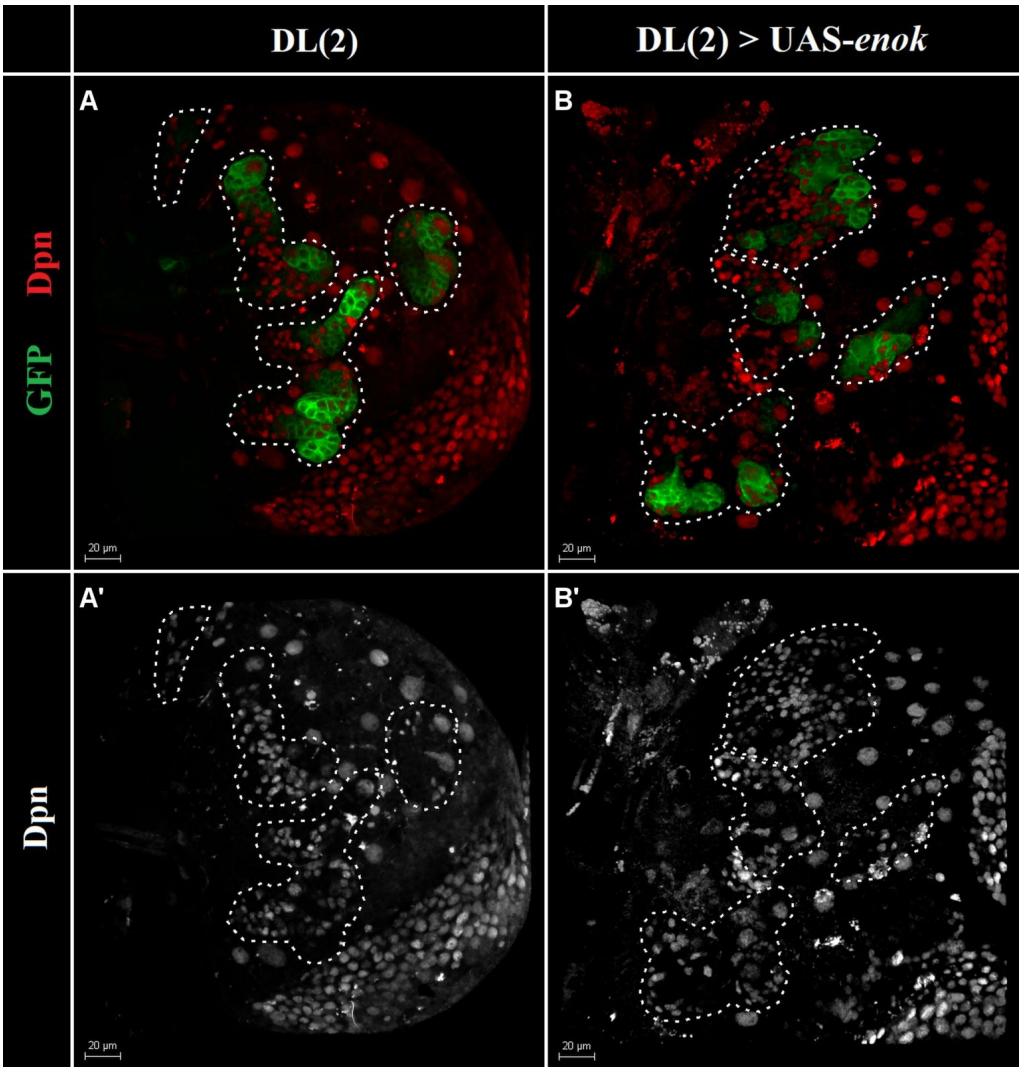


Figure 7. Br140, the scaffold protein of the Enok HAT complex is also necessary for type II NB maintenance

Knockdown of *br140* by RNAi leads to reduction in number of type II NBs. (A) and (B) 3D projection of stage L3, dorsal, larval, CB from control (DL(1)) and UAS-*br140*-RNAi driven by DL(1), respectively, stained for Dpn. Type II NB lineages are marked by white dotted lines. (A') and (B') Dpn channel from (A) and (B) respectively. (C) Quantification of phenotype shown in (A) and (B). RNAi mediated knockdown of *br140* leads to reduction in number of type II NBs from (8±0) in controls to (1.4±0.54). $p < 0.001$.

3.5 Overexpression of *enok* results in supernumerary Dpn+ cells

To further study the role of *enok* in NB development, I decided to overexpress *enok* in NBs. Overexpression of *enok* using DL(1) results in early (stage L1) larval lethality. To circumvent this issue I used two different strategies. First, I used a more specific DL(2), driver line to overexpress *enok*. This led to more than 8 (11.6 ± 2.9 (p <0.01)) type II NB lineages per brain lobe in stage L3 larvae (Figure 8). Second, since DL(1)'s expression becomes CNS specific only during late L2 stage in order to avoid non CNS expression of DL(1), I used DL(1) along with temperature sensitive Gal80 (Zeidler et al., 2004) under the ubiquitously active promoter of tubulin (*alphaTub84B*). The temperature sensitive Gal80 is active at lower temperature (T_{low} = 18° C) and hence there is no Gal4 activity at this temperature. Whereas at higher temperatures (T_{high} = 29° C) the Gal80 becomes inactive and the Gal4 can drive the expression of genes under UAS promoter. I also found out that the $Gal80^{ts}$ is still active at 25° C and represses Gal4 activity in the CNS. Hence, I decided to use 25° C as T_{low}. The larvae were allowed to grow at 25°C and then shited at various developmental time points to T_{high} = 29° C. After trying out various timepoints for the temperature shift, I found out that allowing the larvae to grow at 25° C until 60 hours after larval hatching, and then shifting them to 29° C and dissecting the CNSs shortly before pupa formation maximises both, the chances of larvae reaching the wandering L3 stage, and the time the larvae are at Gal4 permissive temperature. When the larvae were stained with Dpn after following the scheme above, they showed supernumerary Dpn+ cells in both type I and type II NB lineages (Figure 9 and 10). Wild type, type I NBs have only one Dpn+ cell in each NB lineage, whereas *enok* overexpressing type I NB lineages of thoracic VNC have (6.3 ± 2.6 (p <0.001)) Dpn+ cells per lineage . However, since it is difficult to distinguish one type II NB lineage from the other in these larvae, total number of Dpn+ cells in type II lineages per brain lobe were conted. Overexpression of *enok* resulted in increase in number Dpn+ cells in type II NB lineages per brain lobe from (189 ± 6.5) in control to (228 ± 20), (p < 0.001).



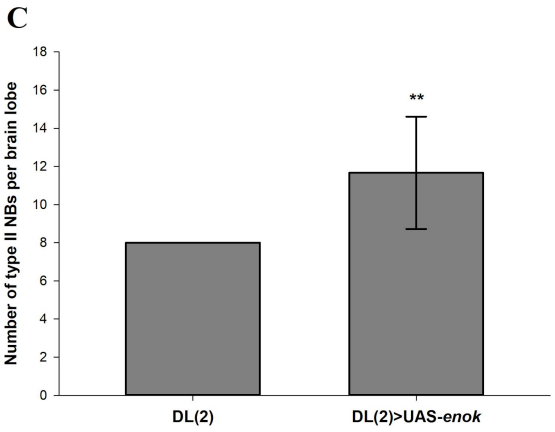
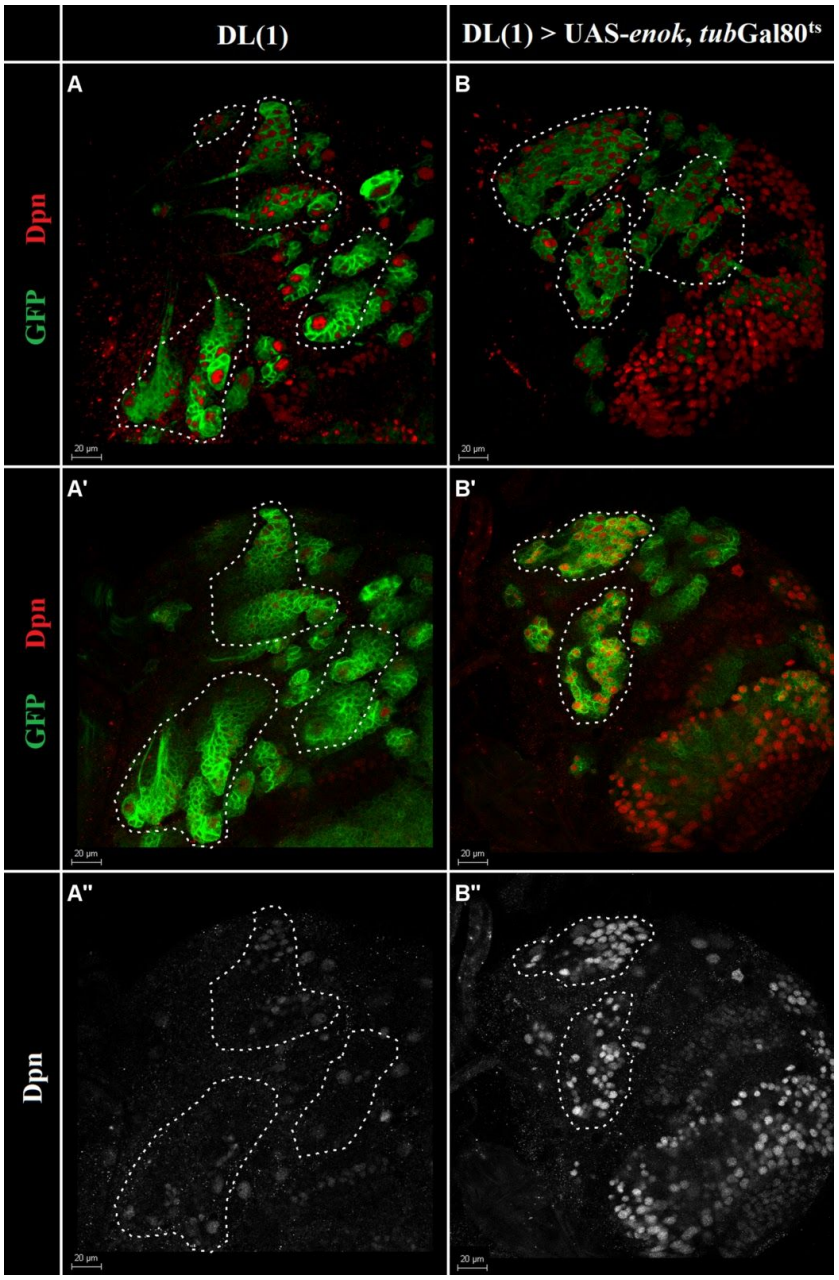


Figure 8. Overexpression of *enok* leads to more than eight type II NB lineages per lobe

(A) and (B) 3D projection of stage L3, dorsal, larval CB from control (DL(2)) and full length *enok* under UAS control driven by DL(2), respectively, stained for Dpn. Type II NB lineages are marked by white dotted lines. (A') and (B') Dpn channel from (A) and (B) respectively. (C) Quantification of phenotype depicted in (A) to (C). Overexpression of *enok* increases the number of type II NBs from (8) in control to (11.6±2.9). $p < 0.01$.



