# Genetic Regulation of *Drosophila* Mushroom Body Neuroblast Neurogenesis by Notch and Eyeless

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**Project Summary**

Neural stem cells are the building blocks of the developing brain. Without strict regulation of stem cell progenitors, errors in proliferation can arise, leading to disease. Understanding the genetic and molecular (intrinsic and extrinsic) mechanisms that underlie stem cell regulation is crucial to developing therapeutics to treat disease. In this dissertation, I investigated the role of two conserved neural stem cell regulatory genes, Eyeless and Notch in the regulation of *Drosophila* mushroom body neuroblasts (MBNBs). Chapter one begins with an in-depth review of mushroom body neuroblasts from their origins, neurogenic period regulation by intrinsic and extrinsic cues, and termination. I also proposed future directions that could expand our knowledge in the field of MBNB neurogenesis. Chapter two experimentally showed how loss of Notch signaling pathway affects mushroom body neuroblasts. We found that without Notch and its ligand Delta, MBNBs experience premature elimination due to misregulation of their early temporal factor Imp. MBNBs are lost early via apoptosis in Delta knockdown animals. Due to this premature loss of MBNBs and Notch signaling in the MBNB progeny, the resulting mushroom body structure is also severely disrupted. In addition, I investigated how Eyeless operates as a MBNB specific factor to create lineage specific differences between MBNBs and other CBNBs. This work has helped increase the understanding of how MBNBs maintain a longer Imp positive proliferative window.

In chapter three, I investigated the role of the MBNB specific factor Eyeless in MBNB neurogenesis. I found that Ey functions to regulate MBNBs through late acting temporal factors Syncrip (Syp) and Ecdysone-induced protein 93 (E93). Loss of Ey signaling also leads to diminished ecdysone receptor (EcR) expression, a component

key to proper timing of MBNB termination at the end of their neurogenic period. Without Ey, MBNBs experience defects in autophagic cell death, leading to their persistence into adulthood. I also found that errors in autophagy initiation in Ey knockdown animals may be due in part to decreased regulation of the master autophagy regulator microphthalmia inducing transcription factor (Mitf). This chapter reveals novel roles for Eyeless in regulating the termination of MBNBs.

Chapter four summarizes findings for Eyeless and Notch in regulating MBNB neurogenesis and also summarizes future avenues of research. Overall, my work highlights how MBNBs are controlled by Notch and Eyeless in the fly brain and furthering our understanding of NSC regulation.

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Finally, I'd like to thank my late grandparents Frank and Nancy for being the inspiration to pursue scientific research. Their kindness to others is something I seek to emulate in my daily life, and I know they would be proud of all I have achieved.

**List of Abbreviations**

20E: 20hydroxyecdysone

ALH: After larval hatching

APF: After pupal formation

ASD: Autism spectrum disorders

Ase: Asense

ATG: Autophagy-related genes

Babo: Baboon

Br: Broad

BTB-ZF: Broad complex, tramtrack, bric-a-brac zinc finger

Cas: Castor

CB: Central brain

CBNB: Central brain neuroblast

CG: Cortex glia

Chinmo: Chronologically inappropriate morphogenesis

ChIP-seq: Chromatin immunoprecipitation sequencing

CNS: Central nervous system

Cx: Calyx

Dac: Dachshund

Dl: Delta

Dlg: Discs large

Dpn: Deadpan

E(spl): Enhancer of split

E93: Ecdysone induced protein 93

EcR: Ecdysone receptor

Edu: 5-ethynyl-2´-deoxyuridine

Ey: Eyeless

FasII: Fasciclin II

FBS: Fetal Bovine Serum

FE: Freshly eclosed

FH: Freshly hatched

GFP: Green fluorescent protein

GMC: Ganglion mother cell

Grh: Grainyhead

Hb: Hunchback

Imp: Insulin-like Growth factor II mRNA binding factor

Insc: Inscuteable

KC: Kenyon cells

Kr: Kruppel

Mamo: Maternal gene required for meiosis

MARCM: Mosaic analysis with a repressible cell marker

MB: Mushroom body

MBNB: Mushroom body neuroblast

Mira: Miranda

Mitf: Microphthalmia inducing transcription factor

N: Notch

NB: Neuroblast

NDD: Neurodevelopmental disease

NE: Neuroectoderm

NGS: Normal goat serum

NICD: Notch intracellular domain

NSC: Neural stem cell

PBS-T: Phosphate buffered saline- triton

PCNA: Proliferating cell nuclear antigen

Pd: Pedunculus

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

Pros: Prospero

RFP: Red fluorescent protein

RHG: Reaper hid grim

Rx: Retinal homeobox

Scrib: Scribble

scRNA-seq: Single cell RNA sequencing

Syp: Syncrip

Tll: Tailless

Toy: Twin of eyeless

TF: Transcription factor

tTF: Temporal transcription factor

TS: Temperature sensitive

UAS: Upstream activating sequence

Wor: Worniu

# Chapter 1: A review of mushroom body neuroblasts and their neurogenic period


## Abstract

The mushroom body neuroblasts (MBNBs) are a unique subset of neural stem cells (NSCs) found in the developing brain of *Drosophila melanogaster*. This lineage consisting of 4 NSCs, termed neuroblasts (NB), per hemisphere and are responsible for producing the daughter progeny, called kenyon cells, that form the evolutionarily conserved center for learning and memory known as the mushroom body (MB). MBNBs experience a proliferation period different than any of the other ~96 NBs in the same brain hemisphere, despite occupying tissue that receives similar extrinsic signaling. How do 4 discrete cells form to create such an important structure such as the MB while simultaneously maintaining an identity independent of other stem cells that are seemingly very similar? In this review, I consolidate the current research on MBNBs, how they arise during embryogenesis, the factors that specify them, and the interplay of intrinsic and extrinsic signaling that allows their regulation to differ from CBNBs. In addition, I will discuss how MBNBs are eliminated at the end of neurogenesis and some areas for research moving forward. This review consolidates decades of neuroblast research to help explain what creates lineage specific differences in the NSCs of the *Drosophila* brain. A deeper knowledge of the regulation of NSCs by highly conserved cell signaling pathways will provide a scaffold for understanding neurogenesis across taxa moving forward.



**Key Terms:** Neurodevelopment, neurogenesis, neural stem cell, mushroom body neuroblast, mushroom body, temporal patterning, apoptosis, autophagy

## Introduction

The complex process of neurogenesis occurs in diverse developing organisms across the animal kingdom from invertebrates to mammals, yet maintains similar regulatory mechanisms (Brand & Livesey, 2011; Lichtneckert & Reichert, 2005). Undifferentiated stem cells interpret cellular signals, combine intrinsic and extrinsic cues, and correctly regulate when proliferation begins and eventually ceases (Zhao & Moore, 2018). This integration of signals allows neural stem cells (NSCs) to form the molecularly and cellularly distinct progeny required for a fully functional brain (Homem & Knoblich, 2012). To more holistically understand why neurodevelopmental diseases (NDDs) such as autism spectrum disorder (ASD), macrocephaly, and microcephaly arise, it is key to have a complete knowledge of the regulation of neural stem cells during early development (Freitas et al., 2014; Robinson et al., 2020; Sacco et al., 2018). Studying neurogenesis in the human brain to understand NDD is complex, however model organisms with similar tissue types and conserved genomes provide an opportunity to discover functions than translate across divergent taxa.

In the common fruit fly, *Drosophila melanogaster,* neurogenesis is studied due to the discrete number of neural stem cells, easily identifiable cellular markers, and abundant genetic tools (Doe, 2008; Homem & Knoblich, 2012). In this review, I elaborate on the role of an important subpopulation of *Drosophila* NSCs, known as neuroblasts (NBs), called mushroom body neuroblasts (MBNBs). MBNBs are a distinct population of NBs due to their small population (four per brain hemisphere, eight total), prolonged proliferation window, diverse progenies made, and regulatory mechanisms (Lee et al., 1999; Sipe & Siegrist., 2017). In recent decades, a great deal of work has

been done to further understand why MBNBs are capable of different periods of proliferation compared to other NBs in the *Drosophila* central brain (CB). Despite this work, there is a lack of a comprehensive review on MBNBs and questions related to regulation, proliferation, and neurogenesis remain unanswered. Fully uncovering the mechanisms that govern MBNB proliferation for instance will provide insight into NSC regulation, with the potential to gain understanding in both invertebrates and mammals through conserved gene networks.

While MBNBs make up a small number of the total cells in the CB, their intrinsic and extrinsic cues are comparable to other central brain neuroblasts (CBNBs). However, their neurogenic period is distinct, leaving many open questions as to how NBs are differentially regulated (Liu et al., 2015). This review seeks to consolidate established knowledge in the field of *Drosophila* neurobiology along with recent findings on MBNBs and their role in developing the fly brain to understand NSC regulation across taxonomic scales. We also identify areas for more research and experiments we believe would further elucidate how intrinsic and extrinsic cues determining MBNB fate are regulated. A full understanding of MBNB regulation would be a needed expansion into the field of developmental neurobiology.

## Mushroom Body Neuroblasts (MBNB) Identity

In the developing *Drosophila* brain, there are ~100 neuroblasts per hemisphere of the central brain (CB) region. Of those 100 NBs, 4 are mushroom body neuroblasts (MBNBs), the stem cell lineage responsible for forming the center of learning and memory known as the mushroom body (MB) (Ito & Hotta, 1992; Lee et al., 1999).

Neuroblasts have two main patterns of stereotyped asymmetric cell divisions that classify them as either type I or type II NB (Boone & Doe, 2008). MBNBs are considered a type I neuroblast lineage and all four cells are thought to be identical in the number and types of neurons they produce (Ito et al., 1997). While interesting, type II NBs have extensive research understanding their function and will not be a focus in this review. Type I NBs divide asymmetrically to both self-renew and produce a daughter cell called a ganglion mother cell (GMC). To maintain stem cell like fate during this division, the NB maintains apical proteins Inscutable (Insc) and Par complex consisting of aPKC, Par-6, and Bazooka, while the GMC receives basal determinants Numb, Brat, Prospero (Pros), and Miranda (Mira) (Rolls et al., 2003; Betschinger et al., 2006). The apical positioning of the Par complex is responsible for retaining the NB's stem cell identity (Yu et al., 2006). GMCs then symmetrically divide to give rise to post-mitotic neurons and glia. In the case of MBNBs, their GMCs divide to produce kenyon cells (KCs), which in total contribute ~2,000 of the adult neurons per hemisphere in the *Drosophila* brain (Lee et al., 1999). All NBs express the NB transcription factor Worniu (Wor) but MBNBs also express type I NB specific transcription factors asense (ase) and deadpan (dpn) (Doe, 2008) (Fig 1B). In addition, MBNBs also express the MB specific factors retinal homeobox (Rx), tailless (Tll), and Eyeless (Ey) (Kraft et al., 2016; Kurusu et al., 2009; Noveen & Hartenstein, 2000). MBNB specific factors, cellular size, and positioning allow MBNBs to be differentiated from other CBNBs in early embryogenesis (Table 1).

A key difference between MBNBs and other type I NBs of the CB is the proliferation period (Fig 2). MBNBs divide continuously from embryogenesis to just before eclosion into adulthood (Truman & Bate, 1988; Lee et al., 1999). Unlike all other

CBNBs, besides one lateral NB, MBNBs do not undergo a period of quiescence during larval phases as evidenced by their continuous expression of proliferation markers like BrdU (Ito & Hotta, 1992). This extended proliferative window is key to the MBNBs successful production of distinct progeny that form the mushroom body (MB), an evolutionarily conserved structure for learning and memory across Arthropods, primarily insects, and some Crustaceans (Farris & Sinakevitch, 2003; Heisenberg, 2003; Strausfeld et al., 1998). MBNB daughter cells are known as kenyon cells (KCs) which are sequentially born to form the tri-lobed MB structure (Crittenden et al., 1998). These KCs form the MB lobes beginning with the gamma lobe, the alpha/beta prime lobes, and finally the alpha/beta lobes with additional axonal pruning throughout this process (Ito et al., 1997; Kurusu et al., 2002; Lee et al., 1999). How the MBNBs produce the MB will be discussed in greater detail in later sections.

Previous work has shown that MBNBs avoid quiescence through the MBNB specific factor, Eyeless which allows MBNBs to proliferate independent of nutrient status (Sipe et al., 2017). However, it remains unclear what other intrinsic and extrinsic factors may contribute to the prolonged proliferative window experienced by MBNBs outside of Eyeless. Other work suggests that the importance of building the MB structure is one of the driving factors as to why MBNBs are differentially regulated from other CBNBs. A key to MBNB differential regulation is the interplay of the intrinsic and extrinsic signaling. Below, we discuss the intrinsic and extrinsic signaling of MBNBs.

MBNB Intrinsic Regulation

Sequential divisions of the neuroblasts create the diverse cell types that make a functional *Drosophila* central nervous system (Bayrakatar & Doe, 2013; Holguera & Desplan 2018). A key aspect that generates the diversity of NBs in the brain are spatial and temporal dynamics (El-Danaf et al., 2023; Li et al., 2013). Where a NB physically is positioned in the brain and the integration of the intrinsic and extrinsic cues it receives will dictate its proliferative fate, in both vertebrates and invertebrates (Chen & Konstantinides, 2022). In *Drosophila,* neurogenesis begins in embryogenesis, when CBNBs are specified by delaminating from the neuroectoderm (NE) at stage 9 (Fig 1.C; Younossi-Hartenstein, 1996). The four MBNBs specifically arise from four independent but adjacent progenitors in the mitotic domain B of the neuroectoderm and express the same proneural genes achaete, scute, and lethal of scute (Kunz et al., 2012). A key difference from other CBNBs in specification occurs through Notch signaling, which functions through lateral inhibition to keep one NB proneural while the others surrounding it remain epithelial (Egger et al., 2007). However, in MBNBs, this mechanism of Notch signaling does not appear to be necessary for specification, despite Notch pathway being active in these cells (Kunz et al., 2012).

After specification, temporal patterning, the sequential changes in gene expression that occur within a NB, is immediately employed to regulate proliferation (Doe, 2017; Li et al., 2017). MBNBs divide continuously through embryogenesis but it remains unclear if they experience the same temporal transcription factor (tTF) cascade as the other type I neuroblasts consisting of hunchback (Hb) → kruppel (Kr) → pdm → castor (cas) (Ishikki et al., 2001; Kunz et al., 2012). During embryogenesis, MBNBs can

be distinguished from other CBNBs by their expression specific markers of Eyeless (Ey), twin of eyeless (Toy), and dachshund (Dac) as well as their increased size (Kunz et al., 2012; Kurusu et al., 2000; Noveen et al., 2000). These factors are considered key to the embryonic MBNB temporal cassette and their identity along with seven-up (Svp) and Retinal homeobox (Rx) (Table 1; Kunz et al., 2012; Noveen et al., 2000).