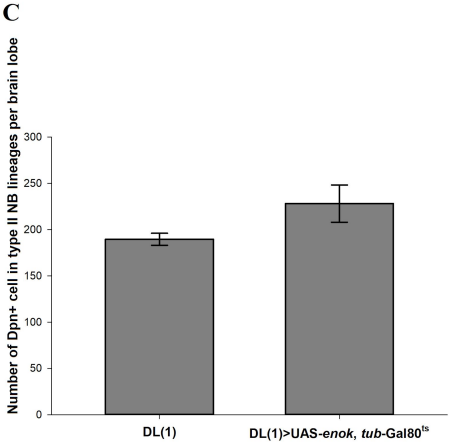
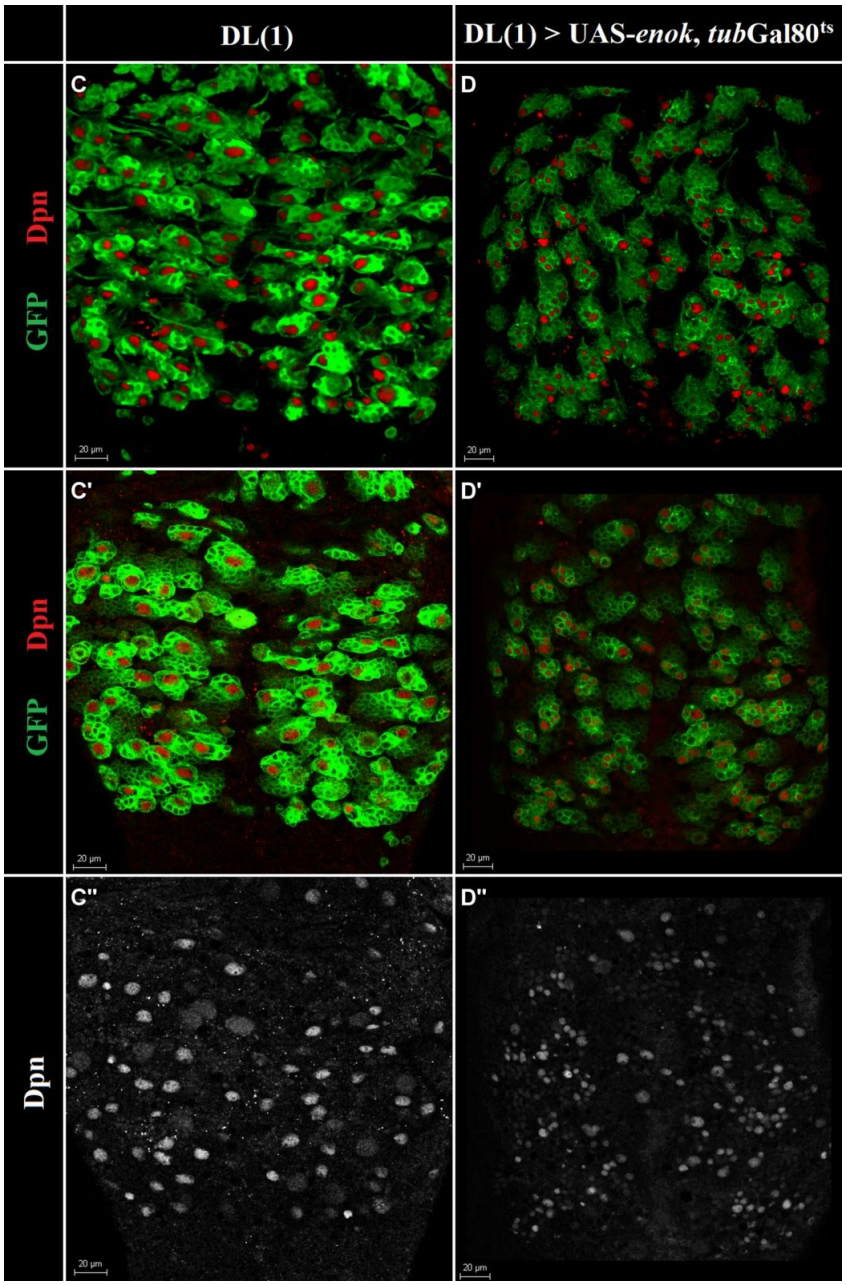


Figure 9. Overexpression of *enok* using DL(1) leads to supernumerary Dpn+ cells in the type II NB lineages

Overexpression of *enok*, using the strategy described previously, results in production of supernumerary Dpn+ cells in type II NB lineages. (A) and (B) 3D projection of dorsal, stage L3, CB from control (DL(1)) and UAS-*enok* driven by DL(1), *tub*-Gal80^{ts}, stained for Dpn. (A') and (B') single confocal slices from (A) and (B) respectively. (A'') and (B'') Dpn channels from (A') and (B'), respectively. (C) Quantification of phenotype shown in (A) and (B). Overexpression of *enok* leads to increase in Dpn+ cells in type II NB lineages per brain lobe from (189±6.5) in controls to (228±20). $p < 0.001$



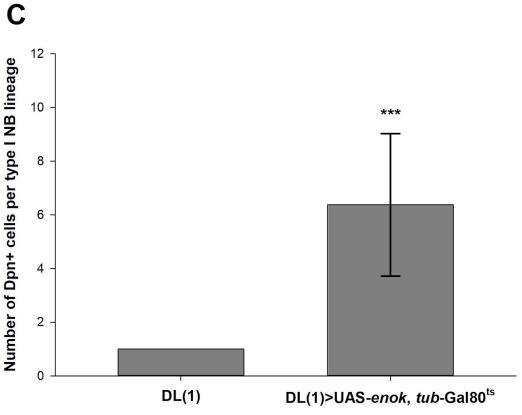


Figure 10. Overexpression of *enok* using DL(1) leads to supernumerary Dpn+ cells in the type I NB lineages

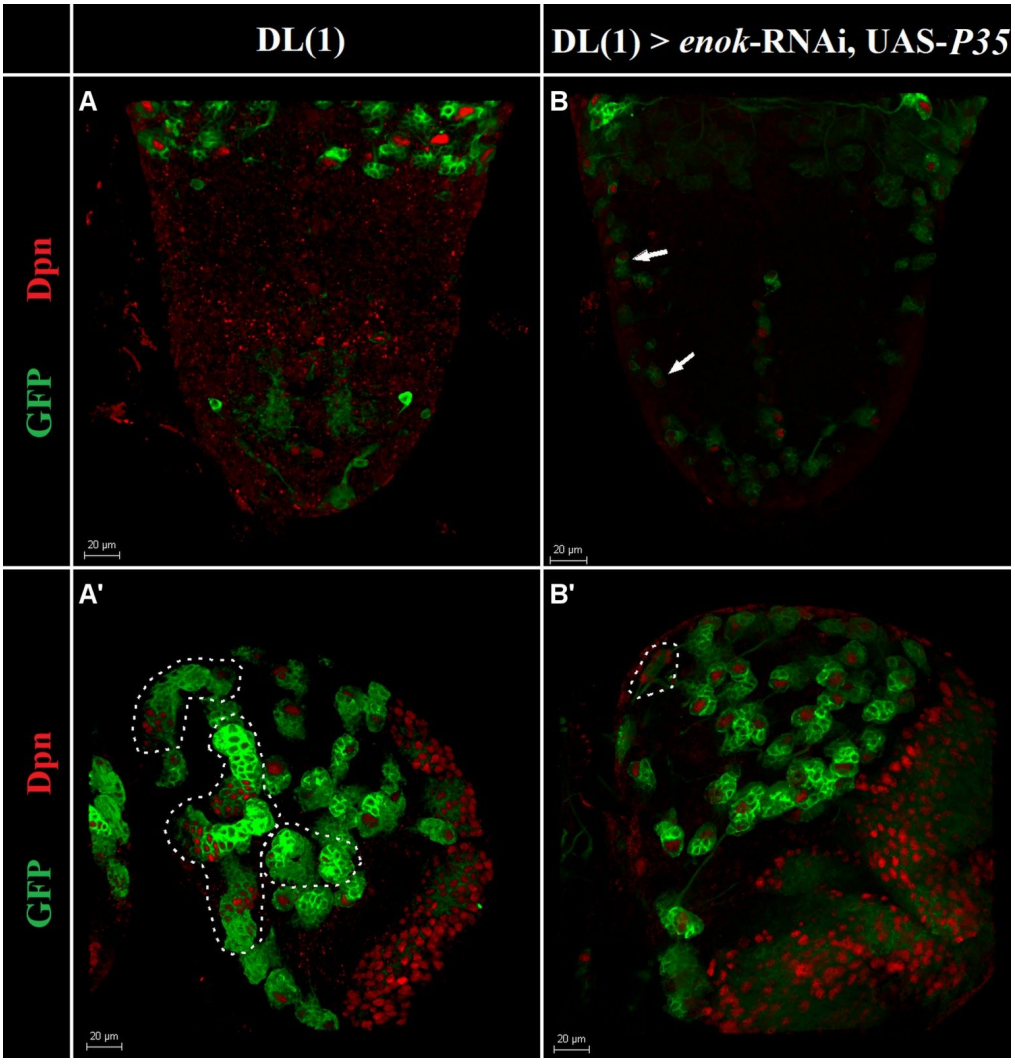
Overexpression of *enok*, using the strategy described previously, results in production of supernumerary Dpn+ cells in type I NB lineages. (A) and (B) 3D projection of thoracic region of stage L3, VNC from control (DL(1)) and UAS-*enok* driven by DL(1), *tub*-Gal80^{ts} , stained for Dpn. (A') and (B') single confocal slices from (A) and (B) respectively. (A'') and (B'') Dpn channels from (A') and (B'), respectively. (C) Quantification of phenotype shown in (A) and (B). Overexpression of *enok* leads to increase in Dpn+ cells in type I NB lineages from (1±0) NB per lineage in contro to (6.3±2.6) NBs per lineage. $p < 0.001$

3.6 Three possible mechanisms for loss of type II NB is *enok* RNAi knockdown

There are three possible mechanisms by which *enok* knockdown can lead to loss of type II NBs. First, *enok* knockdown causes cell death of type II NBs. Second, the type II NBs undergo premature terminal differentiation. Third the type II NB switch fate and become type I NBs. To distinguish between these three possibilities, I performed the following experiments.