Spatial factors and chromatin accessibility dictate which temporal window the neuroblast exists in and therefore which progeny they produce (Sen et al., 2019). One such spatial factor, the homeodomain transcription factor Ey, and its associated genes Toy and Dac, are known to regulate neuron number produced by MBNBs during embryonic development, creating roughly 2,000 progeny total in the resulting MB structure (Callaerts et al., 2001; Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000). As MBNBs progress into larval stages, Ey remains a MBNB specific factor, however Dac becomes restricted to MBNB progeny (Martini et al., 2000; Noveen et al., 2000). Other homeobox domain transcription factors are also seen in the MBNBs from late embryogenesis until pupal stages including the orphan nuclear receptor Tll and Rx. Tll is expressed in most procephalic NBs in embryonic stages but becomes restricted to MBNBs in late embryogenesis and remains highly expressed in the MBNBs and GMCs from birth until pupal stages (Kurusu et al., 2009). Tll is key to prolonged MBNB proliferation as loss of Tll leads to premature elimination of MBNBs (Kurusu et al., 2009). Rx is seen in MBNBs beginning embryonic stage 13 and is essential for maintaining MBNBs as well as their progeny (Kraft et al., 2016). Markers such as Tll and Rx are very useful in differentiating MBNBs from other CBNBs in larval phases.

While the above listed homeodomain transcription factors remain important for maintaining MBNB identity, the key temporal cassette that regulates all CBNB divisions during larval and pupal development consists of Insulin-like Growth factor II mRNA binding factor (Imp) and Syncrip (Syp) (Table 1). Imp is an early factor, thought to reciprocally inhibit its competing late factor Syp (Liu et al., 2015). Both Imp and Syp are RNA binding proteins and their changes in expression level through development contribute to the intrinsic mechanisms that regulate what progeny a NB produces and when (Fig 1.A). It is thought Imp and Syp play a large role in determining lineage size and eventual elimination of NBs (Guan et al., 2024; Liu et al., 2015). In MBNBs, the Imp window is longer which allows for a longer period of proliferation and a later elimination than other CBNBs (Liu et al., 2015; Yang et al., 2017).

In early larval development, Imp expression is high while Syp remains low, leading to the production of gamma neurons and some alpha/beta prime neurons (Liu et al., 2015). When Imp gene expression is knocked down using RNAi, Syp is upregulated earlier and gamma cell fates are lost (Liu et al., 2015) Around early to mid-pupal stages, Imp levels begin to decrease as Syp levels increase, leading to remaining alpha/beta prime neuron production followed by alpha/beta neuron production from late pupal stages until adult eclosion (Liu et al., 2015). If Syp gene expression is knocked down, Imp can persist, causing inappropriate long term gamma neuron production (Liu et al., 2015). Imp also positively regulates downstream effectors in the kenyon cells like Chronologically Inappropriate Morphogenesis (Chinmo) and Lin-28 (Zhu et al., 2006). Alternatively, Syp has been shown to regulate Broad (Br) and Let-7 (Ray et al., 2022; Islam & Erclik, 2022; Yu & Lee, 2007). When Imp or Syp are disrupted, the KC progeny

made are disrupted in the amount of number and type produced, highlighting the importance of this temporal cassette in the developing brain (Liu et al., 2015).

Chinmo is a BTB zinc-finger protein regulated by Imp and found to be highly expressed in early born gamma neurons but absent in late born alpha/beta neurons (Zhu et al., 2006). Chinmo is inhibited by increased Syp expression, which leads to its gradual decrease in expression through larval stages (Jacob et al., 2008; Yang et al., 2017). As Chinmo levels decrease, Maternal gene required for meiosis (Mamo) levels increase to specify alpha/beta prime neurons in late larval and early pupal stages (Liu et al., 2019). The transition from Imp+ MBNBs producing Chinmo+ progeny is in part mediated by the micro-RNA let-7 which allows for the opening of the Syp mediated window of proliferation during the larval to pupal transition (Kucherenko et al., 2012; Wu et al., 2012). As Syp level continue to rise, MBNBs make one final switch to producing the alpha/beta KCs which form the alpha/beta lobes of the MB structure (Lin, 2023). These late born neurons are Broad+ (Br) and Chinmo- (Maurange et al., 2008; Zhu et al., 2006). The resulting structure is a trilobed mushroom body where the MBNBs remain on the dorsal surface, creating a dendritic field called the calyx, and project their axons through the brain in a structure called the pedunculus towards the ventral surface (Fig 1D). Additional pruning occurs as MB neurons make their ultimate connections for a mature functional adult brain (Bu et al., 2023).

Outside of the above-mentioned intrinsic growth cues, there exists others that also play a role in controlling MBNB development. Myc, a conserved transcription factor, promotes cell growth and self-renewal in MBNBs (Betschinger et al., 2006). Early temporal Imp interacts with Myc by binding its mRNA transcript to stabilize it and

promote cell division in larval stages of MBNB development. This stabilization increases Myc half-life 2.5-fold in MBNBs compared to other type I CBNBs (Samuels et al., 2020). Another factor that regulates the early Imp window of proliferation is Notch signaling. When Notch signaling is lost by decreasing expression of its ligand Delta, MBNBs prematurely terminate and experience a shorter Imp window (Branham et al., 2024). The interplay of temporal patterning, cell-cell communication pathways, and cellular growth signals allows MBNBs to continue to proliferate for a longer period than other CBNBs.

*MBNB Identity & Intrinsic Regulation Summary*

MBNBs arise from four distinct but adjacent proneural cells in the NE and do not require Notch signaling or lateral inhibition for their specification. This is in part due to MBNBs being fated early and expressing MBNB specific factors Rx, Tll, and Ey rather than arising from a proneural field like other CBNBs. MBNBs undergo an embryonic temporal cassette different from most other CBNBs as they do not express Hb. MBNBs continuously divide through larval stages, avoiding a period of quiescence and nutrient-dependent reactivation through Eyeless dependent uncoupling of proliferation and nutrient cues. MBNBs divide longer than other CBNBs because their temporal cassette of Imp and Syp is shifted, potentially to allow for a longer period to produce the MB. MBNBs experience a longer Imp window than other CBNBs and transition to Syp later in pupal stages, allowing for a longer proliferative window as Imp can stabilize the growth factor Myc for an extended period. These distinct periods of temporal regulation

allow for sequential production of daughter kenyon cells that form the final MB structure required for learning and memory in the fly.

MBNB Extrinsic Regulation

Intrinsic temporal programs and eventual elimination of MBNBs are coordinated with specifically expressed extrinsic cues. In most CBNBs, a period of nutrient regulated quiescence occurs early and exit from quiescence involves the CBNBs responding to intrinsic cues and extrinsic systemic insulin/PI3K signaling to reactivate (Britton & Edgar, 1998; Chell & Brand, 2010). However, MBNBs are able to proliferate independent of nutritional cues and therefore PI3K signaling due to the MBNB specific spatial factor Eyeless, the ortholog to mammalian Pax-6 (Sipe & Siegrist, 2017). CBNBs are also under hormonal regulation by Ecdysone, a steroid hormone crucial for regulating developmental timing as well as gene regulation. Ecdysone is released in pulses from the prothoracic gland and converted to its active form, 20-hydroxyecdysone (20E) in the hemolymph (Yamanaka et al., 2012). Responding to ecdysone is dependent on the expression of its receptor EcR and the associated Mediator complex (Homem et al., 2014).

Upregulation of the EcR in CBNBs is mediated by intrinsic signaling from Svp and allows the NB to become competent to ecdysone signaling (Syed et al., 2017). This is an important step that shows the coordination of intrinsic and extrinsic signaling as ecdysone signaling has been shown to be one of the key factors regulating the Imp to Syp transition in CBNBs (Syed et al., 2017). While it is not known if ecdysone plays a similar role in mediating the Imp to Syp transition in MBNB temporal pattering, it is

known that the MB neurons express EcR themselves to regulate neuronal remodeling of the MB structure in larval and pupal stages (Boulanger et al., 2011; Lee et al., 2000). Further work is needed to understand what regulates EcR in MBNBs to allow their competency to extrinsic ecdysone signaling to change through development, and if Ecdysone is a factor in progressing the temporal cassette in MBNBs.

External growth factors also play a known role in controlling MBNB development. One key factor that regulates the MBNB and its proliferation is the physical space they reside in, known as the glial niche. The MBNB niche is a specialized microenvironment made of cortex glia (CG) and has been shown to signal to the neuroblast itself to promote cell division (Rujano et al., 2022). Activin signaling from the surrounding glia is key to ensuring all KC fates are achieved. Activin signaling involves Myoglianin binding its receptor Baboon (Babo) on the MBNB to reduce Imp levels and lead to the production of Mamo+ alpha/beta prime neurons (Rossi & Desplan, 2020). Another glial derived signal dsmurf, an E3 Ubiquitin ligase, activates downstream hedgehog (Hh) signaling non-autonomously by targeting Hh receptor Patched, which typically functions to inhibit Hh signaling (Huang et al., 2013). Activation of Hh signaling promotes MBNB cell cycle exit while also providing FasII stabilization in alpha/beta lobes (Yang et al., 2021). In all, non-cell autonomous signals from the glial niche help to regulate MBNB proliferation, termination, and progeny identity to form the fully functional mushroom body structure.

MBNB Termination

Despite global cues from the steroid hormone ecdysone that cause all other CBNBs to terminate in early pupal stages, MBNBs do not stop proliferation until late pupal stages (Ito & Hotta, 1992; Truman & Bate 1988). In addition, while most NBs undergo Prospero (pros) dependent cell cycle exit and terminal differentiation, MBNBs undergo cell death via parallel pathways of apoptosis and autophagy (Fig 1E; Maurange et al., 2008; Siegrist et al., 2010). It remains unclear what creates lineage specific differences in termination mechanisms and timing, but it is possible a MBNB specific factor regulates the NBs competency to Ecdysone signaling to induce elimination later than in other CBNBs.

Base level autophagy occurs across neuroblasts in the developing *Drosophila* brain but increases towards termination (Rogov et al., 2014). Increased autophagy leading to elimination of MBNBs is initiated by falling levels of the growth factor PI3K and increased transcription of autophagy-related genes (ATGs) (Zirin & Perrimon, 2010). This induces the formation of the early phagophore, an organelle-like structure that forms in the cytoplasm (Mizushima et al., 2011). The phagophore then matures by sealing its edges and forming the autophagosome, a double-membrane vesicle that engulfs target cargo within the cytosol (Klionsky & Codogno, 2013). The autophagosome fuses with the lysosome to form autolysosomes. These acidic autolysosomes are then able to bulk degrade their engulfed cargo (Feng et al., 2014; Mulakkal et al., 2014).

MBNBs first transition towards elimination in late pupal stages when levels of the growth signaling pathway Phosphoinositide 3-Kinase (PI3K) drop in response to the late

acting tTF ecdysone induced protein 93 (E93) (Pahl et al., 2019; Siegrist et al., 2010). Inhibition of PI3K signaling leads to reductive cell divisions, decreasing the size of the NB which is key to initiating termination mechanisms in late pupal stages (Maurange et al., 2008). E93 expression is induced by upregulation of EcR and subsequent ecdysone binding and is further stabilized by late acting temporal factor Syp (Pahl et al., 2019). It has been shown that E93 is required for proper autophagy, and it is thought E93 which is a transcription factor may bind ATGs to initiate autophagy, however whether E93 regulates autophagic genes or which genes E93 regulates requires deeper investigation (Pahl et al., 2019). It has been shown that E93 functions to regulate autophagy in other *Drosophila* tissues such as the midgut and salivary glands (Lee et al., 2000; Lee & Baehrecke, 2001). If this role of E93 functioning as a transcription factor to directly induce expression of autophagy inducing genes were conserved in MBNBs, it would increase our understanding of termination initiation.

A theory of why MBNBs undergo the parallel mechanisms of elimination of autophagy and apoptotic cell death is that cells must first degrade self-renewal factors in the MBNB before termination of the progenitor. If autophagy is inhibited, MBNBs are able to transiently persist, meaning the degradation of stem-cell factors is important for elimination (Siegrist et al., 2010). The *Drosophila* central nervous system lacks a typical phagocytic macrophage so autophagy may function to break down stem cell factors, allowing for more efficient termination of MBNBs through apoptosis.

In parallel to autophagy, MBNBs undergo reaper, head involution defective (hid), and grim (RHG) dependent apoptosis (Siegrist et al., 2010). This is different from other CBNBs which experience Prospero (pros) dependent cell cycle exit during termination.

It remains unclear how apoptosis is initiated in MBNBs but may be induced by decreasing PI3K levels and increasing nuclear localization of the transcription factor FOXO (Siegrist et al., 2010). In addition, E93 has been shown to initiate apoptosis in *Drosophila* salivary glands and may play a similar role in the CNS (Zhang et al., 2023). While it is unclear why both autophagy and apoptosis are required, it is key that MBNBs undergo both mechanisms. If autophagy and apoptosis are blocked, MBNBs can inappropriately continue to divide long term into adulthood which can lead to excess neurons with mis-projected axons incorporating unnecessarily into neuronal structures (Siegrist et al., 2010).

*MBNB Extrinsic Regulation & Termination Summary*

MBNBs are regulated by extrinsic cues like steroid hormone signaling from Ecdysone, and signals from the surround glial niche. The surrounding cortex glia provide support to the NB and send signals to the NB that regulate temporal windows and eventual elimination. MBNBs terminate in late pupal stages prior to adult eclosion. Termination occurs through parallel pathways of RHG dependent apoptosis and autophagy induced by late acting temporal factor E93. If both of these processes are impeded, MBNBs can avoid elimination and persist long term into adulthood. More research is needed to understand why both of these mechanisms are employed as well as what is upstream to activate these pathways. It is also unclear why MBNBs terminate later than other CBNBs and through a different mechanism than Pros-dependent cell cycle exit. Further insight into what MBNB factors may establish these lineage specific differences would be of great interest to the field.

MBNBs Moving Forward

While a great deal of research has led to the findings above, there still remain questions about MBNBs that are of interest to further our understanding of stem cell biology. For instance, a more complete temporal cascade for other neuroblasts in embryogenesis is known, but MBNBs do not have a well understood temporal patterning mechanism until Imp/Syp are active in larval stages. As temporal patterning is used as a method for generating neuronal diversity, perhaps MBNBs do not require as strict of a temporal cassette in embryogenesis since they only produce a few types of progenies through development. In addition, MBNBs can be tracked as individuals during embryonic development based on Ey, Toy, and Dac expression, but as they enter larval and pupal stages, they are virtually indistinguishable. It remains unclear if the four MBNBs are truly molecularly identical or if they have differences post embryogenesis. Single-cell RNA-sequencing (scRNA-seq) experiments are lacking for MBNBs which would reveal the expression profile for many genes and help understand the general timing of development. A scRNA-seq experiment in early larval phases could help unravel when MBNBs become molecularly identical if they truly are. The current limitation of this proposed sequencing is how to sort MBNBs from other NBs in order to sequence them, but some methodology has been developed including robotic cell picking (Liu et al., 2015).

Despite no scRNA-seq MBNB transcriptome currently existing, excellent bulk RNA-seq data of the MBNB transcriptome exists in larval and early pupal phases (Liu et al., 2015). However, there is still a need for a late pupal phase transcriptome. This could

reveal more insight into the genes upregulated leading towards termination that may play a role in activating autophagy and apoptosis. An experiment of this nature could also provide more understanding of what other factors may be involved in mediating the Imp to Syp transition as currently it is only known that Imp and Syp reciprocally inhibit each other to maintain their distinct temporal windows. It would also be interesting to perform chromatin immunoprecipitation sequencing (ChIP-seq) of E93 at these late pupal time points to directly assess if E93 is functioning as a transcription factor to induce gene expression of autophagy inducing or proapoptotic genes.

Another major question remains in understanding why MBNBs and other CBNBs have such different proliferation windows. MBNBs have a longer Imp window which contributes to their longer neurogenic period and is maintained by Notch signaling (Branham et al., 2024). What regulates these neurogenic windows in MBNBs to persist longer is still unknown and would be of great interest to the field. Further investigation into MBNB specific factors of Ey, Rx, and Tll could provide answers to how NB lineages are differently regulated. In addition, why do MBNBs experience parallel pathways of autophagy and apoptosis rather than cell cycle exit and terminal differentiation? Again, understanding if some MBNB specific factor plays a role in maintaining temporal factor windows, inducing the Imp to Syp transition, or mediating the MBNB's competency to external cues would reveal a great deal as to how lineage specific differences are maintained in the *Drosophila* central brain.

Overall, the MBNBs are a unique lineage within the *Drosophila* CNS and provide an excellent model for studying NSC regulation. With a clearly defined neurogenic

period, known molecular markers, and genetic tools developed in *Drosophila*, understanding neurogenesis by studying MBNBs is highly achievable.

## Figures

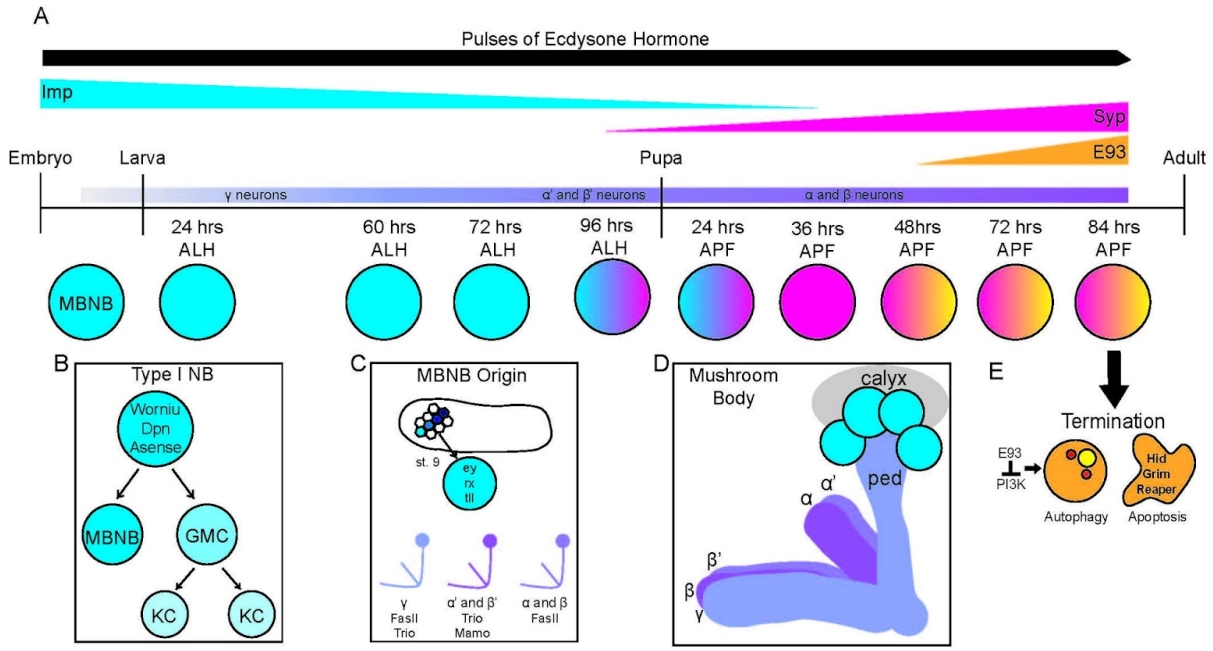


**Fig 1. MBNBs undergo stereotyped divisions regulated by intrinsic and extrinsic signaling**. **A**) Development of MBNBs begins in embryogenesis. Pulses of ecdysone steroid during different phases engages molting to transition through life stages. RNA binding proteins Imp and Syp make up a temporal cassette to induce patterning of progeny. The changes in expression of temporal genes allows for sequential production of neurons that form the mushroom body. As development continues, gamma neurons then alpha/beta prime, and finally alpha/beta neurons are produced. **B**) MBNBs are classified as type I NBs and undergo asymmetric cell division where they self-renew as well as produce a ganglion mother cell (GMC). This GMC then undergoes symmetric division to form two daughter kenyon cells. The identity of the MBNB is considered type I by the markers Worniu, Deadpan (dpn), and Asense (Ase). **C**) MBNBs originate from four adjacent proneural cells in the neuroectoderm of the embryo at stage 9 of

embryonic development. The MBNB is positive for the markers Eyeless (Ey), Retinal homeobox (Rx), and Tailless (Tll). MBNBs continue to proliferate and sequentially produce gamma neurons which are positive for markers FasII and Trio, alpha and beta prime neurons which are positive for markers Trio and Mamo, and finally alpha and beta neurons which are FasII positive. **D**) The resulting structure from MBNB proliferation is the Mushroom Body (MB). This is a tri-lobed clonal structure that consists of MBNBs that project their axons through the brain to the ventral surface through a structure called the pedunculus. MBNBs themself remain on the dorsal surface and make up a dendritic field called the calyx. **E**) MBNB termination occurs through parallel pathways of apoptosis and autophagy at the end of pupal phases. As a final pulse of ecdysone prepares the pupa to eclose into an adult, Ecdysone induced protein 93 (E93) expression reduces levels of growth factor PI3K and initiates autophagy. Meanwhile, pro-apoptotic genes reaper, hid, and grim (RHG) induce cellular death through apoptosis.

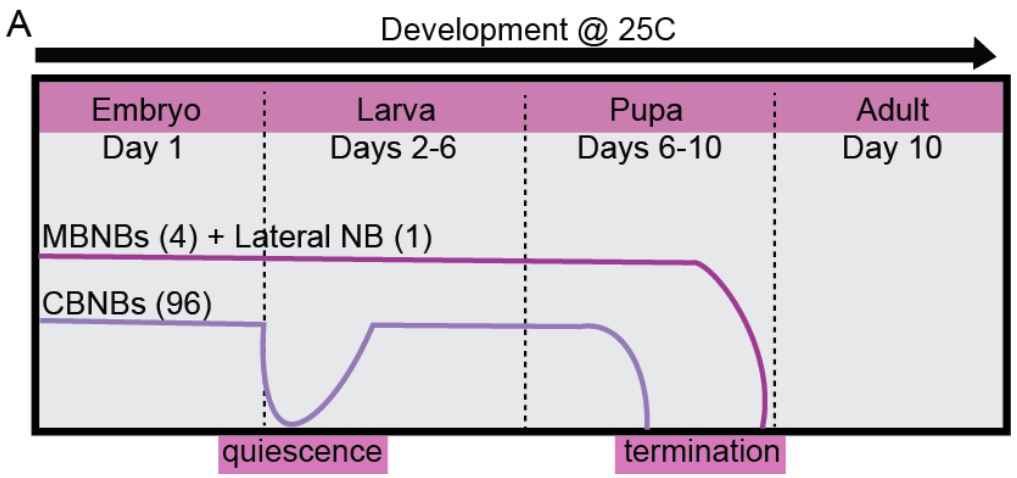


**Fig 2. Different NB lineages through development.** A) Of the ~100 CBNBs of the *Drosophila* CB, 4 MBNBs and 1 lateral NB proliferate continuously from embryogenesis until just before adulthood (~10 dys). Alternatively, the other 96 CBNBs proliferate in embryogenesis, then enter a period of quiescence that requires responding to nutrient dependent cues to exit. After exiting quiescence, the CBNBs proliferate until mid-pupal phases when they are eliminated.

**Table 1. MBNB related genes and their functions.**

| Gene | Function | Reference |
|---|---|---|
| Broad (Br) | Early MB Neuron temporal factor | Maurange et al., 2008 |
| Chronically inappropriate morphogenesis (Chinmo) | Early MB neuron temporal factor | Zhu et al., 2006 |
| Dachsund (Dac) | Embryonic MBNB factor/ MB neuron factor | Kunz et al., 2012; Kurusu et al., 2000 |
| Ecdysone induced protein 93 (E93) | Late temporal factor | Pahl et al., 2019 |
| Eyeless (Ey) | MBNB specific factor | Kurusu et al., 2000; Noveen et al., 2000; Sipe & Siegrist, 2017 |
| Insulin-like growth factor II (Imp) | Early temporal factor | Liu et al., 2015 |
| Let-7 | Late MB neuron temporal factor | Wu et al., 2007 |
| Maternal gene required for meiosis (Mamo) | Middle MB Neuron temporal factor | Samuels, et al., 2020 |
| Retinal homeobox (Rx) | MBNB specific factor | Kraft et al., 2016 |
| Seven-up (Svp) | Embryonic MBNB temporal factor | Kunz et al., 2012 |
| Syncrip (Syp) | Late temporal factor | Liu et al., 2015 |
| Tailless (Tll) | MBNB specific factor | Kurusu et al., 2009 |
| Twin of Eyeless (Toy) | Embryonic MBNB factor/ MB neuron factor | Kunz et al., 2012; Kurusu et al., 2000; Noveen et al., 2000 |

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## Chapter 2: Notch controls early temporal factor expression to control timing of mushroom body neuroblast apoptosis

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**Abstract**

The neurogenic period, where neural stem cells (NSCs) proliferate to produce molecularly distinct progeny in the developing brain, is a critical time of growth in many organisms. Proper brain development is crucial for survival and requires strict regulation of NSC divisions along a set developmental timeline. In *Drosophila* NSCs, known as neuroblasts (NBs), cell intrinsic programs involving temporal patterning genes integrate with extrinsic cues to control periods of rapid growth. Without regulation, NSCs can under proliferate leading to diseases like microcephaly and autism spectrum disorders or over proliferate leading to macrocephaly and tumors. We know programs that involve sequentially expressed temporal patterning genes function to control timing of proliferation and elimination of NSCs. However, many aspects of how these temporal programs are regulated remain unclear. What genes may be upstream of temporal patterning genes to regulate known temporal programs that control when certain progeny are produced have not been fully identified, leaving a gap in our understanding.

To address these questions, we carried out a large-scale RNAi screen aimed at identifying genes required for NSC elimination. We identified Notch and its ligand, Delta. When Notch pathway activity is reduced in NSCs, we found premature elimination of an important subset of neuroblasts called the mushroom body neuroblasts (MBNBs). These MBNBs produce the neurons responsible for formation of the evolutionarily conserved structure called the mushroom body (MB), which is involved in olfactory based learning and memory. Animals in which Notch pathway activity is reduced in NSCs also experienced defects in MB structure. Furthermore, we determined that temporal patterning is disrupted primarily through loss of early temporal factor

expression. Finally, we assess how Eyeless (Ey), a Pax6 ortholog and MBNB specific factor, functions to control Notch signaling in a lineage specific manner. In this work, we find that cell signaling pathways that involve the receptor Notch and its ligand Delta function to regulate NB proliferation in *Drosophila melanogaster* by regulating early temporal factor expression. In addition, we identify a strong genetic interaction between Notch and Ey in the central brain that promotes MBNB neurogenesis.

**Key Terms:**

Temporal patterning, neural stem cell, neuroblast, mushroom body, Notch, Delta, Imp, Syp, Eyeless, neural development

## Introduction

Stem cell division during development is a fundamental biological process that occurs across all organisms and requires strict regulation for appropriate tissue construction. In the developing central nervous system (CNS), proper timing of neural stem cell (NSC) proliferation during development is paramount to the formation of morphologically correct and functional neural tissues (Hartenstein & Wodarz, 2013; Matsubara et al., 2021). If NSC divisions are not regulated, underproliferation can occur leading to reduced neuron numbers and microcephaly (Courchesne et al., 2018; Gilmore & Walsh, 2013). Conversely, over proliferation of NSCs can result in excess growth leading to macrocephaly. Both scenarios of under and over proliferation can lead to neurodevelopmental disorders, including autism spectrum disorders (ASD) (Parenti et al., 2020; Katsimpardi & Lledo, 2018; Courchesne et al., 2018). Therefore, initiating divisions as well as terminating divisions of NSCs at proper points in development are both equally important for brain and neural tissue formation.

It is understood that cell intrinsic factors integrate their signaling with extrinsic cues to regulate NSC proliferation. Timed expression of specific temporal patterning genes allows for production of molecularly distinct progeny through development to form all the cell types needed for a functional brain. Transient activation of temporal patterning genes is controlled by temporal cassettes consisting of temporal transcription factors (tTFs). These tTFs respond to extrinsic cues such as steroid hormones and surrounding glial cell signals (Liu et al., 2019; Rossi & Desplan, 2020; Syed et al., 2017; Sood et al., 2023). It is crucial to understand how systems of intrinsic and extrinsic cell signaling interact to regulate gene expression and ensure correct proliferation and

differentiation of NSCs in the developing brain, as well as how they initiate termination of NSCs when development is complete.

The regulation of neurogenesis through temporal patterning is seen across species, including in the fruit fly *Drosophila melanogaster*. The neural development of the fruit fly brain is tightly regulated to correctly pattern the growing brain throughout life stages. NSCs in *D. melanogaster* are known as neuroblasts (NBs) and have distinct subtypes that produce the varying progeny needed for the adult fly brain (Bayraktar & Doe, 2013; Doe, 2017; Islam & Erclik, 2022; Rossi et al., 2017). A particular neuroblast subset of interest within the central brain are the mushroom body neuroblasts (MBNBs). MBNBs give rise to the evolutionarily conserved mushroom body (MB) structure known to function in learning and memory, particularly when integrated with olfaction (Kunz et al., 2012; Kurusu et al., 2000; Lee et al., 2020, Lin, 2023). The four MBNBs of each fly central brain hemisphere actively proliferate throughout development, from embryonic stages until late pupal stages (Ito & Hotta, 1992; Kunz et al., 2012). This differs from other central brain neuroblasts (CBNBs) that enter a period of quiescence during early larval stages, reactivate in response to nutritional cues to divide again, then permanently terminate their divisions during early pupal stages (Hartenstein & Wodarz, 2013; Nassel et al., 2015).

MBNBs are type I neuroblasts which means they asymmetrically divide to both self-renew and produce an intermediate progenitor called a ganglion mother cell (GMC). GMCs then symmetrically divide to produce neurons, called Kenyon cells (KCs), that are sequentially produced, forming the distinct γ (early born), α'/β' (middle born), and α/β (late born) neurons of the mushroom body (Ito et al., 1997; Liu et al., 2015). KCs

extend their dendrites to an area on the dorsal surface called the calyx, and their axons traverse the brain through the peduncle and bifurcate to form the different lobes. Intrinsic temporal programs determine what type of KC is produced when in response to changes in temporal programs, triggered by extrinsic signaling cues (Heisenberg, 2003; Rossi & Desplan, 2020). The final MB is a five-lobed structure consisting of ~2,000 neurons produced from the four MBNBs of each hemisphere. After forming this structure, MBNBs are eliminated via parallel pathways of both apoptosis and autophagy prior to adulthood (Siegrist et al., 2010; Pahl et al., 2019).