3.7 Overexpression of *P35* fails to rescue the *enok* knockdown phenotype

In order to check whether *enok* knockdown leads to apoptosis of type II NBs, pan-caspase inhibitor, *P35* (Hay et al., 1994), was overexpressed in the *enok* RNAi knockdown background. It is known that abdominal segment NBs undergo Abdominal-A mediated apoptosis (Bello et al., 2003). Overexpression of *P35* rescues the apoptosis of abdominal NBs in both wt (DL(1)) and *enok* knockdown background, but fails to rescue the loss of type II NBs in *enok* knockdown larvae (Figure 11). This suggests that loss of type II NB in *enok* knockdown CNSs does not occur via caspase mediated apoptosis. However other modes of apoptosis, and non-apoptotic cell death could not be excluded.



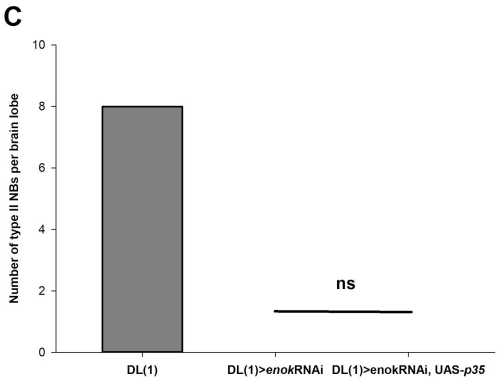


Figure 11. Overexpression of *P35* does not rescue the loss of type II NBs caused by *enok* RNAi knockdowns
Overexpression of *P35* rescues the abdominal NBs in the VNC, which are known to undergo apoptosis, but it fails to rescue loss of type II NBs cause by RNAi mediated *enok* knockdown. (A) and (B) 3D projection of abdominal region of VNCs, from,wandering L3 larvae from DL(1) and DL(1) driving both *enok* RNAi and *P35*, under UAS control. White arrows show rescued abdominal NBs. (B) and (B') 3D projection of, dorsal, stage L3, CBs, from DL(1) and DL(1) driving UAS-*enok*-RNAi and UAS-*p35*. Type II lineages are marked with white dotted lines. (In B' the white dotted lines mark remaining cells of a type II lineage in which the NB is missing from the lineage). (C) Quantification of the phenotype shown (B) and (B') from this figure and b from figure 3B. After *P35* overexpression, the number of NBs stays same (0) as it was in RNAi mediated knockdown of *enok* alone.

3.8 *Enok* knockdown converts type II NBs into type I NBs

Next, I decided to check whether loss of *enok* leads to type II to type I NB fate conversion. Type I NBs have nuclear Ase and cytoplasmic Pros. Progeny of type I NBs, GMCs, have nuclear Pros. Type II NBs, on the other hand, are negative for both nuclear Ase, cytoplasmic and nuclear Pros. The immediate progeny of type II NBs, the imINPs are negative for nuclear Pros. In my immunohistochemistry experiments, it was difficult to see the weakly expressed cytoplasmic Pros even in the wild type, type I NBs. However, presence of nuclear Ase in type I NBs, and absence of Ase in type II NBs could be readily seen. Similarly, presence of nuclear Pros in the progeny of type I NBs (GMCs) and absence of nuclear Pros in the progeny of type II NBs (imINPs) was also clearly visible. Hence, in order to check whether type II NBs in *enok* knockdown CNSs convert to type I NB fate, I decided to use nuclear Ase in the NBs and nuclear Pros in their progeny as markers of type I NB fate. Indeed RNAi knockdown of *enok* using DL(2) led to upregulation of nuclear Ase in type II NBs (Figure 12). Furthermore, Vanessa Zurawski, F2 student in our lab, observed that loss of *enok* using DL(2) led to nuclear Pros in the progeny of type II NBs, whereas control type II NB, progeny were negative for nuclear Pros (Figure 13). Taken together, the results suggest that after *enok* knockdown, type II NBs convert to type I NB fate.

In DL(2), the Gal80 is under *ase* promoter. We observed that *ase* got upregulated in type II NBs upon *enok* knockdown. This would lead to Gal80 expression in type II NBs. Which in turn would lead to loss of Gal4 activity and loss of GFP expression. Indeed we see a progressive reduction in GPF strength in *enok* knockdowns, which eventually leads to disappearance of type II lineages.

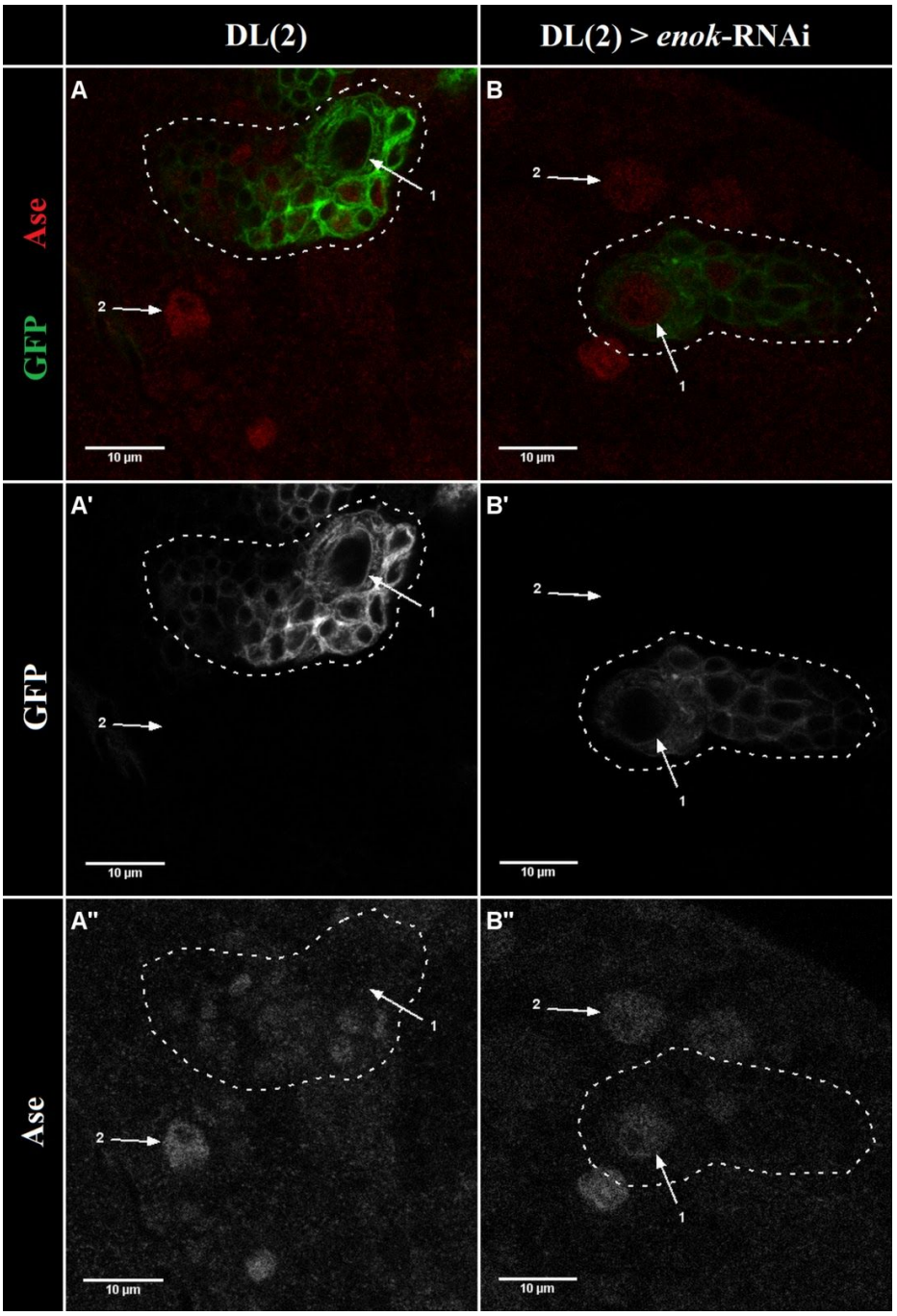


Figure 12. RNAi mediated *enok* knockdown results in type II NBs expressing type I NB marker Ase
Knockdown of *enok* via RNAi driven by DL(2) leads to nuclear Ase in type II NBs. Ase is not expressed in type II NBs in controls (DL(2)). (A) and (B) Type II NB lineages from 50 ALH larvae from DL(2) and UAS-*enok*-RNAi driven by DL(2), respectively, stained for Ase. Type II lineages are marked by white dotted lines. Arrow 1 and 2 mark type II and type I NBs respectively. (A') and (B') GFP channel from (A) and (B) respectively. (A'') and (B'') Ase channel from (A) and (B) respectively.

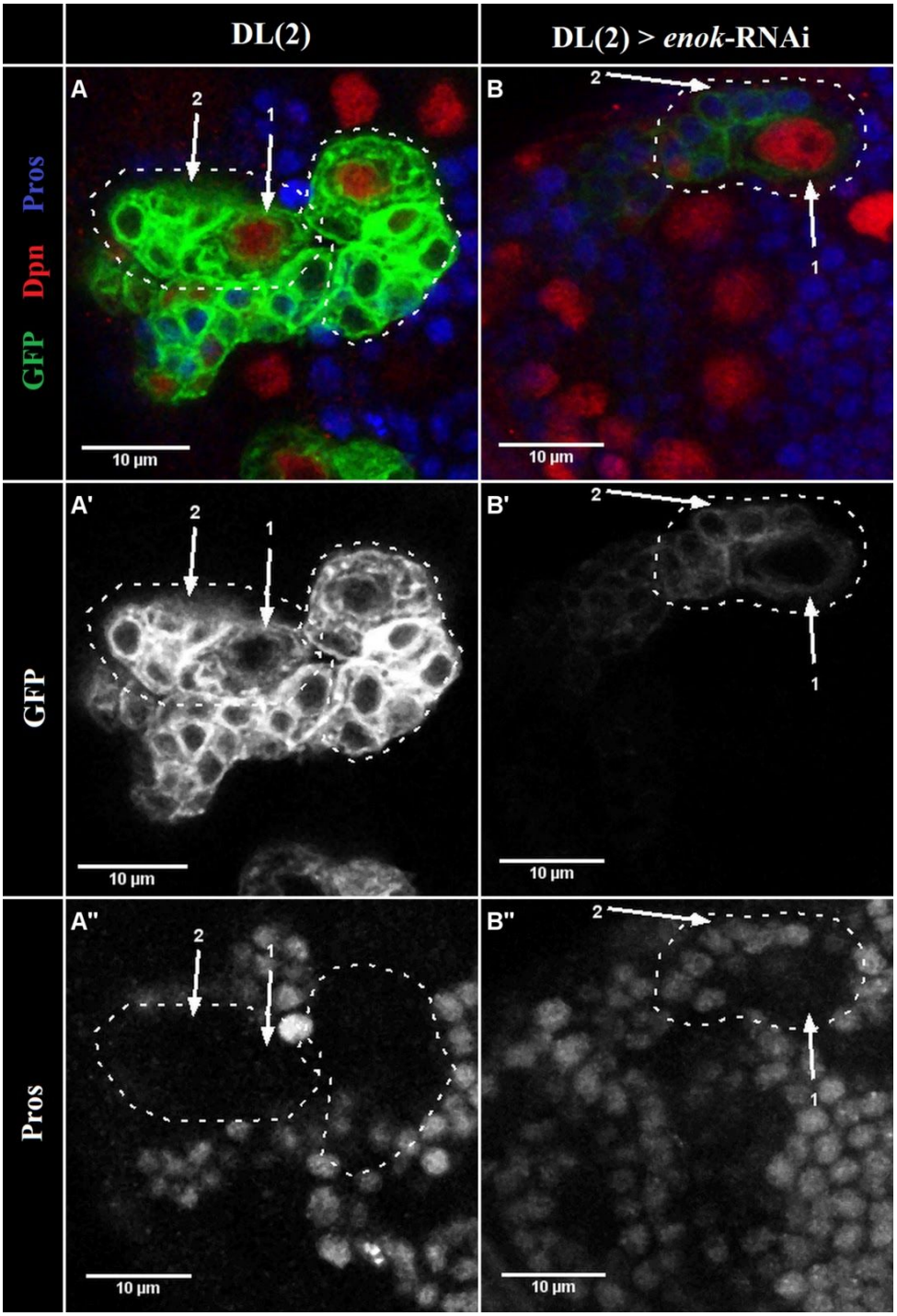


Figure 13. RNAi mediated *enok* knockdown results in nuclear Pros in imINPs.
Knockdown of *enok* via RNAi driven by DL(2) leads to nuclear Pros in imINPs. Pros is not expressed in imINPs in controls (DL(2)). (A) and (B) Type II NB lineages from 50 ALH larvae from DL(2) and UAS-*enok*-RNAi driven by DL(2), respectively, stained for Pros and Dpn. Parts of type II lineages, NB and imINPs, are marked with white dotted lines. Arrow 1 and 2 mark type II NB and imINPs respectively (A') and (B') GFP channel from (A) and (B) respectively. (A'') and (B'') Pros channel from (A) and (B) respectively.

3.9 Overexpression of *enok* results in type I NB to type II NB conversion

Knockdown of *enok* in type II NBs converts them to type I NBs fate. I further wanted to check if overexpression of *enok* results in type I NBs converting to type II NB fate. In other words, I wanted to check whether the supernumerary Dpn+ cells observed in type I lineages after *enok* over expression are due to type I to type II fate conversion. To test this, I overexpressed *enok* in type I NBs using the DL(1) combined with *tubulin*-Gal80^{ts} and the scheme described earlier, and stained for both Dpn and Ase. In control VNCs, there are no Dpn+Ase-. But in *enok* overexpressing VNCs Dpn+Ase- type II NB like cells are present (Figure 14). Suggesting that overexpression of *enok* converted the type I NBs to a type II NBs fate.

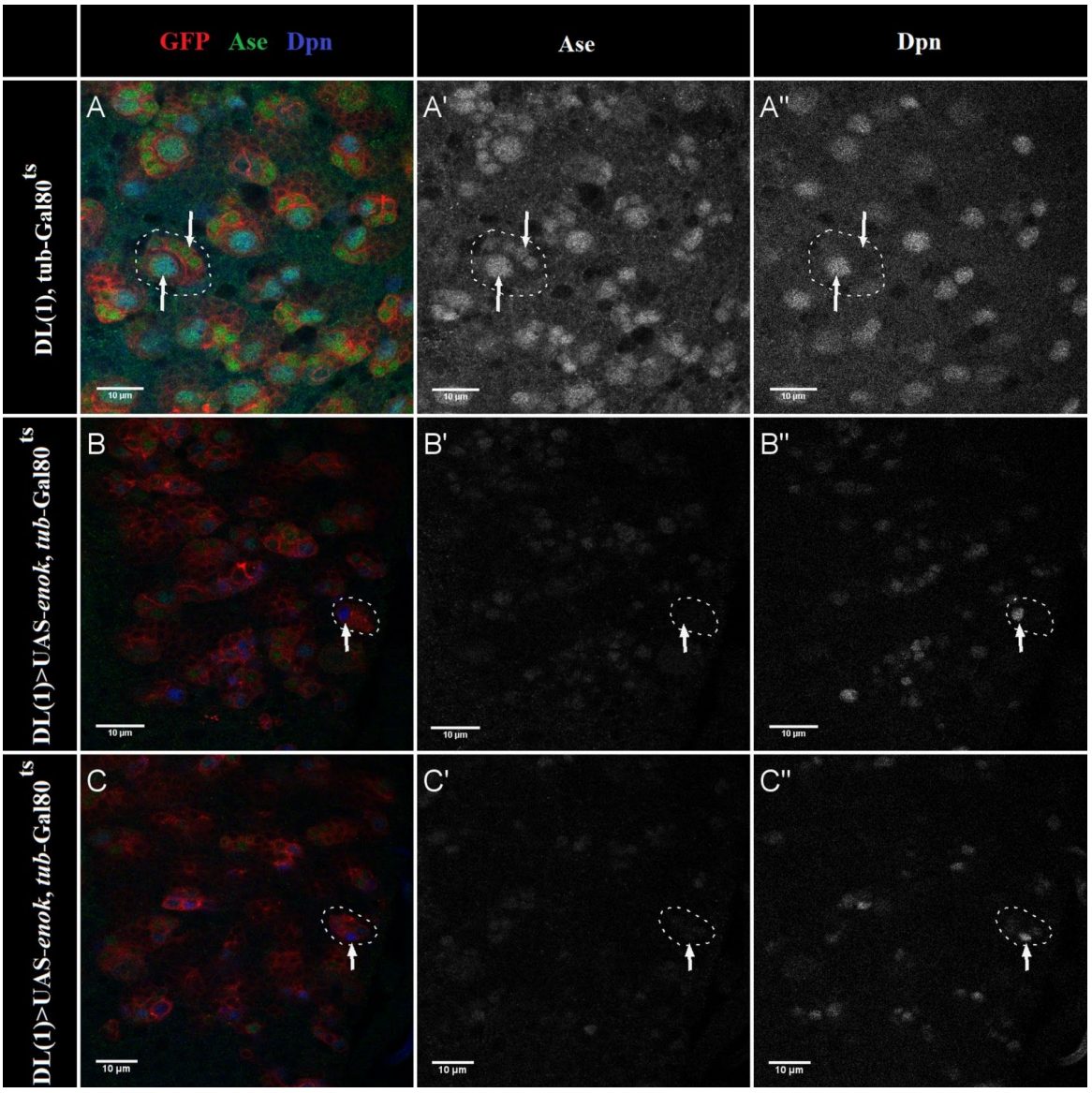


Figure 14. Overexpression of *enok* results in Dpn+Ase-, type II neuroblasts like cells in type I NB lineages.

Overexpression of *enok*, using the previously described scheme, leads to Dpn+Ase- type II NB like cells in type I NB lineages of the thoracic VNC. (A) to (C) Single confocal slice of VNC thoracic region of stage L3 larvae from control (DL(1), *tub*-Gal80ts,(A)) and DL(1)>UAS-enok, *tub*-Gal80ts ((B) and (C)), stained for Dpn and Ase. NB lineages are marked with white dotted lines. Arrow points to Dpn+ type I NB in (A) and to Dpn+Ase- cells in (B) and (C). (A') to (C') Ase channel from (A) to (C) respectively. (A'') to (C'') Dpn channel from (A) to (C) respectively

3.10 ChIP-Seq to find out Enok targets in type II NBs

Enok is a histone acetyltransferase. Histone acetyltransferases typically acetylate the histone tails in the promoter regions of their target genes, which leads to opening of chromatin and increased transcription of their targets. Enok knockdown led to type II NBs converting to type I NB fate. We hypothesized that Enok might be doing this via positively regulating the expression of one or more genes that are necessary for maintaining type II NB fate. In order to test our hypothesis we decided to find out the targets of Enok in type II NBs by performing type II NB specific ChIP-Seq analysis, using antibody against Enok protein. Berger et al., 2012, had used NB size and the strength of NB specific Gal4 driver driven GFP to FACS sort stage L3, larval NBs. Similarly in order to get a pure population of type II NBs we decided to FACS sort the type II NBs from DL(2), stage L3 larvae. To perform a ChIP-Seq experiment using the conventional method requires at least a million cells per replicate (Agrawal et al., 2014). There are a total of 16 type II NBs in each larval CNS. Assuming a 10% FACS efficiency (Berger et al., 2012) and requirement of at least two replicates of the ChIP-Seq experiment, I would have needed to dissect more than a million larval CNSs. Since this was not feasible, we considered two alternative possible ways to do it. One, was to implement an unconventional ChIP protocol that requires considerably less number of cells. Recently it was shown that a new ChIP-seq protocol, called favored amplification and recovery via protection, FARP-ChIP-Seq, was shown to require only 500 mammalian cells (Zheng et al., 2015). This method uses bacteria as carrier for cells and biotinylated synthetic DNA as carrier for the chromatin to prevent the loss of cells and DNA of interest, respectively. Furthermore the DNA of interest is selectively amplified using PCR by blocking the amplification of biotinylated DNA using an amplification blocker oligo against the synthetic biotinylated DNA. Dr Junaid Akhtar, a postdoctoral fellow in our lab, tried to establish this protocol in our lab but could not successfully do it in time. It has been shown that overexpressing the activated form of Notch receptor *NICD*, in type II NBs leads to exponential growth of type II NBs and leads to hyperplasia. The cells from the hyperplasia maintain the expression of Dpn and are Ase negative suggesting that they resemble type II NB fate more than the type I NB fate (Zacharioudaki et al., 2012). Since

this was our second and only alternative we decided to use FACS sorted NBs from NICD overexpressing CNSs using DL(2). Using this method we were able to get one million FACS sorted type II NB like cells from 200 larval CNSs. ChIP was performed using one million cells per replicate. An antibody against Enok that has previously been used for ChIP-qPCR experiment (Huang et al., 2014) Afte I supplied the FACS sorted type II NB like cells, the ChIP experiment was performed by Dr Junaid Akhtar and bioinformatic analysis of the data was performed by Dr Anne Bicker. The two replicates show excellent agreement with each other. The peaks were assigned a peak shape score based on peak height and peak shape. Our goal was to test the hypothesis that Enok binds and upregulates the expression of one or more genes that are necessary for type II NB fate maintenance. To find these targets I decided to look at top 150 peaks according to the peak score. The number 150 was chosen arbitrarily and if I were not to find any targets that are important for type II NB development I decided I would check the the next 150 and so on. Out of these 150 peaks most of the peaks lie in the promoters or introns of 37 genes (Figure 15 and Table 2). Recently a ChIP-Seq experiment was performed on *Drosophila* whole embryos using a tagged version of Br140, the scaffold protein of Enok HAT complex (Kang et al., 2017). Surprisingly 25 out of these 37 genes were present in the top 500 genes that were bound by Br140. This suggested that Enok binds to similar targets during different developmental stages and tissues. Recently a NB type specific transcriptome has been published (Yang et al., 2016). In this study the authors found that transcripts for six genes (*Sp1, Dll, Six4, Optix, pnt, btd*) are enriched only in type II NBs and not in other NB types. Surprisingly all the six genes are present in the top 37 targets listed in table one.