Temporal patterning genes like hunchback (Hb), kruppel (Kr), pdm, castor (cas), and grainy head (Grh) have been shown to regulate periods of embryonic neurogenesis in non-MBNB CBNBs, while in MBNBs retinal homeobox (Rx), Eyeless (Ey), and Tailless (Tll) confer identity (Brody & Odenwald, 2000; Isshiki et al., 2001; Kohwi & Doe, 2013; Kurusu et al., 2009; Li et al., 2013; Noveen et al., 2000). It is currently known that an opposing gradient of RNA binding proteins Insulin-like growth factor II (Imp) and Syncrip (Syp) are expressed in MBNBs and are responsible for patterning MBNB progeny (Liu et al., 2015; Rossi et al., 2021). During early developmental stages when Imp is expressed, chronologically inappropriate morphogenesis (Chinmo) and Lin-28 positive γ neurons are produced, whereas in later stages when Syp is expressed, Let-7 and Broad (Br) positive progeny are produced (Doe, 2006; Islam & Erclik, 2022).

Chinmo is a BTB zinc-finger (BTB-ZF) transcription factor whose levels are high in early born γ neurons but decrease to no expression in late born α/β neurons due in part to activity of the microRNA Let-7 (Zhu et al., 2006; Wu et al., 2012; Liu et al., 2015; Islam & Erclik, 2022). As levels of early factor expression decrease in MBNBs, late

factor expression increases. Syp represses early factor expression and promotes even later temporal factor expression and during the overlap of temporal factors (low Imp and increasing Syp). During this period of temporal factor overlap, α'/β' neurons are produced that are maternal gene require for meiosis (Mamo) positive (Rossi & Desplan, 2020). Mamo is a BTB-ZF transcription factor whose expression is stabilized by the late factor Syp (Liu et al., 2015; Rossi & Desplan, 2020). Finally, expression of Ecdysone induced protein 93 (E93) is positively regulated by Syp and ecdysone steroid hormone signaling. E93 inhibits MBNB PI3-kinase activity to induce autophagy and prime MBNBs for elimination via apoptosis (Siegrist et al., 2010; Pahl et al., 2019).

While it is known that Imp, Syp and their targets function to determine types of progenies produced over time, it still remains unclear how forward progression through temporal programs are controlled. To better understand the regulation of temporal patterning, we carried out a targeted RNAi screen aimed at identifying genes required to terminate MB neurogenesis on time. From this screen, we identified the transmembrane receptor, Notch and its ligand, Delta.

Notch binds to its membrane bound ligand Delta which is expressed on neighboring cells. After ligand binding, Notch is proteolytically cleaved allowing for the nuclear translocation of the Notch intracellular domain (NICD) to the nucleus to regulate gene expression (Schnute et al., 2018; Kandachar & Roegiers, 2012; van Teetering et al., 2009). Notch signaling is evolutionarily conserved and defects in Notch pathway activity are known to cause developmental defects and cancer. During development, Notch plays well known roles in regulating binary cell fate choices (Sato et al., 2016). Notch also controls cell cycle exit to promote NB quiescence during the embryonic to

larval transition and regulates elimination of CBNBs (Sood et al., 2022; Sood et al., 2024). Here we report a role for Notch and its associated ligand Delta in temporal patterning and timing of termination of MB neurogenesis.

## Results

Notch Pathway Knockdown Results in Premature Loss of MBNBs

MBNBS are known to actively proliferate from embryogenesis until 84 to 90 hours after pupal formation (APF), shortly before adult eclosion. To understand the role that Notch pathway signaling plays in maintenance of this division period, we knocked down Notch (N) and its ligand Delta (Dl) in all neuroblasts by driving expression of UAS-*NRNAi* or UAS-*DlRNAi* with a neuroblast specific driver worGal4 (hereafter referred to as NB Gal4) and examined MBNB number during pupal phases. At 48h APF, no difference was observed in the number of MBNBs between *NRNAi* and control animals (Fig. 1A-B, G, MBNBs marked with arrowhead). However, a significant reduction of MBNBs was observed following Delta knockdown (Fig. 1C, 1G). By 72h APF we found a significant reduction of MBNBs in both Notch and Delta knockdown brains compared to control animals (*NRNAi*, 3.7 ± .13, *DlRNAi*, 2.1 ± .31 MBNBs compared to controls, 4 ± .19 MBNBs) (Fig. 1D-F, G). Note the presence of other CBNBs in *DlRNAi* animals (see discussion). Furthermore, cell size based on the average diameter was reduced in MBNBs still present in *NRNAi* and *DlRNAi* brains compared to controls. At 48h APF, all genotypes tested showed MBNBs with an average diameter size of 10 μm. However, by 72h APF, *NRNAi* brains had MBNBs with an average diameter of 8.5 ± .14 μm and *DlRNAi* brains with an average of 7.0 ± .27 μm. (Fig. 1H).

The observation of significantly smaller sized MBNBs in *NRNAi* and *DlRNAi* animals suggests that these MBNBs still undergo normal reductive cell size divisions before their elimination. Reductive cell division occurs in wild type animals in preparation for apoptotic cell death as levels of PI3K decrease. Indeed, at 72h APF in *DlRNAi* brains, we observed morphological characteristics of apoptotic cell death in MBNBs (Fig 1F inset). To further confirm these results, Notch loss of function allele Notch$^{55e11}$ MARCM clones were employed to assess how a null allele for Notch would affect MBNB survival. At 48h APF we found that the MBNB was absent in the Notch$^{55e11}$ MARCM clone, while MBNBs were still present outside the clone (Supp. 1). We conclude that Notch pathway signaling regulates timing of MB neurogenesis, and when Notch pathway activity is attenuated, MBNBs are prematurely lost, possibly through apoptosis.

*DlRNAi* MBNBs undergo premature cell death

Because observed phenotypes of early termination and reduced cell size were more penetrant in *DlRNAi* lines than *NRNAi*, we chose to focus on *DlRNAi* animals for the subsequent analyses. In wild type animals, MBNBs are eliminated via parallel pathways of autophagy and apoptosis during late pupal stages whereas other CBNBs terminally differentiate during early pupal stages (Siegrist et al., 2010; Pahl et al., 2019; Maurange et al., 2008). When Notch pathway activity is reduced, MBNBs are lost prematurely with some displaying blebbing cell morphology consistent with apoptosis. To determine whether *DlRNAi* MBNBs are eliminated via cell death as in control animals, or change their mechanism to terminal differentiation, we co-expressed an

inhibitor of apoptosis (UAS-miRHG) in *DlRNAi* animals. In one day old *DlRNAi* adults, no MBNBs are observed (Fig. 2A, C). Yet in *DlRNAi* animals that co-express an inhibitor of cell death, we observed a significant number of MBNBs (Fig. 2B, C). On average, 2.5 ± .29 MBNBs were seen in one day old adults compared to 0 MBNBs in brains with *DlRNAi* alone (Fig. 2C). We conclude that MBNBs undergo apoptosis when Notch pathway activity is reduced, however MBNB elimination via apoptosis occurs prematurely.

Temporal Windows are Shifted in Notch Pathway Knockdown

MBNBs as opposed to other CBNBs are known to experience a longer period of Imp expression (Liu et al., 2015). This prolonged Imp expression allows for extended proliferation, partially accounting for the longer life cycle of MBNBs compared to other CBNBs. In CBNBs, the Imp to Syp transition is mediated by the steroid hormone, ecdysone (Doe, 2017; Syed et al., 2017). However, it remains unclear whether this is true for MBNBs and if other factors are required. After observing premature loss of MBNBs in both *NRNAi* and *DlRNAi*, we hypothesized that temporal patterning may be altered given that MBNBs are also lost prematurely when Imp is reduced (Liu et al., 2015). To test this hypothesis, we employed heat shock flippase clone constructs which allows for expression of *DlRNAi* and a fluorescent protein in a subset of MBNBs. This technique provides a control wild type MBNB alongside a *DlRNAi*, GFP expressing MBNB clone for direct comparison within the same brain hemisphere. Brains were staged upon hatching, heat shocked, and then imaged at 72h after larval hatching (ALH).

Imp expression in *DlRNAi* clones was reduced compared to wildtype MBNBs (Fig. 3A, yellow arrowhead). Approximately 54% of MBNBs expressing *DlRNAi* maintained Imp expression compared to 93% of control MBNBs (Fig. 3E). This result indicates that Notch/Delta signaling positively regulates Imp expression. Because Imp and Syp reciprocally inhibit one another (as Imp decreases, Syp increases), we tested whether premature loss of Imp would lead to precocious Syp expression (Liu et al., 2015). We again used *DlRNAi* clones to assess Syp expression at 72h ALH. As expected, no Syp expression was seen in controls as the Imp window is still active at this time (Fig. 3B, red arrowhead). However, precocious Syp expression was also never observed in MBNB *DlRNAi* clones (Fig. 3B, yellow arrowhead, n = 6). We do see Syp expression in whole brain knockdown of Dl at 48h APF, leading to the assumption that Syp expression may just be delayed without Dl (Fig 3D). We conclude that Notch/Delta signaling positively regulates early temporal patterning by promoting Imp expression. Moreover, additional cues are likely required to promote Syp, most likely being yet unknown signals that are independent of early temporal factor Imp expression.

Since early temporal patterning is altered in *DlRNAi* MBNB clones, we next asked whether late temporal patterning is also altered. At 48h APF, control MBNBs express low levels of E93 but high levels of E93 in their Kenyon cell progeny (Fig. 3C, red arrowhead and C', red outline). However, at 48h APF in *DlRNAi* clones, E93 was not detected in MBNBs nor in their Kenyon cell progeny (Fig. 3C, yellow arrowhead and 3', yellow outline). This is drastically different from the 100% of control MBNBs expressing E93 at this time point (Fig. 3F). Previous work has shown that expression of both the intrinsic temporal factor Syp and the extrinsic cue Ecdysone is important for promoting

E93 expression in MBNBs at late developmental stages prior to termination (Pahl et al., 2019). This loss of E93 expression at late pupal stages in *DlRNAi* clones further shows Notch signaling pathway is key to maintaining the temporal cassette of MBNBs.

Mushroom Body Defects are seen in Notch Pathway Knockdown

Proper mushroom body morphology is dependent on MBNBs proliferating and producing the correct subtype of neuronal progeny at distinct timepoints of the neurogenic period. We found that when MBNB proliferation is disrupted by altering Notch pathway signaling, the MB structure is severely affected. We stained freshly eclosed adult brains with the MB marker Fasciclin 2 (FasII) that labels the γ, α/β neurons and a membrane marker Scribble (scrib). Compared to controls, we see in *DlRNAi* animals that late born α/β neurons are absent and early born γ neurons make up a large portion of the structure (Fig. 4A-D). While γ cells are present, the γ lobe does not look like it has undergone the same amount of axonal pruning as would be expected at the adult stage (Fig. 4D-D'') (Yu & Schuldiner, 2014). These abnormal looking structures appear to result from defects in gamma neuron differentiation based on morphology (Figure 4C, yellow arrowhead). MB neurons are experiencing defects in molecular identity and number. The survival of these neurons is affected by loss of Delta which would account for absence of late born kenyon cell types. Observed morphological defects show the drastic effect Notch pathway disruption can have on important structures in the adult brain.

Following our results found in figure 2, we wanted to test if blocking death in DeltaRNAi animals by using the UAS-miRHG construct would restore some of the MB

structure in adult animals. We found that when apoptosis was inhibited, the MB morphology is not identical to control but appears to have better organization of the γ neurons than in DeltaRNAi brains (Fig. 4E-F''). In addition, we see a slight increase in the production of late born, α and β neurons, enough to form an α lobe to some extent (Fig. 4F). While we did not observe a complete rescue of MB morphology, this finding further emphasizes that when death is blocked in a Delta knockdown animal, MBNBs are able to persist longer. Their lineage is then able to better survive and can integrate into the MB structure.

Eyeless as an Upstream Regulator of Notch

One of the most perplexing questions from this research is how Notch activity loss so differently affects MBNBs and other CBNBs. This work has shown that loss of Notch in MBNBs leads to a skewed temporal cassette with the early factor Imp being lost prematurely, leading to premature termination of these 4 MBNBs. However, we have observed an opposing phenotype in the other ~96 CBNBs in the same brain hemisphere. Non-mushroom body CBNBs are able to persist in Notch knockdown animals, primarily through extension of their early temporal window of Imp expression (Sood et al., 2024). To try and understand these lineage specific differences in response to Notch activity, we again turned to our RNAi screen and found of our candidate genes, a MBNB specific gene of interest Eyeless (Ey).

Ey is a paired box homeodomain transcription factor known for its role in eye development (Martini et al., 2000; Noveen et al., 2000). It is highly conserved and known as Pax6 in mammals (Callearts et al., 2001). To assess the role of Ey in

mediating Notch activity in MBNBs we used the heat-shock flippase induced clone system again, with UAS-*EyRNAi*. In this experiment, clones express *EyRNAi*, a fluorescent protein, and a Notch activity reporter enhancer of split (E(spl))-GFP. We found that at 48h APF and 72h APF *EyRNAi* clones had a reduction in fluorescence from the E(spl)GFP reporter, suggesting that Notch activity is reduced compared to control MBNBs in mid to late pupal stages (Fig 5A-C). At 24h APF there is only a slight decrease in E(spl)GFP in *EyRNAi* clones with 90% of clones being GFP positive. At 48h APF the percent of MBNBs expressing E(spl)GFP was greatly decreased with only 16.6% of *EyRNAi* clones are positive for GFP while 100% of control MBNBs are positive for GFP (Fig 5B). Finally, at 72h APF this percent increases with 40% of *EyRNAi* clones are positive for GFP while again 100% of MBNB controls are positive. This has led us to conclude that the MBNB specific factor Ey plays a role in regulating Notch activity during mid to late pupal phases, and may be a key gene in establishing lineage specific differences between MBNBs and other CBNBs.

We next wanted to further assess how Ey may contribute to regulating Notch pathway activity and create lineage specific differences between MBNBs and other CBNBs. Unpublished data from the Siegrist lab has shown that Ey knockdown affects MBNB neurogenesis. If Ey, a MBNB specific factor, regulates Notch, what would occur if Ey were expressed in other CBNBs? We hypothesize that if Ey is overexpressed while knocking down Delta, the *DlRNAi* phenotype seen in non-MBNB CBNBs may be lost. To test this, we constructed another heat-shock flippase line that when induced, would overexpress Ey and simultaneously express *DlRNAi*. Resulting CBNB clones would have Ey overexpression, *DlRNAi*, and GFP. We assessed non-MBNB CBNBs at 48h

APF where in controls we see no dpn+ cells as they have been eliminated by this timepoint however in *DlRNAi* animals we see ectopic dpn+ cells at 48h APF (Sood et al., 2024). The non-MBNB CBNB clones we observed were dpn+ 70% of the time and dpn- 30% of the time (Fig 6A). *DlRNAi* alone results in Dpn+ clones 50% of the time and dpn- 47% of the time (Sood et al., 2024). This was an encouraging result that Ey overexpression in non MBNB CBNBs may regulate Notch signaling to alter the *DlRNAi* phenotype of ectopic CBNBs but would require further investigation.