It has previously been shown that *pnt* and *btd* are both necessary for maintenance of type II NB lineages (Komori et al., 2014). Loss of *pnt* and *btd* converts the type II NBs into type I NBs. Hense, *pnt* and *btd* could be the possible mediators of *enok* function in maintaining type II NBs. In order to uncover whether any of other genes listed in table-- might be possible mediators of *enok* function I knocked them down individually using RNAi driven by DL(2). Surprisingly none of these genes affected type II lineages.

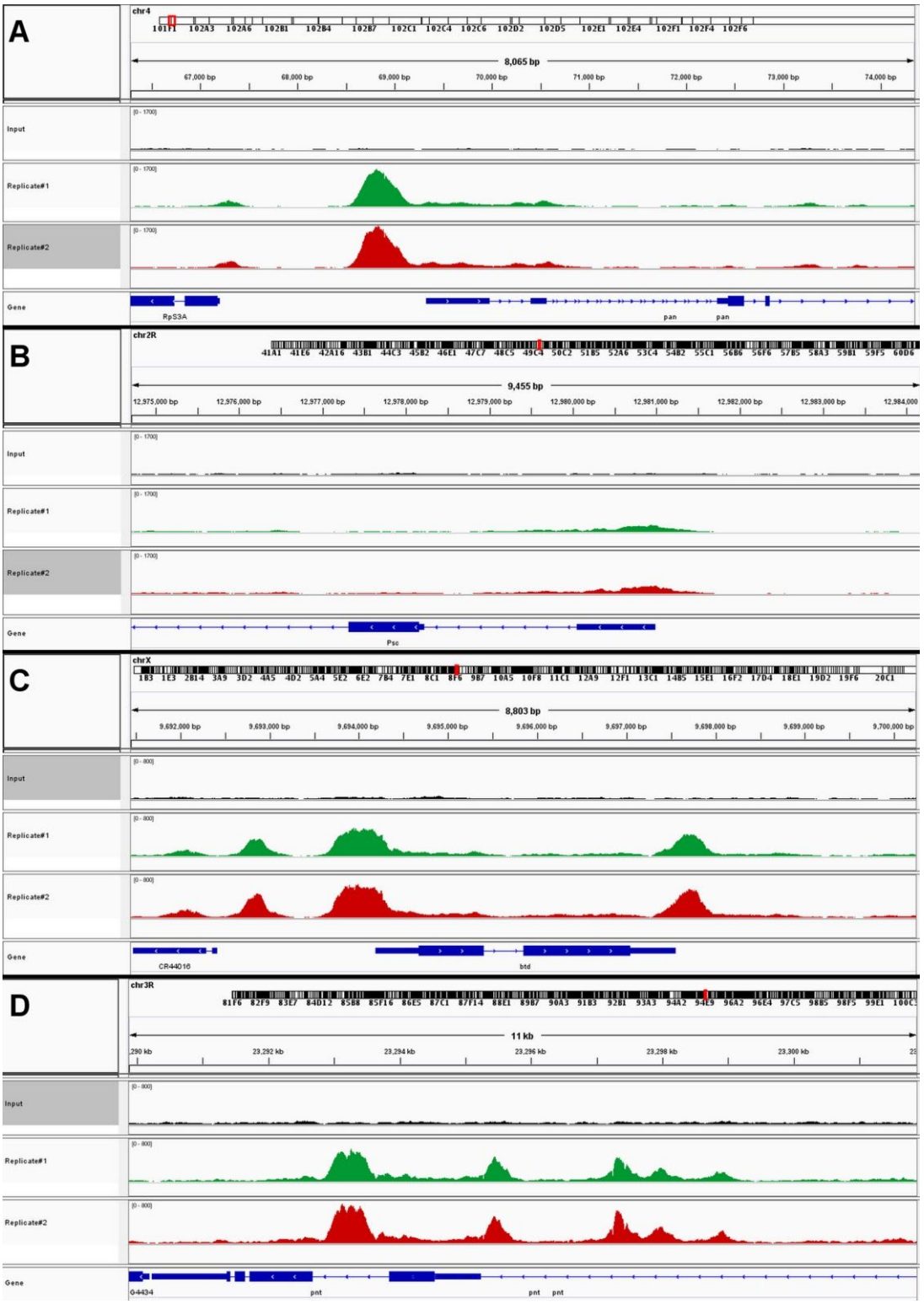


Figure 15. ChIP-Seq tracks

IGV screenshots for two replicates of Enok ChIP-Seq and input are shown for four genomics regions around four genes pan, psc, btd, and *pnt* (A) to (D) respectively. The peaks shown (tallest in each panel) (A) (B) are the first and the 150th peaks according to peak shape score. For comparison of peak heights, data range scale of (A) and (B) are kept same.

1	pan	14	pnt	27	CG2993
2	Neu2	15	iab-8	28	Tll
3	grn	16	corto	29	Optix
4	Dll	17	CG9722	30	vvl
5	slp2	18	so	31	run
6	Gaphaq	19	CG12413	32	Six4
7	slp1	20	hth	33	CG6966
8	SoxN	21	noc	34	raw
9	IA-2	22	btd	35	Dfd
10	eya	23	Tis11	36	opa
11	Atpalpha	24	CG1124	37	Psc
12	sphinx	25	EcR		
13	Sp1	26	CG31472		

Table 2. Nearest genes to the first 150 Enok ChIP peaks

Enok ChIP peaks were arranged in decreasing order of peak shape scores. List of genes nearest to the first 150 peaks (the peaks lie either on the promoters of the these genes or in the introns of these genes). The first peak lies on the promoter of pan gene whereas the 150th peak lies on the promoter of psc gene (Figure 15A and 15B) The genes listed here were screened for phenotype in type II NB development

3.11 *Enok* positively regulates the expression *pnt* and *btd*

Next, in order to check whether *pnt* and *btd* are indeed regulated by *enok,* I performed qRT-PCR experiments. This was done on FACS sorted type II NBs from DL(2)>UAS-NICD (control) and DL(2) >UAS-NICD, UAS-enok-RNAi (experiment). Loss of enok resulted in (58.01 ± 11.2)% decrease in *pnt* transcripts, whereas it resulted in (29.9 ± 8.3)% decrease in *btd* transcripts. Confirming that *enok* positively regulates the expression of *pnt* and *btd* both (Figure 16).

3.12 Overexpression of *pnt* rescues the *enok* knockdown phenotype

Since *enok* knockdown resulted in a more pronounced reduction in *pnt* transcript levels, I decided to check whether overexpression of *pn*t in *enok* knockdown background rescues the loss of type II NB phenotype. Indeed, overexpression of *pnt* in *enok* knockdown background driven by DL(2) rescues the NB number from 0 in *enok* knockdown to (6.3 ± 0.8) (Figure 17). Suggesting that function of *enok* in maintaining type II NBs is mediated via *pnt*.

A

Delta-Ct(control)	Delta-Ct(experiment)	Delta-Delta-Ct	Fold change	
0.186	1.63	1.444	0.367546834	Bioloical replicate #1
0.051	1.426	1.375	0.385552706	
0.091	1.443	1.352	0.391748594	
0.044	1.414	1.37	0.386891248	
-0.1	1.524	1.624	0.324434692	Bioloical replicate #2
0.373	1.183	0.81	0.570381858	
1.046	1.746	0.7	0.615572207	
-0.025	1.661	1.686	0.310787417	

B

Delta-Ct(control)	Delta-Ct(experiment)	Delta-Delta-Ct	Fold change	
4.974	5.634	0.66	0.632878297	Bioloical replicate #1
5.217	5.616	0.399	0.758383773	
4.836	5.311	0.475	0.71946679	
5.012	5.412	0.4	0.757858283	
4.906	5.213	0.307	0.808320869	Bioloical replicate #2
4.955	5.551	0.596	0.66158572	
4.735	5.089	0.354	0.782411782	
4.517	5.375	0.858	0.55171687	