Finally, we wanted to continue testing the hypothesis that Ey creates the lineage specific differences between MBNBs and other CBNBs, allowing different NBs to respond differently to Notch signaling, by assessing how activating Notch signaling in an Ey null animal would impact proliferation and termination of MBNBs. A line was constructed combining NB Gal4 UAS driven Notch intracellular domain (NICD), the active form of Notch, in an Ey null animal. We attempted to raise these animals to mid-pupal stages in order to dissect and assess MBNBs late but quickly found that this combination was lethal. Larvae of this genotype were dying between hatching and 24h ALH. In addition, many larvae were observed to have issues hatching from their egg cases, as we could see their mouth hooks moving and the larvae wiggling, but they were rarely able to pry themselves out of their case and properly hatch.

We decided to dissect 24h ALH larvae by assisting them from their egg case at FH and allowing them to develop for 24 hours. We observed extremely small brains in these animals, with only the 4 MBNBs visible via a very diminished dpn signal (Fig 7A). We next fed these animals Edu to assess if the MBNBs were proliferating. At FH stages after being fed Edu for 3 hours, only the MBNBs were proliferating (Fig 7B). We next fed

animals at 21h ALH and dissected at 24h ALH and found that there was no Edu incorporation in the MBNBS (Fig 7C). From this we concluded the MBNBs are dying at or around 24h ALH in the combined Ey null, activated Notch construct. This suggested to us that the Notch and Ey genetic interaction is much stronger than previously understood and requires more research to fully uncover how lineage specific differences are created between MBNBs and other CBNBs.

## Discussion

Notch activity is lineage-specific

Here we report that Notch pathway signaling regulates the proliferative window of MBNBs in the developing *D. melanogaster* brain. We found that when Notch pathway components are knocked down using RNAi lines against Notch receptor and its ligand Delta, MBNBs prematurely terminate, and the MB structure is disrupted. This is due to a shift in the known Imp-Syp-E93 temporal cassette. Rather than having a long period of Imp+ proliferation from larval to mid-pupal stages, *DeltaRNAi* animals lose Imp expression early. Despite the loss of this early temporal factor, we do not see expression of its reciprocally inhibitory partner Syp. This was a very interesting finding as it is currently unknown what mediates the Imp to Syp transition besides their reciprocal inhibition (Liu et al., 2015). In other CBNBs this transition is mediated by ecdysone hormone signaling (Syed et al., 2017). More research to assess if this holds true in MBNBs would further our understanding of progression through the temporal cassette during neurogenesis.

We also identify that without Notch signaling, E93 expression is also delayed. This could be due to the delay in Syp expression, as Syp is required to stabilize E93 in late pupal phases (Pahl et al., 2019; Syed et al., 2017). E93 is dependent on Ecdysone pulses and the expression of the EcR (Pahl et al., 2019). Notch and ecdysone have been shown to interact in wing imaginal tissues to regulate D/V patterning (Jia et al., 2016). In the CB, ecdysone signaling and Notch signaling may also function together to coordinate the neurogenic period of MBNBs by advancing the temporal program. It would be of interest to further investigate EcR in Notch pathway knockdown to assess exactly why E93 expression is delayed.

We have shown that the Notch pathway positively regulates temporal patterning programs in a lineage specific manner as the effects seen in MBNBs are different than other CBNBs (Sood et al., 2024). At late timepoints when Notch signaling is perturbed, primarily through Dl knockdown, we also see a large amount of other CBNBs persisting past their typical termination time point (Fig. 1F). This highlights differences between NB lineages in the central brain and provides insight as to how Notch pathway may regulate temporal patterning in MBNB to allow for a longer proliferative window.

Apoptosis accounts for premature MBNB elimination

Due to shifts in temporal patterning, primarily through extinction of Imp expression prematurely, MBNBs are cycling through their neurogenic period faster leading to early elimination. We assessed how MBNBs may be undergoing premature termination by blocking the apoptotic cell death pathway with a synthetic transgene against pro-apoptotic genes reaper, hid, and grim. We found that MBNBs were able to

persist into adulthood when death was blocked in a *DlRNAi* animal and showed expression of the proliferative marker PCNA:GFP. We also assessed how the premature loss of the MBNBs affects the important MB structure by staining the α, β, and γ neurons. We found that without Delta expression, we lose the late born α and β progeny resulting in a misformed MB. However, when we again block death by combining UAS-miRHG with *DlRNAi* we see more of this later born progeny and a more correctly formed γ lobe than in *DlRNAi* brains alone. This result showed that premature elimination of MBNBs in the *DlRNAi* animal is due to RHG dependent apoptosis.

Eyeless contributes to lineage specific differences in Notch activity

Finally, we assessed what lineage specific factor could cause such different phenotypes of premature elimination in MBNBs and ectopic persistence in other CBNBs. The evolutionarily conserved gene, Ey is specific to MBNBs, and we have previously observed phenotypes of disrupted neurogenic periods in animals expressing *EyRNAi* (unpublished). Here we find that in *EyRNAi* conditions, Notch signaling is reduced as seen through the activity reporter E(spl)GFP. In addition, when we overexpress Ey in non-MBNB CBNBs with *DlRNAi* we see some dpn negative clones, showing that activating Ey in a NB lineage that does not typically express this factor may change how it responds to Notch signaling. Finally, we activated Notch signaling in Ey null animals and found this to be lethal. This unexpected result showed that the interaction between Notch and Ey is stronger than it was previously thought to be. This finding provides an excellent opportunity for future research into the role of Ey in

regulating Notch activity in MBNBs, and how lineage specific differences between CBNBs arise.

Together, this has led us to conclude that the Notch pathway is imperative for proper MBNB proliferation and resulting MB morphology. Notch and its ligand Delta interact with the known temporal program of MBNBs to regulate when each type of progeny is produced and in the correct volume. Eyeless functions as a MBNB specific factor to differentially regulate how CBNBs respond to Notch signaling through the neurogenic period (Fig 8). This work also offers an opportunity to understand how other well-known signaling pathways may play a role in regulation of neurodevelopment through downstream temporal patterning genes. This is a novel component of MBNB temporal regulation that has not yet been recognized and allows for a deeper understanding of the genes involved in intrinsic control of neuroblast proliferation.

## Methods

### Fly Stocks:

Fly stocks utilized, and their source are listed in key resources. Stocks generated were verified by PCR, antibody staining, and dominant markers.

### Animal Husbandry:

All animals were reared in uncrowded conditions at 25°C. Animals were staged from freshly hatched for larval time points and white pre-pupae for pupal time points.

### Clonal Induction:

Flp-Frt and MARCM clones were heat shocked at 37°C for 10-30 minutes during the first larval instar after staging at freshly hatched.

### Edu analysis:

Larval animals were fed Edu for 3 hours directly after hatching or at 21h ALH. Animals were then fixed and dissected. Primary antibodies were applied for 24 hours and washed followed by Alexa-Fluor conjugated secondary antibodies for another 24 hours (Supplemental Table 2). Secondary antibodies were washed and the Edu reaction was completed followed by more washes. Brains were stored overnight in glycerol solution and then imaged. Animals were quantified as being Edu+ or Edu- for each time point.

### Immunofluorescence and Confocal Imaging:

Larval, pupal, and adult brains were dissected according to Pahl et al., 2019. Dissected brains were fixed in a solution of 4% paraformaldehyde in PEM buffer for 20 mins (larvae) or 30 mins (pupae), followed by a series of washes in PBST (1X PBS + 0.1% Triton X-100). Blocking solution of 10% normal goat serum (NGS) in PBST was

applied and tissues were stored at 4°C overnight. Primary antibodies were applied and washed followed by Alexa-Fluor conjugated secondary antibodies (Supplemental Table 2). Secondary antibodies were washed, and brains were placed in anti-fade glycerol solution overnight prior to imaging. Z-stacks were taken of each central brain hemisphere by a Leica SP8 laser scanning confocal microscope using a 63x/1.4 oil immersion objective.

**Software and Data Analysis:**

Images were analyzed using Fiji ImageJ and processed using Adobe Photoshop. Figures were assembled using Adobe Illustrator. MBNBs were identified by nuclear dpn staining and superficial location along the dorsal surface of the brain. Cell size was calculated using Image J's line tool to draw a cross hair across the NB cell body and the average of those two values was recorded in Graphpad Prism. Sample sizes are listed within the bars of all charts. All data is represented as mean ± standard error of the mean and statistical significance was determined using unpaired two-tailed student's t-tests or ANOVAs in Graphpad Prism 9.

## Figures

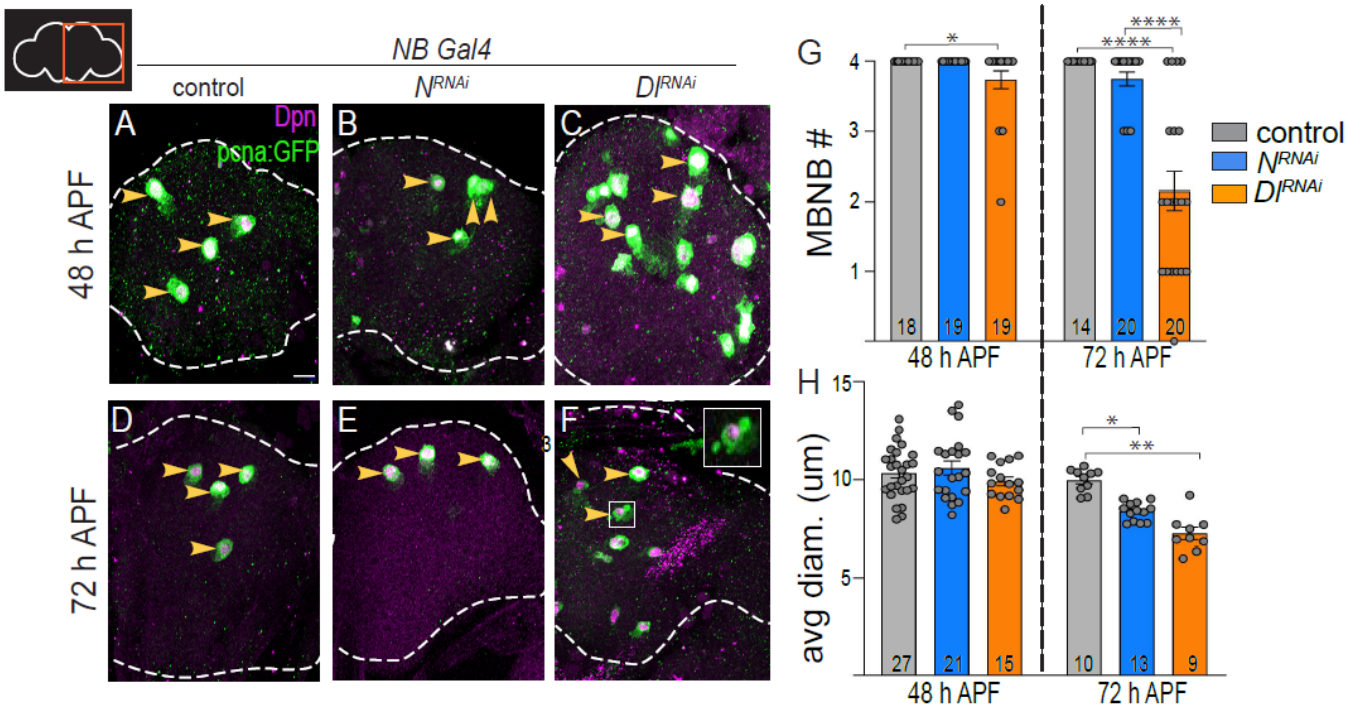



**Figure 1. Notch pathway knockdown results in premature loss of MBNBs.**

(**A-F**) Cartoon in top left indicates brain hemisphere imaged for this and all subsequent figures. Maximum intensity projections of the right brain hemisphere with pupal timepoints and genotypes as listed. Antibodies used are indicated in the top right of panel A and the hemisphere is outlined in a white dashed line. Yellow arrows indicate MBNBs, unmarked NBs are other non-MBNB central brain NBs. The inset (**F**) indicates a MBNB undergoing premature cell death. Scale bar = 20 μm. (**G**) Histogram represents the mean number of MBNBs present at the stated time points for each genotype. Each data point represents a separate brain hemisphere with the n represented within the bar. (**H**) Histogram represents the average MBNB diameter at the stated time points for each genotype. Each data point represents an individual MBNB with the n represented within the bar. (**G-H**) Error bars represent the SEM, alpha = 0.05, analyzed with a one-

way ANOVA followed by a test for multiple comparisons. Significance is indicated with asterisks.

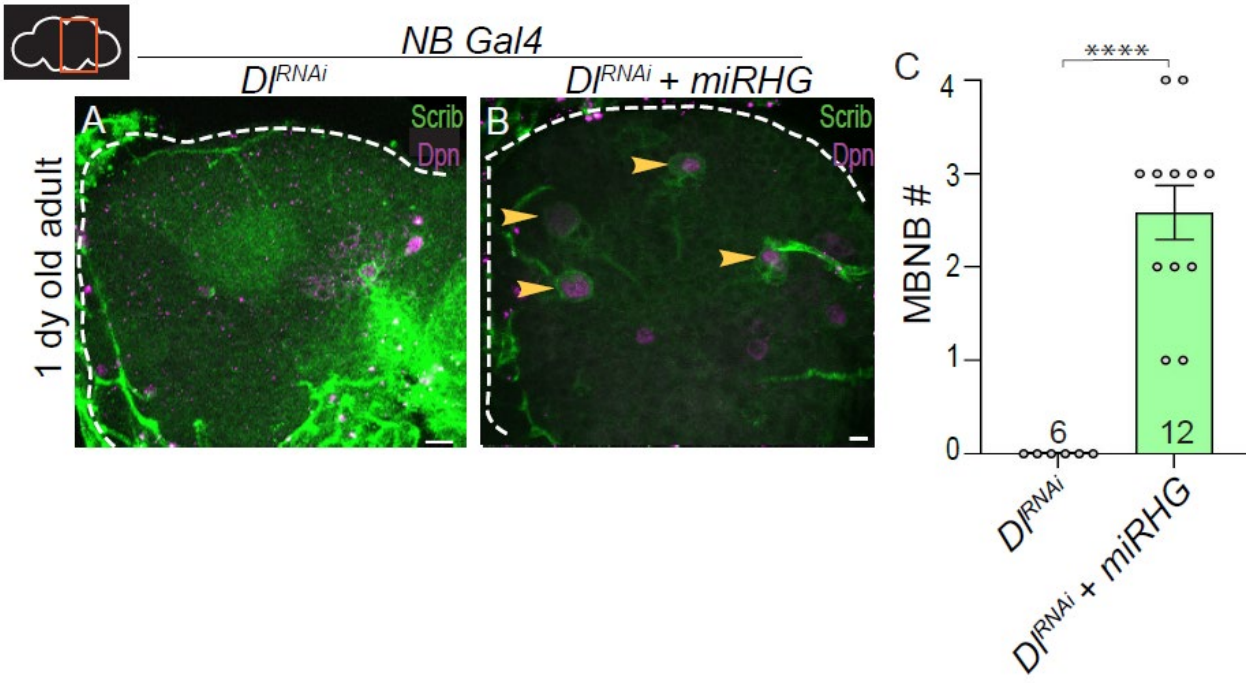



**Figure 2. Blocking apoptotic cell death in a DeltaRNAi animal allows persistence of MBNBs.**

(**A**) Maximum intensity projection of the right hemisphere of 1-day old adult brains of the specified genotypes. Scale bar = 10 μm. (**B**) Maximum intensity projection of the right hemisphere of freshly eclosed brains of the specified genotype. Persisting MBNBs indicated with yellow arrowheads. Scale bar = 10m. Markers used are indicated under the corresponding panels. (**C**) Histogram represents the average number of persisting MBNBs at the FE one day old time point in DeltaRNAi and DeltaRNAi + UAS-miRHG brains. Each data point represents one brain hemisphere for a total of 12 brain hemispheres from 6 different animals. Data was analyzed using an unpaired, two-tailed Student's t-test, alpha = 0.05. Error bars represent the standard error of the mean. Significance is indicated with asterisks.