C

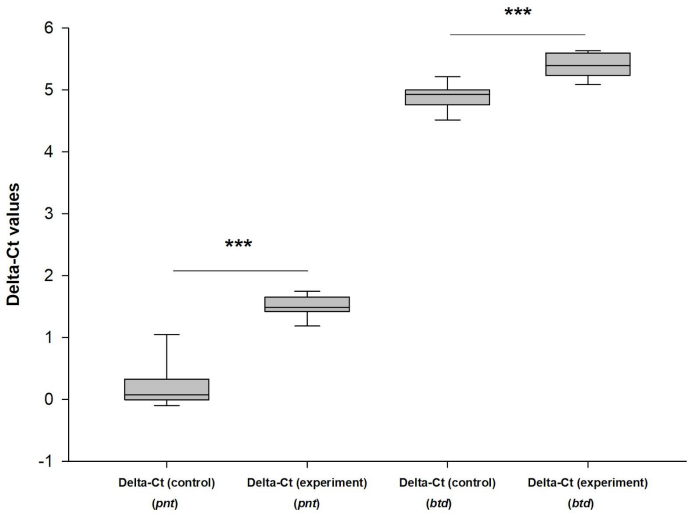
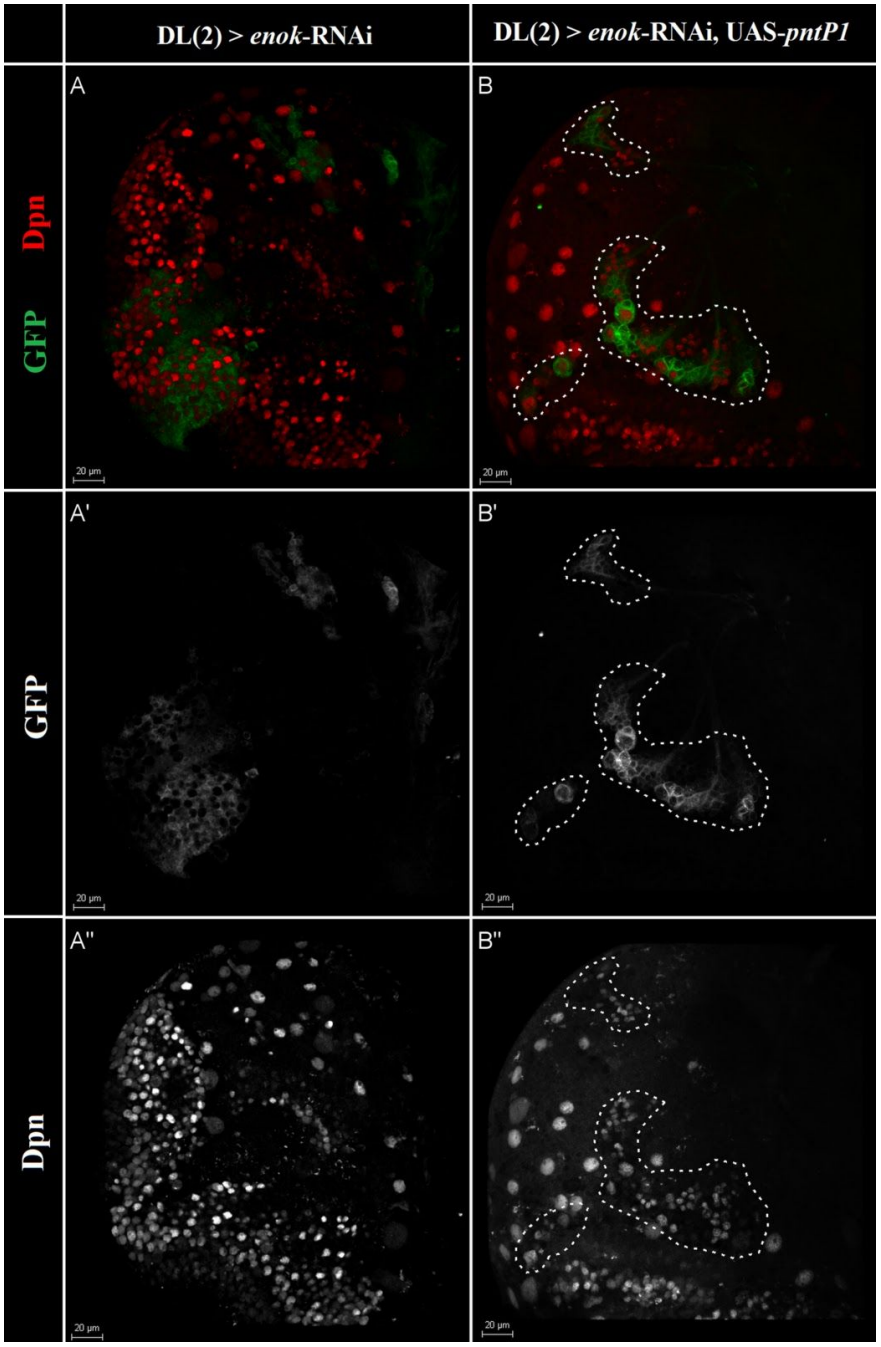


Figure 16. RNAi knockdown of *enok* knockdown leads reduction in transcript levels of both *pnt* and *btd*
Compared to NBs from DL(2)>NICD, DL(2)>*NICD*, *enok*-RNAi NBs show a reduced level of both *pnt* and btd transcript. (A) and (B) show the Delta-Ct, Delta-Delta-Ct and fold change values for *pnt* and *btd* respectively. In *enok* knockdowns *pnt* is reduced to (41.91±11.2 %) (a reduction of ~58%) whereas btd is reduced to (70.91±8.7 %) (a reduction of ~29%). (C) Delta Ct values from (A) and (B) are plotted. $p < 0.001$



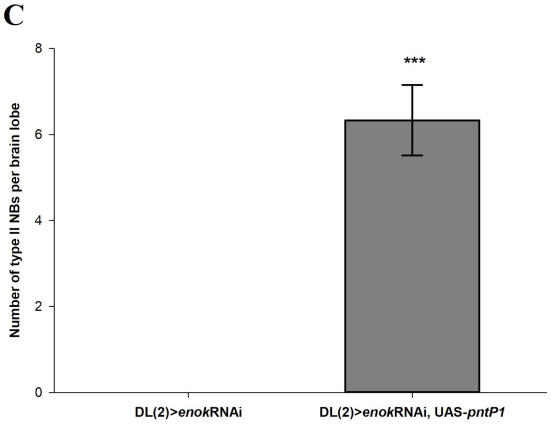


Figure 17 . Overexpression of *pntP1* rescues the loss of type II NB phenotype caused by *enok* knockdown

(A) and (B) 3D projection of, dorsal, larval stage L3, CBs, from UAS-*enok*-RNAi drive by DL(2) and UAS-*enok*-RNAi, UAS-*pntP1* driven by DL(2), respectively, stained for Dpn. White dotted lines mark the type II NB lineages.

(A') and (B') GFP channel from (A) and (B) respectively. (A'') and (B'') Dpn channel from (A) and (B) respectively. (C) Quantification of phenotypes shown in (A) and (B). *pntP1* overexpression in the enok rnai background increases the number of type II neuroblasts from (0) in enok knockdowns to (6.3 ± 0.8). $p < 0.001$

4. Conclusion

I performed an RNAi mediated knockdown screen to identify the role of histone post-translational modifier (HATs, HDACs, HMTs, and HDMs) in development of type II NBs in *Drosophila* larval CNS. Out of the 26 candidates screened, three candidates, *enok*, *ash1* and *HDAC1* gave a loss of type II NB phenotype upon RNAi mediated knockdown. For *enok,* I confirmed the loss type II NB phenotype uncovered in the screen, by using a second RNAi line and a null mutant for *enok*. Overexpression of *enok* in the *enok* RNAi knockdown background rescued the loss of type II NB phenotype. Antibody staining against the Enok protein showed that Enok is ubiquitously expressed in the cells of CB and is nuclearly localized. Enok expression was higher in NBs compared to their progeny. However there was no significant difference in Enok expression levels between type I and type II NBs. RNAi mediated knockdown of *br140*, the scaffold protein for the *enok* HAT complex, resulted in a similar loss of type II NB phenotype, suggesting that function of *enok* in maintenance of type II NBs is dependent on the HAT complex. Overexpression of *enok* resulted in supernumerary Dpn+ cells in both type I and type II NB lineages. Overexpression of caspase inhibitor *P35* in *enok* knockdown background did not rescue the loss of type II NBs, indicating that the loss of type II NBs was not mediated via caspase mediated apoptosis. Upon *enok* knockdown, type II NBs and their progeny, imINP start expressing type I NB and GMC marker, Ase and Pros respectively. This suggests that *enok* knockdown converts type II NBs in type I NBs. Moreover, overexpression of *enok* in type I NBs converts them to type II NBs. By performing ChIP-seq analysis on FACS sorted type II NB like cells, with specific antibody against Enok we found out that Enok binds to several type II NB specific genes. Performing qRT-PCR analysis showed that *enok* positively regulates *pnt* and *btd*, two genes, whose loss of function phenotype, similar to *enok*, is conversion of type II NB into type I NB. I further was able to show that overexpression of *pnt* in *enok* knockdown background rescues the loss of type II NB phenotype. Thus, we conclude that *enok* maintains type II NB identity via binding and positively regulating genes that are necessary for type II NB maintenance.

5. Discussion

5.1 Place of *enok* in the genetic network that governs the type II NB identity

Type I NBs are marked by presence of nuclear Dpn and nuclear Ase. *Ase* is a positive regulator of *pros* and type I NBs express cytoplasmic Pros. During division, type I NBs segregate Pros to only one progeny, that is, the GMC. In GMCs Pros gets nuclearly localized, wherein it facilitates cell cycle exit by down regulating genes like E2F and CycE. It also initiates differentiation program by positively regulating neuronal markers like Fasciclin 1 and Fasciclin 2 (Choksi et al., 2006). The central difference between type I and type II NBs is that the progeny of type I NBs undergo cell cycle exit by dividing only once into differentiated neurons, whereas the progeny of type II NBs continue to divide several times before exiting cell cycle. Type II NBs achieve this by not expressing nuclear Pros in their progeny. This is achieved in part by preventing *ase* from being expressed in the type II NB (Homem et al., 2012). PntP1 and Btd, proteins that are only expressed in type II NBs play central role in repressing *pros* expression in type II lineages partly via repressing *ase* (Zhu et al., 2011; Komori et al., 2014). Here we show that Enok binds to both *pnt* and *btd* genomic loci and positively regulates their transcription. Loss of *enok* results in reduction in *pnt* and *btd* expression leading to (likely mediated via *ase*) *pros* expression in type II NB lineages. Which in turn would lead to their progeny dividing only once to produce terminally differentiated neurons (Figure 18).

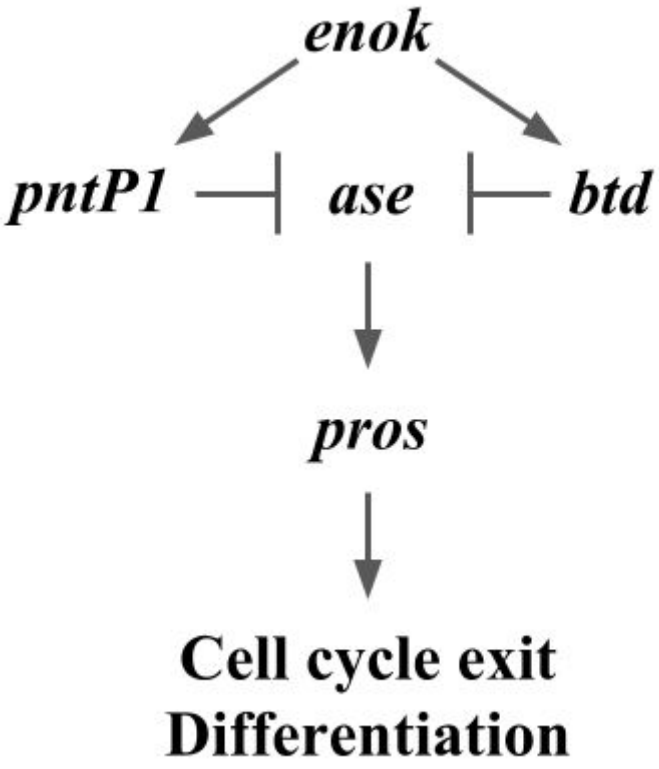


Figure 18. *Enok* in the genetic network that regulates development of type II NB lineages
The schematic shows the position *enok* occupies in the genetic network that regulates the development of type II NB lineages.

5.2 About Necessity and sufficiency of *enok* in maintaining type II NB identity

In this work we present evidence to demonstrate that *enok* is necessary for maintenance of type II NBs. RNAi mediated knockdown of *enok* leads to conversion of type II NBs into type I NBs. ChIP-Seq and qRT-PCR analysis showed that Enok directly binds to *pnt* and *btd* loci in the genome and positively regulates their transcription. In the larval central bain, *btd* and *pntP1* are only expressed in type II liages. Both *pntP1* and *btd* have previously been shown to be necessary for type II NB maintenance and loss of either *pntP1* or *btd* results in type II to type I NB conversion (Zhu et al., 2011; Komori et al., 2014). I further show that overexpression of *pntP1* significantly rescues the loss of type II NB phenotype in *enok* RNAi knockdowns, suggesting that function of *enok* in maintenance of type II NBs is mediated via *pntP1*. I also show that Enok is expressed in both type I and type II NBs and there is no significant difference between the level of Enok protein between type I and type II NBs. If Enok is expressed in both type I and type II NBs, then this begs a question, that why Enok is a positive regulator of *pntP1* only in the type II lineages? I can think of two possible explanations for this. First, Enok may require another factor which is expressed only in type II NBs. This implies that Enok is necessary, but not sufficient for expression of *pntP1*. A slightly different, but related second explanation could be, that *pntP1* locus in type II and type I NBs may exist in two different epigenetic states, one permissive for Enok binding and other not (or to a lesser extent), respectively. I have also shown that *enok* overexpression in type I NBs leads to type I NBs converting to type II NBs suggesting that *enok* is sufficient for giving type II NB identity. This is apparently contradictory to the previous observation. One possible way to explain the apparent contradiction may lie in the level of case of wild type NBs and NBs overexpressing *enok*. Wild type levels of *enok* are necessary but not sufficient to maintain type II NB identity, but excess *enok* resulting from *enok* overexpression is able to override the not so permissive epigenetic state or pnpt1 locus or override the necessity for another factor. Hence wild type levels of *enok* are necessary but not sufficient for preventing type II NB to type I NB conversion, whereas excess levels of *enok* are sufficient to convert type I NBs into type II NBs.

5.3 Specificity of *enok* overexpression phenotype

Around 30 hours after pupa formation, most of the wild type, type I and type II NBs exit cells and either undergo apoptosis or terminal differentiation into neurons. This cell cycle exit is regulated by combination of effect of temporal series of transcription factors and effect of Ecd mediated changes in NB metabolism (Homem et al., 2014). In *brat* mutant larvae, the imINPs fail to mature and revert back to type II NB fate (Bello et al., 2008). This leads to exponential growth in type II NB number and tumorigenesis. *Brat* mutant NBs survive till adulthood, suggesting that they are resistant to mechanism that ensure timely cell cycle exit. Overexpression of *enok* converts type I NBs to type II NB fate. Presence of more than one type II NBs in one *enok* overexpressing lineage (Fig 14B and 14C) and presence of more than eight type II NBs in DL(2) mediated overexpression of *enok* (Fig 8) raises the possibility that *enok* overexpression might lead to INPs reverting to type II fate. To see check whether type II NBs in *enok* overexpressing CNSs undergo timely cell cycle exit, I stained the CNS from pupae at 30 hours after pupa formation and observed no significant difference from the wild type control. This suggests that overexpression of *enok* has a very specific effect and the effect is limited to cell fate change. And it does not interfere with the ability of NBs to respond to mechanisms that ensure timely cell cycle exit.

5.4 Enok, PRC1, MBNB NBs

Recently it was shown that Enok binds to members of PRC1 complex (Kang et al., 2017). By doing ChIP-Seq analysis on whole embryos on Br140, the scaffold for Enok HAT complex and comparing it with a ChIP-Seq data for Pc, the authors found out that Pc and Br140 bind to nearly identical sites on the genome. In order to see if PRC1 complex members have a role in type II NB, I knocked down pc and sce using dl(2). Loss of PRC1 complex members did not have an effect on type II NB lineages (data not shown). PRC is a well known repressor and Enok/Br140 HAT complex is known to be a positive regulator. The fact that they bind each other and also bind to similar loci on the genome is puzzling. It will be interesting to see the resultant of combined action of PRC1 and Enok

HAT complex on the transcription levels of a common target. *Enok* has been shown to be important for MBNB proliferation. *Enok* mutants prematurely stop producing neurons leading to smaller mushroom bodies. Recently in an article (Yang et al., 2016) transcriptomes of MBNBs have been compared to other NBs of the CNS and it was found that *SoxN, slp1,* and *slp2* are selectively downregulated in MBNBs. They further show that ectopic expression of any one of the factors leads to MBNBs prematurely exiting cell cycle. When we performed ChIP-Seq analysis, we found that promoter regions of all three decorated by Enok binding peaks, moreover, when ranked according to peak shape score, all three peaks belong to the group of top 150 peaks. Our ChIP-Seq analysis was performed on NICD overexpressing type II NB like cells. Assuming that Enok binding profile is similar in MBNBs (a fair assumption, considering the fact that the same peaks are also conserved across cell types from embryos to S2 cell (Kang et al., 2017)) it raises the possibility that Enok, a histone acetyltransferase might be involved in negatively regulating the expression of its target gene. It is worth mentioning though that *tll* is a gene known to be necessary for MBNB development, is also a top Enok target. Whether *enok* functions in MBNB via downregulating *SoxN*, *slp1* and *slp2* or via positively regulating *tll,* needs to be examined.

5.5 Future Perspective

HDAC1 was found as one of the positive candidates in the screen performed by me to find out epigenetic factors necessary for the development of type II NBs. Recently it has been published that HDAC1 indeed plays crucial role in maintenance of type II NB lineages (Janssens et al., 2017). Apart from HDAC1, SWI/SNF chromatin-remodeling complex, SET1/MLL HMT complex are also been shown to be necessary for type II NB development (Komori et al., 2014; Eroglu et al., 2014). This highlights the role of epigenetic factors played in the complex process of type II NB lineage progression. In my screen, I also found out that ash1, a HMT is necessary for maintenance of type II NBs. In a recent study (Kang et al., 2017) it was shown that Ash1 binds to the members of Enok HAT complex. It will be interesting to study whether Ash1 and aEnok work together to maintain type II NBs. It was also shown that Enok exclusively acetylates H3K23 reduces

(Huang et al., 2014). Dr Junaid Akhtar is currently in the process of performing ChIP-qRT-PCR analysis on *pntP1* and *btd* loci to check whether *enok* mediated positive regulation of these two genes is mediated via H3K23ac. *Enok, pnt*, and *btd* are conserved from flies to humans. It will be interesting to see if the functional relationship between the three is also conserved in the vertebrates.

6. Materials and methods

6.1 Immunofluorescent staining of larval CNSs

Immunofluorescent staining of larval CNSs was performed by following the protocol described in (Daul et al., 2010). Briefly, required number of larval CNSs of appropriate developmental stage were dissected in PBS. The dissected CNSs were fixed with 4% formaldehyde in PBS for 25 min. The fixed CNSs were given four, 15 min, washes with 0.3% PBTx. The CNSs were blocked in blocking solution (5% BSA in 0.3% PBTx) for one hour. The CNSs were then incubated for 48 hours at 4^{o}C in primary antibody solution (primary antibodies diluted in blocking solution). After incubation in primary antibody solution, four washes of 0.3% PBTx were given to the CNSs. They were further incubated in secondary antibody solution (secondary antibodies diluted in blocking solution) for 24 hours at 4^{o}C. After incubation in secondary antibody solution, CNSs were washed four time in PBTx and then mounted on glass slides using coverslips in Vectashield (Vector Laboratories) mounting medium. The coverslips were sealed using nail polish.

6.2 Flow cytometry

Pure populations of NBs were obtained using fluorescent activated cell sorting (FACS) was used. For larval NB FACS, the method developed by Berger et al., 2012, was used. This method uses size of the NBs (in the NB lineages, NBs are the largest cells) and the strength of UAS-GFP driven by NB specific Gal4 driver line (NBs have the highest strength of GFP in the NB lineages) was used. For ChIP experiments, the NBs were sorted in PBS and were immediately fixed with 1% formaldehyde in PBS and stored at -80^{o} C. For extraction of total RNA, the NBs were sorted directly in to TRI reagent (Sigma-Aldrich) and stored at -80^{o} C.

6.3 ChIP-Seq analysis

Fixed cells (1 million FACS sorted NBs per replicate) were resuspended in 140mM RIPA (10mM Tris-Cl pH 8.0, 140mM NaCl, 0.1mM EDTA pH8.0, 1% Triton X-100, and 0.1% SDS) and subjected to 14 cycles of sonication on a bioruptor (Diagnode), with 30 Secs "ON"/ "OFF" at high settings. After sonication samples were centrifuged at 14,000 g for 10 minutes at 4°C and supernatant were transferred to a fresh tube. The extracts were incubated overnight with 2 µg of anti-Enoki antibody (a kind gift from Workman lab) at 4°C with head over tail rotations. After overnight incubations blocked beads were added to the tubes and further incubated for 3 hours to capture the antibodies. The beads were centrifuged at 1000 g and were washed as following; once with 140mM RIPA (10mM Tris-Cl pH 8.0, 140mM NaCl, 0.1mM EDTA pH8.0, 1% Triton X-100, and 0.1% SDS), four times with 250mM RIPA (10mM Tris-Cl pH 8.0, 250mM NaCl, 0.1mM EDTA pH8.0, 1% Triton X-100, and 0.1% SDS) and twice with TE buffer pH8.0 (10mM Tris-Cl pH 8.0 and 0.1mM EDTA pH8.0). After the immunoprecipitation samples were RNase-treated (NEB) and subjected to proteinase K treatment for reversal of cross-links, 12 hours at 37°C and at least 6 hours at 65°C. The samples after proteinase K treatment were subjected to phenol chloroform extraction. After precipitating and pelleting, DNA was dissolved in 30 µl of TE. The recovered DNA was converted into libraries using NebNext Ultra II DNA library preparation kit, following manufacturer's protocol. DNA libraries were multiplexed, pooled and sequenced on Illumina HiSeq 2000 platform, generating over 42 Mio. reads for each sample. Sequence reads were filtered for Illumina adapters with the CLC Genomics Workbench 9.5 (Qiagen). Reads were filtered for ambiguities (allowing a maximum of 3 per read), sequencing quality (Phred over 13) and a minimum sequence length of 15 bp. All remaining reads were mapped with the CLC Genomics Workbench 9.5 (Qiagen) to the *Drosophila melanogaster* genome (NCBI). ChIP-peaks were called relative to their input data with the CLC Transcription Factor Peak caller 2.0, with a maximum p-Value of 0.1.