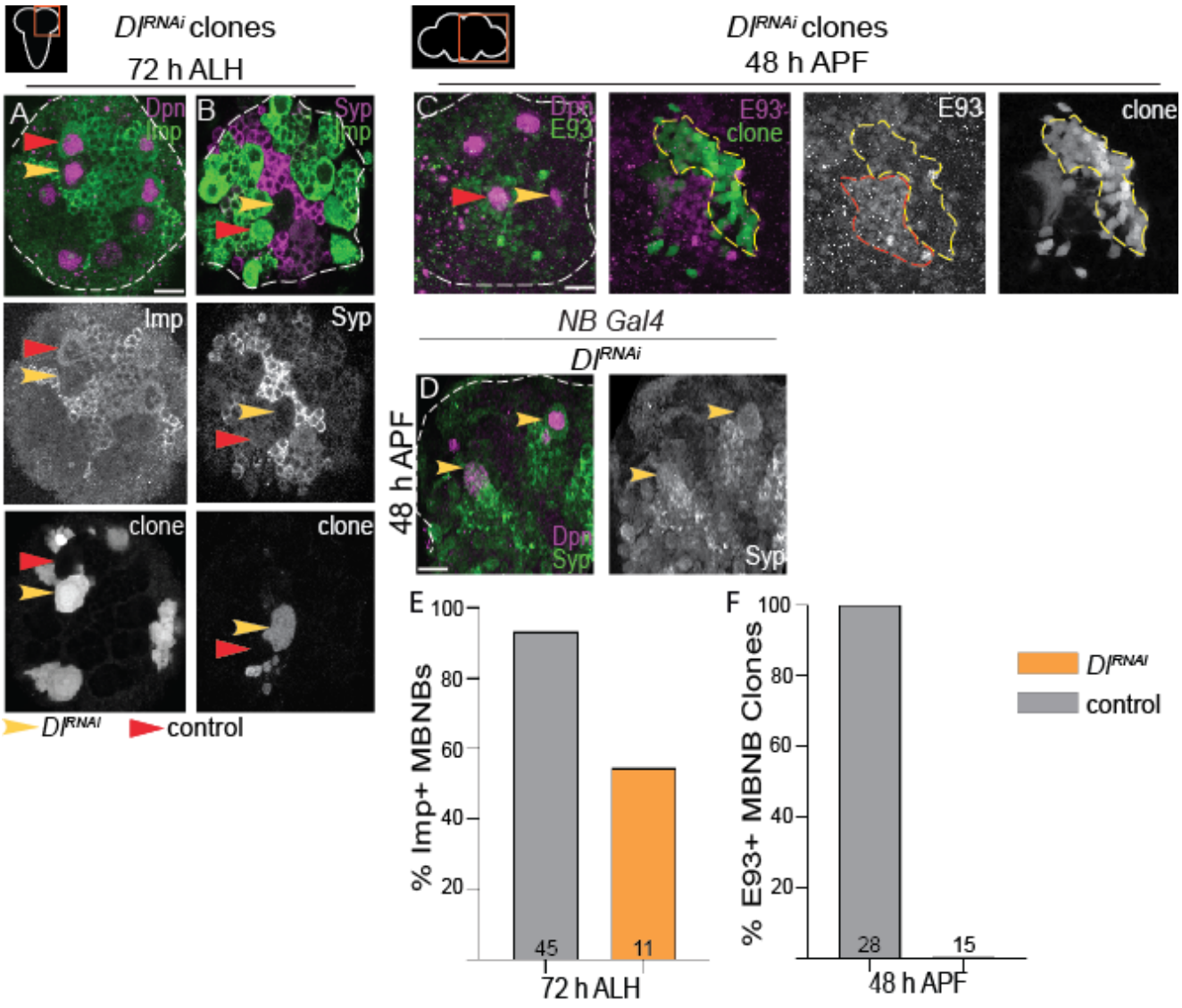



**Figure 3. Delta knockdown alters temporal factor expression in MBNBs.**

(**A-C**) Maximum intensity projections of the right brain hemisphere with larval and pupal timepoints and genotypes as listed. Markers are indicated in the top right of the corresponding panels. Single channel grayscale images provided to show clone MBNB and temporal factor being analyzed. Control MBNBs are indicated with red arrowheads while clones are indicated with yellow arrowheads. Brain hemispheres are outlined with white dashed lines. The Delta clone stained for E93 is outlined in a yellow dashed line.

The control MBNB's E93 is outlined in a red dashed line. Scale bar = 10μm. (**D-E**) Histogram showing the percentage of Imp and E93 positive control MBNBs versus clone MBNBs or progeny. A count of Syp positive cells is not shown as it was zero for both classes of MBNBs. The number of each type of MBNB quantified is listed within the bar. The value was calculated by dividing the number of positive cells by the total number of cells present at the particular time point.

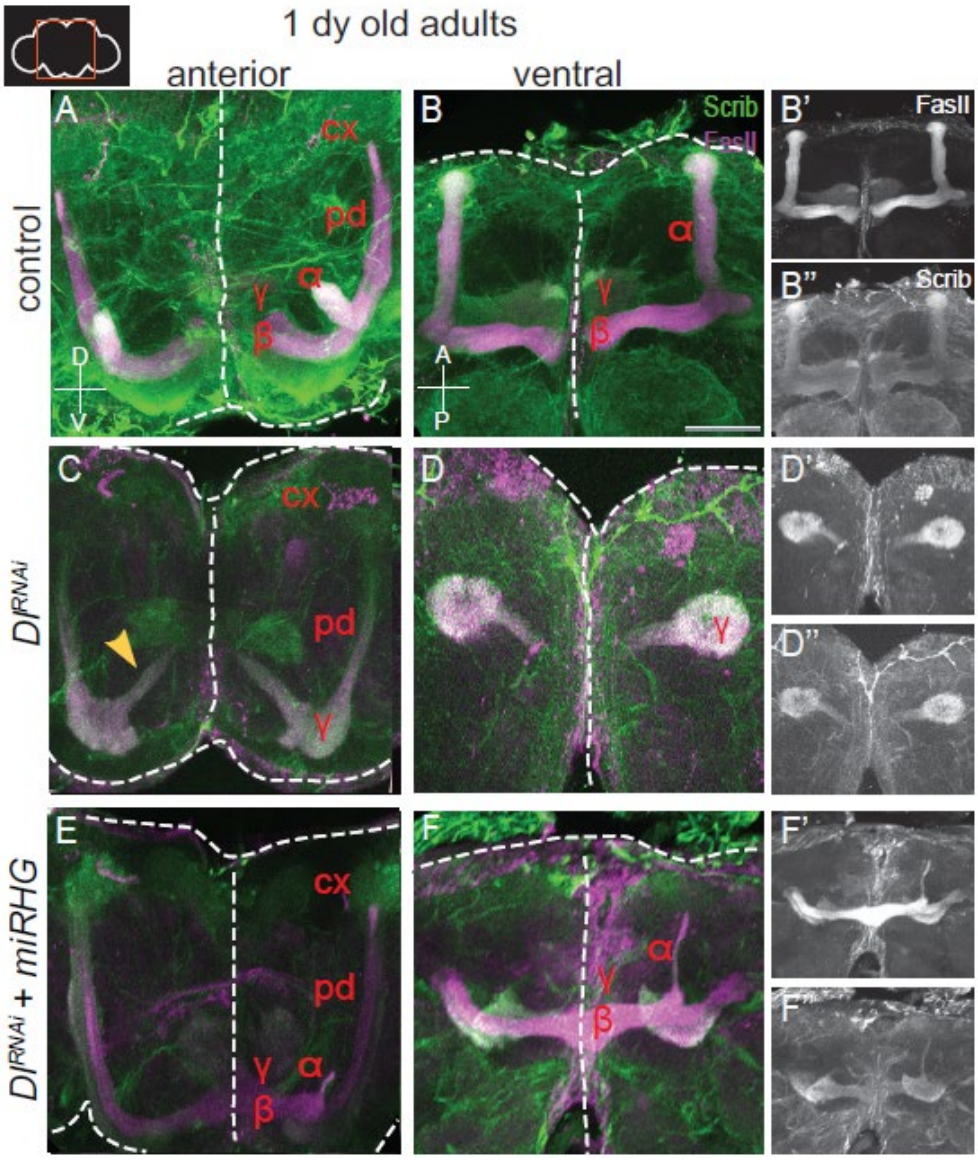



**Figure 4. Premature loss of MBNBs causes severe MB morphological defects.** (**A-F**) Maximum intensity projections of the central brain lobes of one day old adult brains of the specified genotypes. White dashed lines outline the central brain and indicate the midline. Images show expression of alpha, beta, gamma neuron marker fasII and scrib. The α' and β' lobes are not labeled. Red lettering indicates which lobe is present for each genotype. (**A, C, E**) Anterior view lobes are marked to indicate the alpha, beta, gamma lobes as well as the peduncle and calyx when applicable. (**B, D, F**)

Ventral view lobes are marked to indicate the alpha, beta, gamma lobes. (**B'-F"**) Single channel grayscale images show the MB structure from the ventral view for both FasII and Scrib expression. Scale bar = 50 μm. (**A-B**) n= 12. (**C-D**) n=4. (**E-F**) n = 3.

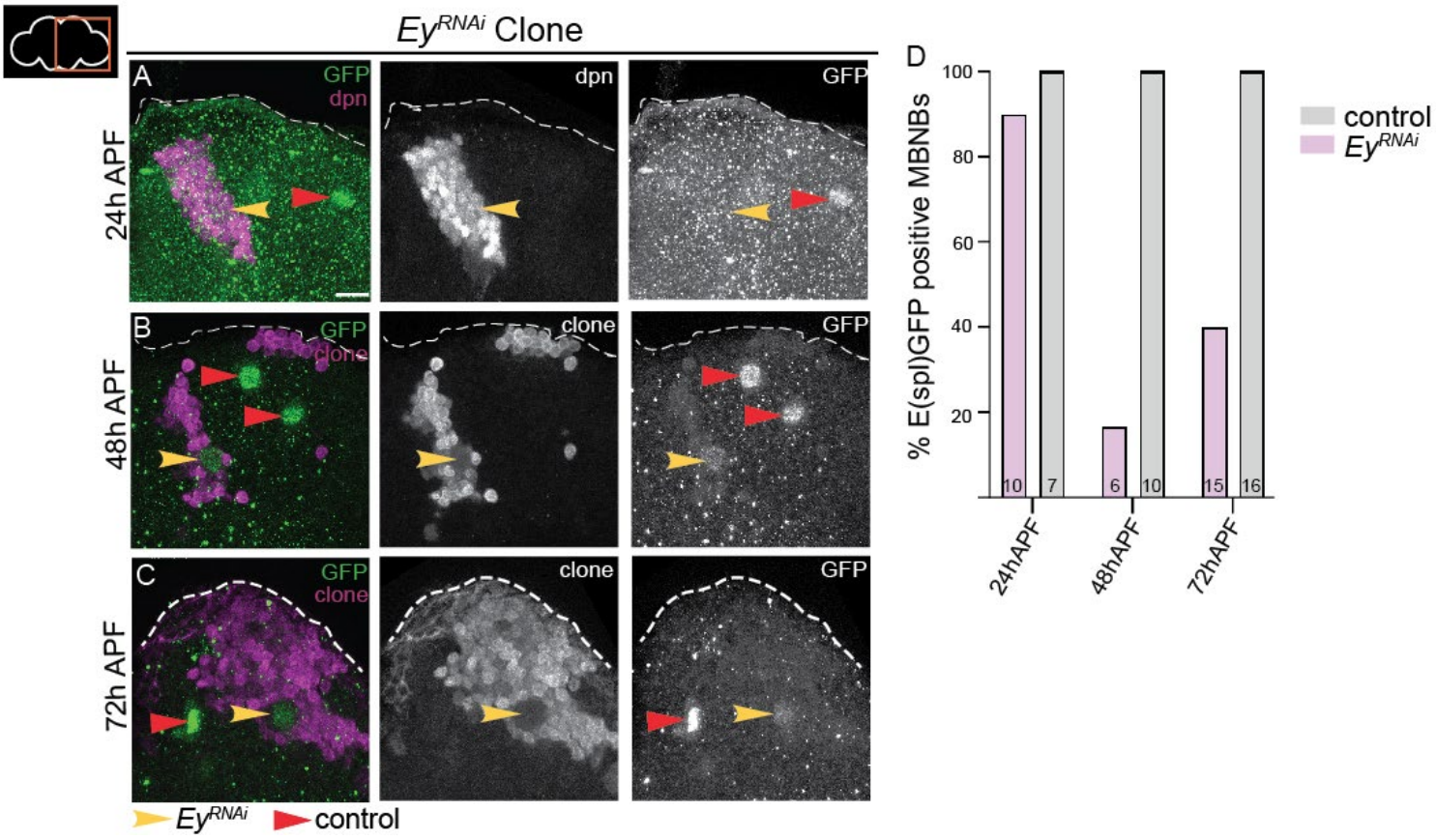



**Figure 5. Eyeless regulates Notch activity in MBNBs.**

(**A-C**) Single z-stack images of the right brain hemisphere at time points as indicated to the left of images. *EyRNAi*; E(spl)GFP MBNBs clones are indicated with a yellow arrowhead while control MBNBs are indicated with a red arrowhead. Brain hemispheres are outlined with white dashed lines. Scale bar = 10 μm. Markers used are indicated within the corresponding panels. (**D**) Histogram showing the percentage of E(spl)GFP positive control MBNBs versus clone MBNBs. The amount of each type of MBNB quantified is listed within the bar. The value was calculated by dividing the number of positive cells by the total number of cells present at the particular time point. **(A)** A total of 5 hemispheres from 4 different animals was quantified. **(B)** A total of 4 hemispheres

from 3 different animals was quantified. (**C)** A total of 9 hemispheres from 5 different animals was quantified.

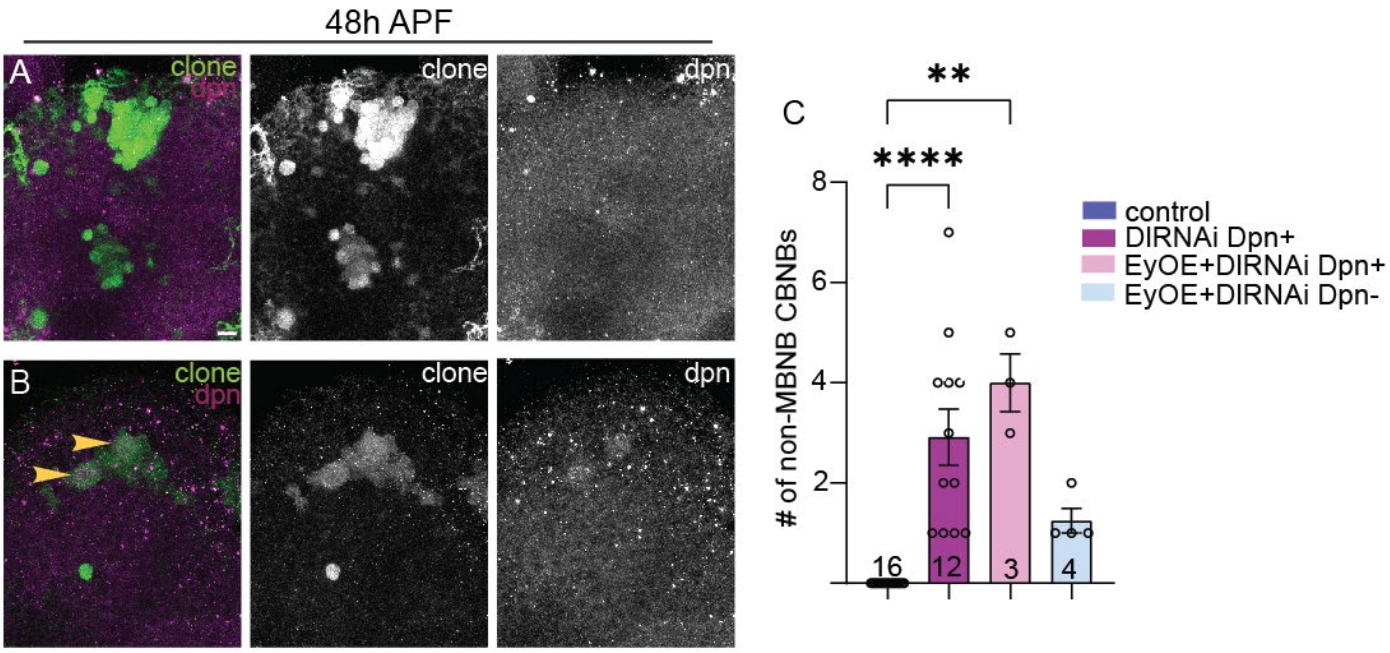



**Figure 6. Ey overexpression with *DIRNAi* results in Dpn- clones.** (**A-B**) Maximum intensity projections of dpn- and dpn+ EyOE;*DIRNAi* clones at 48hAPF. Antibodies used indicated in top right. Scale bar = 10 μm (**B**) Yellow arrowheads indicate dpn+ clones. (**C**) Count of the number of non MBNB CBNBs clones in each genotype. Each point represents one brain hemisphere. N is within each bar. Alpha = 0.05. Error bars represent the standard error of the mean. Number of animals measured:

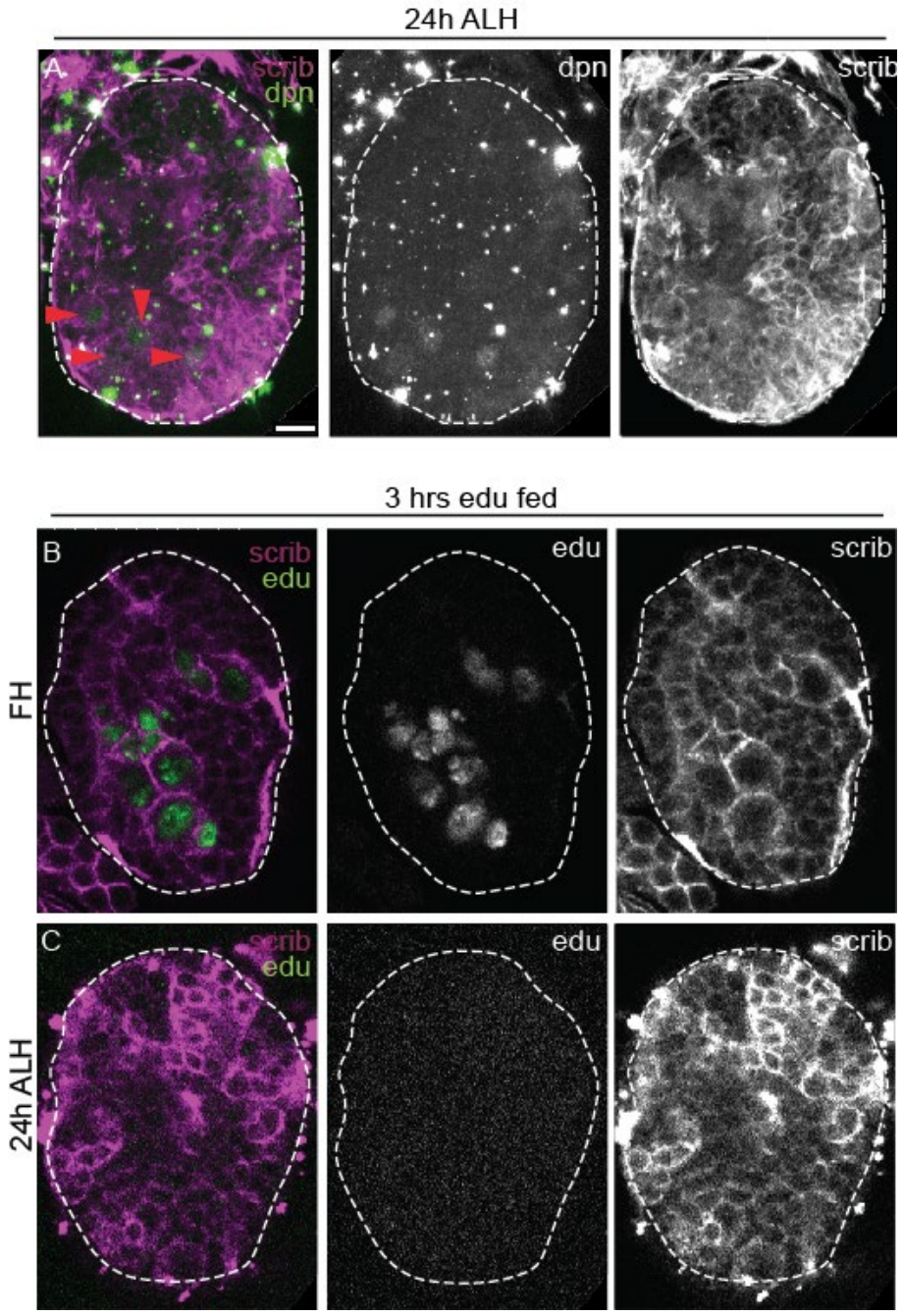



**Figure 7. Ey Null with NICD is lethal.** (**A**) Maximum intensity projection of a 24h ALH larval brain. Scale bar = 10 μm. Red arrowheads identify dpn+ MBNBs. Brain hemisphere outlined in white dotted line. Antibodies used indicated in top right. (**B-C**) Single z-plane images of three-hour Edu fed animals dissected at the specified

timepoints to the left of the panels. Brain hemisphere outlined in white dotted line. Antibodies used indicated in top right.

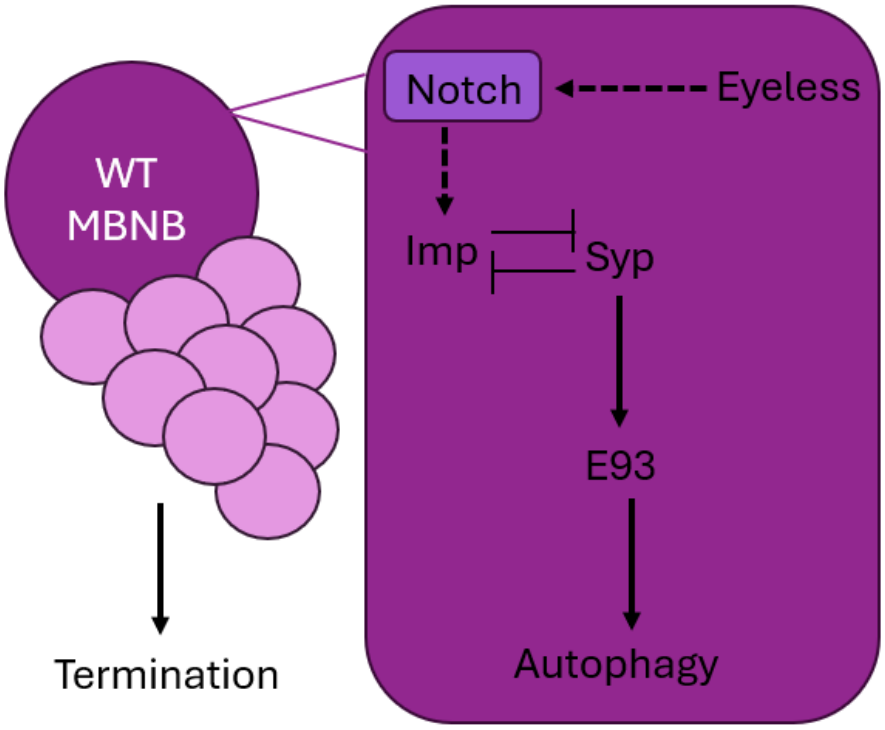
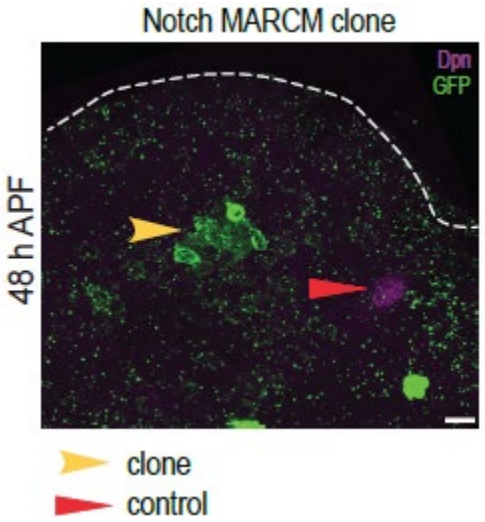



**Figure 8. Notch signaling is regulated by Eyeless and controls downstream temporal patterning to maintain the MBNB neurogenic period.** In MBNBs Notch signaling positively regulates early temporal factor Imp to maintain the longer proliferative window required for MB formation. Eyeless functions as a MBNB specific factor to regulate Notch activity differently than in other CBNBs.

## Supplemental Figures




**Supplementary Figure 1. Notch MARCM clone showing MBNB premature elimination phenotype.**

Maximum intensity projections of MARCM Clones at 48h APF show a premature loss of Dpn+ MBNBs while control MBNBs remain Dpn+. GFP represents the clonal lineage from the eliminated MBNB. Scale bar = 10 μm.

**Table 1. Genotype by figure**

| Figure | Genotype |
|---|---|
| 1A, 1D, 4A-B Control | *WornGAL4/+ (Oregon R); pcnaGFP/ + (Oregon R)* |
| 1B, 1E | *WornGAL4/+; pcnaGFP/UAS-NotchRNAi* |
| 1C, 1F, 3A | *worGAL4/+; pcnaGFP/UAS-DeltaRNAi* |
| 2A-B | *hsFlp; repoGAL80; UAS-DeltaRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-GFP* |
| 2C | *hsFlp; repoGAL80; UAS-DeltaRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* |
| 3B | *WorGal4/+; UAS-DeltaRNAi; UASmiRHG* |
| 4E-F | *WornGAL4/+; pcnaGFP/UAS-DeltaRNai; UASmiRHG/+* |
| 4C-D | *worGAL4,tubGAL80(ts)/+; pcnaGFP/UAS-DeltaRNAi* |
| 5A-C | *hsflp; UAS-EyRNAi; E(spl)GFP/ Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* |
| 6A-B | *Hsflp; UAS-EyO/E; UASDlRNAi/ Act5c-FRT-CD2-FRT-Gal4, UAS-GFP* |

| | |
|---|---|
| 7A-C | *WorGal4 / UAS Notch-ICD; Ey J5.D1/ Ey J5.D1* |
| Supp. 1 | *hsflp, tubgal80, FRT19A/Notch55e11 FRT19A ; tubGal4, UASmCD8GFP/+* |

**Acknowledgements**

Data for Figs 1, 3, 4 (A-D), and supp 1 were generated by CS. Data for Figs 2, 4 (E-F), 5, 6, and 7 were generated by KRB. All figures created by KRB. All data analysis performed by KRB. Manuscript written and edited by KRB. Manuscript edits from SES and SED. Thank you to the Justin Kumar lab for providing Eyeless null reagents. Thank you to Dr. Susan Doyle for assistance with early larval time points in Fig 7. We would like to thank the members of the Siegrist Lab for their help and feedback on this project.

**Future Plans**

This article is currently on BioRxiv at https://doi.org/10.1101/2024.01.31.578279. I plan to submit this to Development or Developmental Biology.

**Key Resources**

| Reagent | Source | Identifier |
|---|---|---|
| **ANTIBODIES** | | |
| rat ant-Dpn (1:100) | Abcam | ab195173 |
| chicken anti-GFP (1:500) | Abcam | ab13970 |
| rabbit anti-dsRed (1:1000) | Clontech | 632496 |
| rabbit anti-Scribble (1:500) | Gift from Chris Q. Doe | |
| guinea pig anti-E93 (1:250) | Gift from Chris Q. Doe | |
| rabbit anti-Imp (1:250) | Gift from Paul MacDonald | |
| rat anti-Imp (1:250) | Gift frorm Claude Desplan | |
| rabbit anti-Syncrip (1:250) | Gift from Chris Q, Doe | |
| mouse anti-Ey (1:100) | DSHB | AB_2253542 |
| goat anti-chicken Alexa 488 (1:300) | Thermo Fisher Scientific | A32931 |
| goat anti-rat Alexa 555 (1:300) | Thermo Fisher Scientific | A48263 |
| goat anti-rat Alexa 647 (1:300) | Thermo Fisher Scientific | A48265 |
| goat anti-rabbit Alexa 405 (1:300) | Thermo Fisher Scientific | A48254 |
| goat anti-rabbit Alexa 555 (1:300) | Thermo Fisher Scientific | A21428 |
| goat anti-rabbit Alexa 633 (1:300) | Thermo Fisher Scientific | A21071 |
| goat anti-guinea pig Alexa 488 (1:300) | Thermo Fisher Scientific | A11073 |

| | | |
|---|---|---|
| goat anti-guinea pig Alexa 555 (1:300) | Thermo Fisher Scientific | A21435 |
| **CHEMICALS** | | |
| SlowFade™ Diamond antifade reagent | Invitrogen | Catalog # S36963 |
| SlowFade™ Gold antifade reagent | Invitrogen | Catalog # S36937 |
| Normal Goat Serum | Thermo Fisher Scientific | Catalog # 31873 |
| Paraformaldehyde 16% solution EM grade | Electron Microscopy Sciences | Catalog # 15710 |
| Triton X-100 | Sigma | Catalog # T9284 |
| Click-iT Edu Proliferation Assay | Invitrogen | Catalog # C10340 |

| **SOFTWARE** | | |
|---|---|---|
| ImageJ/Fiji | Fiji | http://fiji.sc/ |
| LAS X | Leica Microsystems | https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/ |
| Prism 9 | Graphpad | https://www.graphpad.com/scientific-software/prism/ |

| | | |
|---|---|---|
| Photoshop 2022 | Adobe | https://www.adobe.com/products/photoshop.html |
| Illustrator 2022 | Adobe | https://www.adobe.com/products/illustrator.html |

| **EXPERIMENTAL MODEL: *Drosophila melanogastor*** | | |
|---|---|---|
| Oregon R | Bloomington Drosophila Stock Center | 5 |
| *wor-Gal4* | (Albertson and Doe, 2003) | |
| *tubulin-Gal80(ts)* | Bloomington Drosophila Stock Center | 7108 |
| *UAS-Notch RNAi (HMS00001)* | Bloomington Drosophila Stock Center | 33611 |
| *UAS-Delta RNAi (HMS01309)* | Bloomington Drosophila Stock Center | 34322 |
| *UAS-Eyeless RNAi* | Bloomington Drosophila Stock Center | 32486 |
| *UAS-NICD* | (Go et al., 1998) | |
| *UAS-Ey O/E* | From Matt Pahl | |
| *Ey J5.D1* | Gift from Justin Kumar | |
| *E(spl)mg-GFP* | (Almeida & Bray, 2005) | |
| *pcna-GFP* | (Thacker et al., 2003) | |
| *Hsflp (on X)* | Gift from Ishwar Hariharan | |

| | | |
|---|---|---|
| *Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* | Bloomington Drosophila Stock Center | 30558 |
| *Act5c-FRT-CD2-FRT-Gal4, UAS-GFP* | gift from Iswar Hariharan | |
| *Notch55e11 FRT19A* | Bloomington Drosophila Stock Center | 28813 |