6.4 RNA extraction, First strand synthesis and qRT-PCR

For downstream application in qRT-PCR analysis, RNA was extracted from FACS sorted NBs. For each replicate 50,000 NBs were used. The experiment was performed with two biological replicates for each condition (control and experiment). RNA extraction was performed using RNEasy micro kit (Qiagen, Cat No./ID: 74004) and manufactures protocol was followed, which yielded ~500 ng of total RNA per replicate. First strand synthesis was performed using RT2 First strand kit (Cat No./ID: 330404) and manufactures protocol was followed. SYBR-Green based quantification was used for qRT-PCR experiments. Power SYBR Green Master Mix (ThermoFisher) was used. The qRT-PCR experiments were performed in a 96 well format (Bio-Rad; Cat. No.MLL9601). The reaction conditions were as follows: 40 cycles of 95°C for 3 seconds denaturation and 60°C for 30 seconds annealing and extension. The reaction mix was made with 10µl of SYBR Green supermix, 0.1µl forward primer, 0.1µl reverse primer, 10µl cDNA (10ng/µl). All reactions were performed with 4 replicates. The instrument used was the StepOnePlus PCR system from Applied Biosystems and the software utilized for analysis was the StepOne v2.3. Previously described primers (Komori et al., 2014) for *btd* and *pntP1*. Normalization was performed using *Act5c*.

6.5 *Drosophila* husbandry

Fly stocks were maintained on a standard food medium containing yeast flakes, soy flour, corn flour, malt extract as well as sugar syrup. For solidification, agar was added as well as Nipagin and propionic acid for conservation. The fly stocks were maintained at 25°C. All crosses were performed at 29°C unless otherwise mentioned. In cases where precise staging of larval developmental time points was necessary, the crosses were carried out on apple juice agar (2% Agar-agar) and yeast was added after solidification of apple juice agar.

6.6 Image acquisition and processing

Confocal images were acquired on Leica TCS SP5 confocal laser-scanning microscope using 63x glycine immersion objective lens. Z - stacks were with 1 micron step size. Images were processed in Leica Las X software and imageJ. Cell number were manually counted by using marking tool in ImageJ. Figure panels were made using FigureJ tool in ImageJ.

6.7 Fly strains used

Apart from the fly strains mentioned in Table 1 following fly strains were used:

Gene name	BDSC stock number	Gene name	BDSC stock number
pan	53249	Tis11	35434
Neu2	60002	CG1124	57190
grn	34014	EcR	58286
Dll	54804	CG2993	42778
slp2	34634	Tll	34329
Galphaq	36820	Optix	55306
slp1	34633	vvl	50657
SoxN	64930	run	33353
IA-2	44099	Six4	30510
eya	35725	CG6966	51409
Atpalpha	51411	raw	31393
sphinx	55601	Dfd	50792
Sp1	52362	opa	32358
corto	35456	Psc	35297
SoxN	64930	br140	56034
hth	34637	pc	28582
sce	36098		

Strain name	Genotype	Source/referance
DL(1)	UAS-dicer2; insc-Gal4 UAS-CD8::GFP	Neumüller et al., 2011
DL(2)	UAS-dicer2; wor-Gal4 ase-Gal80	Neumüller et al., 2011
tub-Ga80ts	w*; P{tubP-GAL80ts}2/TM2	BDSC #7017
enok-RNAi line #2	P{kk109054}VIE-260B	VDRC #108400
enok(1)	y1 w*; cn1 bw1 enok1/SM6b	BDSC #6285
TM6B,EYFP	TM6B, P{Dfd-EYFP}3, Sb[1] Tb[1] ca[1]	BDSC #8704
UAS-enok	y1 w*; P {UASp-enok}	Michal Pankratz lab
UAS-P35	UAS-P35	Hay et al., 1994
UAS-NICD	y1 w*; P{UAS-N.intra.GS}2/CyO; MKRS/TM2	BDSC #52008
UAS-pntP1	w1118; P{UAS-pnt.P1}3	BDSC #869

6.8 Primary antibodies used

Antigen	Host animal	Dilution used	Source
Dpn	Guinea pig	1 in 2000	Knoblich lab
Dpn	Rao	1 in 100	Abcam
Enok	Rat	1 in 50	Pankratz lab
Enok (used for ChIP)	Guinea pig	1 in 5000	Workman lab
Pros	rabbit	1 in 1000	Knoblich lab
Ase	Guinea pig	1 in 5000	Yan lab

6.9 Consumables

1. Disposal bags: VWR international GmbH, Darmstadt
2. Serological pipette 5 ml, 10 ml: Sarstedt, Nümbrecht
3. Parafilm® M: American National Can Group Inc., Chicago
4. Petri dish Ø 90mm Ø 140mm:VWR international GmbH, Darmstadt
5. Pipette tips 10µl, 200µl, 1000µl: VWR international GmbH, Darmstadt
6. Safe lock tubes 1.5ml, 2.0ml: Eppendorf AG, Hamburg
7. Low-Bind Tubes 1.5ml Eppendorf AG, Hamburg
8. Disposable Gloves Nitrile: Microflex, Reno (USA)
9. Cell culture plates (6 well): Merck Millipore, Billerica (USA)
10. Rubber policeman: Sigma-Aldrich, St-Louis (USA)

11. Nunclon® micro well plates (96 well): Sigma-Aldrich, St-Louis (USA)
12. Microscope slides: Marienfeld-Superior, Lauda-Königshofen
13. Coverslips: Marienfeld-Superior, Lauda-Königshofen
14. Nail polish: COTY Germany GmbH, Mainz
15. Cell strainer: Becton Dickinson Labware, (USA)
16. 96 well plates Bio-Rad, Hercules (USA)
17. Optical adhesive covers: Applied Biosystems, Foster city (USA)

6.10 Chemicals used

1. Sodium phosphate dibasic (Na2HPO4): Carl Roth GmbH & Co. KG, Karlsruhe
2. Sodium dihydrogen phosphate (NaH2PO4):Carl Roth GmbH & Co. KG, Karlsruhe
3. TritonX100 (C14H22O(C2H4O)n): Carl Roth GmbH & Co. KG, Karlsruhe
4. LysinHCl (C6H14N2O2 · HCl): Sigma-Aldrich, St-Louis (USA)
5. Sodium hydroxide (NaOH): Carl Roth GmbH & Co. KG, Karlsruhe
6. Paraformaldehyde (OH(CH2O)nH): Carl Roth GmbH & Co. KG, Karlsruhe
7. Sodium chloride (NaCl): Carl Roth GmbH & Co. KG, Karlsruhe
8. Potassium chloride (KCl): Carl Roth GmbH & Co. KG, Karlsruhe
9. Sodium bicarbonate (NaHCO3): Carl Roth GmbH & Co. KG, Karlsruhe
10. Glucose (C6H12O6): Carl Roth GmbH & Co. KG, Karlsruhe
11. Magnesium chloride (MgCl2): Carl Roth GmbH & Co. KG, Karlsruhe
12. Glycerol (C3H8O3): Carl Roth GmbH & Co. KG, Karlsruhe
13. Vectashield®: Vector Laboratories, Inc., Burlingame (USA)
14. Glutathione (C10H17N3O6S): Sigma-Aldrich, St-Louis (USA)
15. Fetal bovine serum (FBS): PAA Laboratories, Pasching (Austria)
16. Tris (C4H11NO3): Bio-Rad, Hercules (USA)
17. Pen (C9H11N2O4S): Sigma-Aldrich, St-Louis (USA)
18. Strep (C21H39N7O12): Sigma-Aldrich, St-Louis (USA)

6.11 Technical instruments

1. Power-supply units: Power Pack HC, Bio-Rad, Hercules (USA)
2. Refrigerator: Liebherr Premium, Liebherr International AG, Kirchdorf an der Iller
3. Magnetic stirre: IKAMAG® RCT, Janke und Kunkel GmbH & Co. KG, Staufen
4. Centrifuges: Fresco21 centrifuge, Thermo Scientific, Waltham (USA), Sigma 3K20, B. Braun, Melsungen, Micro centrifuge, Carl Roth GmbH + Co. KG, Karlsruhe
5. Microwave:R-201A, Sharp, Osaka (Japan)
6. Photometers: NanoDrop® ND2000 Spectrophotometer, Thermo Scientific, Waltham (USA)
7. Incubators: WTB Binder GmbH, Tuttlingen, Friocell, MMM Medcenter Einrichtungen GmbH
8. Thermal cyclers: X1000 Touch thermal cycler, Bio-Rad, Hercules (USA), StepOnePlus, Thermo Fischer Scientific, Darmstadt.
9. Vortexer: Vortex Genie® 2, Scientific industries Inc., New York (USA)

6.12 Computer programs used

Computer programs used	Purpose
Leica LAS X	Image processing
ImageJ	Image processing
Google Docs	Test editing
Google sheets	Creating tables
SigmaPlot 14.0	Statistical data analysis
StepOne Software v2.3	qPCR data analysis
Integrative Genome Viewr	ChIP-Seq data visualization

6.13 Statistical data analysis

All the obtained data was analysed with SigmaPlot 14.0 (Systat Software Inc.) . In order to comment on the statistical significance of the data Mann Whitney U test was used. Asterisks are used to indicate statistical significance of the presented results (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; ns = $p > 0.05$).

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8. Acknowledgement

I would like to thank:

Dr. Christian Berger, the boss, for giving me the opportunity to be a part of his lab, for listening patiently to every single idea I had, good or bad, and for not rejecting any of them offhandedly, this gave confidence and encouraged me to come up with more.

PD Dr. Joachim Urban, for his invaluable support and for taking care of our lab after Dr Christian Berger had to leave our lab.

Waldemar Kaiser and Rouven Metternich for being the ideal lab mates and friends. I will always cherish our “heated” lunchtime discussions. I would like to thank them both for continuous help throughout the last four years. I would especially like to thank Rouven for his invaluable help in writing of this thesis.

Dr Junaid Akhtar, Vanessa Zurawski and Dr Anne Bicker for performing experiments that provided crucial results for this thesis. I would like to thank Dr Junaid Akhtar for bringing his knowledge and skill of important molecular techniques to our lab.

Prasad Chikte, my partner in crime, for being such an amazing friend and for his continuous help and support throughout the last four years.

All member of the Institute of Genetics family for welcoming me wholeheartedly into the family.

Prof. Dr. Gert Pflugfelder, Prof. Dr. Gerhard Technau, PD Dr. Joachim Urban, and Prof. Dr. Roland Strauss for agreeing to be the examiners for my thesis defence.