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# Chapter 3: Eyeless regulates temporal patterning to control the mushroom body neuroblast neurogenic period in *Drosophila melanogaster*


## Abstract

Neural stem cell (NSC) regulation is key to proper development of a functional adult brain. When NSCs are dysregulated, neural developmental disorders (NDD) can arise, which causes devastating impacts on patients and strains medical systems. Understanding the genetic and molecular underpinnings that control NSC proliferation provides the opportunity to advance therapeutics and potentially treat NDD. *Drosophila melanogaster* provide an excellent model system to study NSC regulation as they have an analogous neural stem cell population called neuroblasts (NB). In addition, the high degree of genome conservation between *Drosophila* and mammals allows for comparisons with relevancy to human health. While it is known that intrinsic and extrinsic signals integrate to regulate NB proliferation and termination, it remains unclear how lineage specific differences in various NB populations are established. In this study, I investigate the *Drosophila* ortholog to Pax6, Eyeless (Ey). Ey is a transcription factor, and master regulator of eye development, but is also expressed in a subset of NBs called mushroom body neuroblasts (MBNBs) and their progeny. I found that Ey regulates temporal patterning through late factors Syncrip (Syp) and Ecdysone-induce protein 93 (E93). Ey also regulates ecdysone receptor (EcR) to control the termination of MBNBs. Additionally, I find that the activity of an autophagy related gene, Mitf is reduced when Ey expression is knocked down. Ultimately, these findings provide

insight into the role of Ey in regulating the MBNB neurogenic period and suggests how autophagy may be initiated at the end of development.

**Key Terms:** Eyeless, Pax6, neural stem cells, temporal patterning, mushroom body neuroblasts, Imp, Syp, E93, EcR, Mitf, autophagy, neural development, neurogenesis

## Introduction

Stem cells are the fundamental building blocks of the developing organism and require strict regulation both intrinsically and extrinsically to properly form a functional system. Neural stem cells (NSCs) are of interest because if not regulated properly, uncontrolled overgrowth can occur or undergrowth, resulting in devastating neural developmental disorders like autism spectrum disorders (ASD), macrocephaly, or microcephaly (Parenti et al., 2020; Katsimpardi & Lledo, 2018; Courchesne et al., 2018). In addition, different neural stem cell subtypes give rise to morphologically and molecularly distinct neural progenies (Hartenstein & Wodarz, 2013). If these lineages do not form properly, incorrect cell types and progeny proportions can be altered, leading to improper cellular connections and disease. Understanding the genetic and molecular mechanisms at play that regulate neural stem cells and create lineage specific differences is key for a deep understanding of wildtype brain development.

To investigate the mechanisms at play in regulating NSCs, we utilize the fruit fly, *Drosophila melanogaster*. *Drosophila* NSCs, called neuroblasts (NB), are a great model for studying stem cell regulation as they are discrete in number, have a stereotyped division pattern, and have a host of genetic tools available with which they can be studied (Homem & Knoblich, 2012). *Drosophila* brain development also occurs solely during juvenile stages, with all NBs eliminated prior to adulthood, similar to mammalian NSCs (Doe, 2017; Islam & Erclik, 2022). This proliferative window as well as the high degree of conserved genes make *Drosophila* an excellent model to further understand stem cell proliferation and brain development.

In the *Drosophila* central brain (CB), there are ~100 NBs per hemisphere. The majority of these central brain neuroblasts (CBNBs) proliferate during embryogenesis, then enter a period of quiescence that requires responding to nutritional cues to reactivate the stem cells in early larval phases (Nassel et al., 2015). However, four CBNBs called mushroom body neuroblasts (MBNBs) are able to avoid nutrient dependent quiescence and continue proliferating while other NBs are dormant. These MBNBs also proliferate longer than other CBNBs, terminating in late pupal stages as opposed to early pupal stages (Ito & Hotta, 1992; Kunz et al., 2012). The MBNB progeny are called kenyon cells (KCs) and sequentially form a tri-lobed structure important for learning and memory called the mushroom body (MB) (Ito et al., 1997; Liu et al., 2015). A key question in *Drosophila* neurodevelopment is how these distinct lineages of NBs are regulated to experience different patterns of proliferation. All CBNBs occupy a similar cellular space in the CB and receive comparable extrinsic cues yet respond differently. To better understand what makes MBNBs unique from other CBNBs, we carried out a RNAi screen aimed at identifying important genes for regulating termination of MBNBs (Pahl et al., 2019).

One candidate from the screen was the gene Eyeless (Ey). Ey is a Pax6 homolog known for its role in eye development in the fruit fly, however it is also expressed in the CB. Ey is a transcription factor that gives MBNB their specific identity during embryogenesis (Callearts et al., 2001). Ey's ortholog Pax6 has been implicated in playing an integral role in mammalian brain development through NSC fate specification (Duan et al., 2013). Based on our preliminary work identifying Ey for its

role in terminating MBNB neurogenesis, I investigated how Ey contributes to regulating the neurogenic period of MBNBs in *Drosophila.*

Ey is a paired domain homeobox transcription factor that is expressed continuously in MBNBs from specification in early embryogenesis until their termination (Kurusu et al., 2000; Noveen et al., 2000). Upon knockdown of Ey, MBNBs experience a dual phenotype where some MBNBs are lost prematurely while others escape elimination and persist into adulthood (Pahl, 2018). When considering how this would be possible for a transcription factor with ubiquitous expression through the neurogenic period, we assess two distinct aspects of MBNB neurogenesis: temporal patterning and termination.

Temporal patterning refers to transient changes in gene expression that occur through neurodevelopment to allow a NB to produce progeny of different fates and identities (Doe et al., 2017). In MBNBs, the main temporal patterning cassette consists of two reciprocally inhibitory RNA binding proteins Insulin-like growth factor II (Imp) and Syncrip (Syp) (Liu et al., 2015). In MBNBs, the early factor Imp window is longer than in other CBNBs, allowing for an extended period of production of daughter KCs that form the gamma lobe of the mushroom body (MB) (Ito et al., 1997; Kurusu et al., 2000; Liu et al., 2015). Next, as Imp levels decrease and Syp levels increase in early pupal phases, MBNBs begin to produce alpha and beta prime neurons (Liu et al., 2015; Rossi & Desplan, 2020). Finally, in later pupal stages as Syp levels peak and Imp is no longer expressed, MBNBs produce their latest born progeny, alpha and beta neurons (Ito & Hotta, 1992; Lee et al., 1999; Liu et al., 2015). Temporal regulation of MBNBs is key to producing the different types of KCs that form the MB (Islam & Erclik, 2022).

While this cassette of Imp to Syp exists in other CBNBs, MBNBs experience high levels of Imp for a longer period of time (Liu et al., 2015). Extrinsic regulation of temporal factors also contribute to changes in gene expression (Rossi & Desplan, 2020). While the Imp to Syp cassette and the phenotypes that arise when these genes are disrupted have been fully characterized, it remains unclear what other factors may initiate the Imp to Syp transition as well as what maintains the longer Imp window (Liu et al., 2015). In other CBNBs changes in ecdysone hormone signaling help to trigger the switch between temporal factors, however it remains unknown what triggers this switch in MBNBs (Syed et al., 2017). This lack of knowledge provides an opportunity for further investigation, with an excellent candidate for establishing these distinctions in MBNBs and other CBNBs being Ey.

After MBNBs produce the ~2,000 KCs needed for a mature MB, they undergo termination through parallel pathways of autophagy and apoptosis (Siegrist et al., 2010; Pahl et al., 2019). While it is known that changes in ecdysone steroid hormone signaling and induction of a late temporal factor Ecdysone-induced protein 93 (E93) triggers elimination of MBNBs, what other genes may initiate autophagy and apoptosis inducing factors is still unknown (Pahl et al., 2019). Since Ey is a master transcriptional regulator, I sought to expand on previous work to assess Ey's role in promoting termination of MBNBs at the end of fly development (Pahl, 2018). In this work, I assessed the EyRNAi phenotype and uncovered that Eyeless plays a significant role in brain development through regulation of both temporal patterning and termination.

## Results

Eyeless knockdown causes persistence of MBNBs

Previous work has shown that Ey plays a role in controlling the timing of MBNBs elimination (Pahl, 2018). Using the Gal4-UAS system, Ey was specifically knocked down in neuroblasts using the driver Worniu (Wor) (hereafter referred to as NB Gal4) (Pahl, 2018). Typically, all MBNBs are eliminated in late pupal stages prior to eclosion into adulthood (Truman & Bate, 1988; Lee et al., 1999). The presence or absence of MBNBs at different pupal stages in these animals was recorded, and we found that compared to control, there was a significant increase in the number of *EyRNAi* MBNBs present at 84h APF (Fig 1A-B) (Pahl, 2018). In addition, some *EyRNAi* MBNBs were even able to persist 1 day into adulthood (Fig 1C) (Pahl, 2018). Based on this phenotype, I wanted to investigate the role Ey plays in regulating MBNB neurogenesis.

To begin to assess what Ey may regulate, I carried out a bioinformatics analysis to identify genetic targets. Currently, there is no chromatin immunoprecipitation sequencing (ChIP-seq) done on Ey targets of the CB. However, there are ChIP-seq analyses of Ey targets in the eye, and bulk-RNA sequencing of MBNBs (Yeung et al., 2018; Liu et al., 2015). Using *R*, I assessed overlap in the significant gene targets of Ey with the list of genes highly expressed in MBNBs. This resulted in a list of seven genes of interest. In order to determine how this would translate to conserved genes in mammals, I used a list of Pax6 target genes found in *Mus musculus* forebrain during neural development and compared it to the seven candidate genes of interest (Xie et al., 2013). This resulted in two candidates that are regulated by Ey, highly expressed in MBNBs, and conserved in mice: Syncrip and Prosap (Supp 2). Syncrip has a role in

temporal patterning that has been well documented (Liu et al., 2015). From this result I hypothesized Ey functions to regulate temporal patterning through the late factor Syp to control the MBNB neurogenic period.

Early temporal patterning is not controlled by Ey

To begin to tease apart the complexity of the Ey phenotype, I assessed how the loss of Ey affects temporal patterning. To do this, heat shock induced flp-frt clones were utilized (see methods & Supp. 4). *Hsflp;UASEyRNAi* virgin females were crossed to *act>>Gal4,UAS GFP* or *act>>Gal4, UAS RFP* males (Table 1). The resulting progeny upon heat shock have a mosaic brain where some NBs will be clones expressing *EyRNAi* and GFP/RFP while other NBs remain wild type.

First, the earliest temporal factor Imp was assessed by fixing and staining late larval phase brains with an Imp antibody at 72h ALH (after larval hatching) and 96h ALH (Fig 2A-D). Compared to controls, there was no difference in Imp expression in the *EyRNAi* clone, with 100% of MBNBs expressing Imp. Next, early pupal phases were assessed, when Imp levels should be diminishing as Syp levels increase. At 24h APF (after pupal formation) there was an observed difference in Imp expression between control and clones with 22% of control MBNBs and 100% of clone MBNBs positive for Imp (Fig 2E-F). Finally, when observing Imp expression at 48h APF, I found that Imp was diminished in the control MBNB as only 42% of MBNBs are positive for Imp in the NB (Fig 2G). However, in the *EyRNAi* clone MBNB, Imp remained present in 100% of the NBs (Fig 2H, 2O).

To follow up on this finding, another tool to visualize Imp activity in the form of an *ImpGFP* protein trap was utilized. At 48h APF in controls, *ImpGFP* was decreased in the MBNB, and *ImpGFP*+ progeny had been pushed away from the MBNB due to birth of Syp+ progeny (Fig 2I). In the *EyRNAi* clone, *ImpGFP* was also decreased in the MBNB but the Imp+ progeny remained close to the MBNB (Fig 2J). Overall, the Imp window was not disrupted but appeared to be extended in *EyRNAi* clones.

Next, I wanted to assess if Syp expression was increasing at the proper time in *EyRNAi* clones. At 48hAPF, Syp expression, as assessed by Syp antibody staining, in control MBNBs and their progeny was high (Fig 2K). In *EyRNAi* clones, Syp was expressed in the NB, but greatly reduced in the progeny, with only 41% Syp+ *EyRNAi* clone progeny compared to 100% of the control progeny (Fig 2L, 2O). At 72h APF, Syp expression *EyRNAi* clone progeny begins to increase with 92% of clone progeny and 100% of control progeny positive for Syp (Fig 2N, 2O). Overall, these results indicate that Ey expression is important to maintain timing of Syp expression. Without Ey, Imp expression is prolonged leading to a delay in Syp.

To verify that this was a delay in Syp expression and not a cell cycle defect, I assessed if *EyRNAi* MBNBs proliferate at the same rate as control MBNBs. To do this, I cultured explants of 48h APF and 72h APF brain tissue with the thymidine analog Edu to measure proliferation according to Keliinui et al. (2022). Brains were cultured for four and eight hours after dissection in supplemented Schneider's media with Edu which incorporates into the DNA during S phase of cell cycle and indicated proliferation (see Methods). After culturing 48h APF brains for four hours *EyRNAi* clones produced 4.33 ± 0.21 average Edu+ progeny while controls produced 4.51 ± 0.17 average Edu+ progeny

(Fig 3A-B, 3G). After eight hours of culturing, 48h APF *EyRNAi* clones produced 6.83 ± 0.31 Edu+ progeny on average and controls produced 6.64 ± 0.20 Edu+ progeny (Fig 3C-D, 3G). Finally, 72h APF *EyRNAi* clones produced 6.0 ± 0.82 Edu+ progeny while controls produced 6.35 ± 0.28 Edu+ progeny after eight hours of culturing (Fig 3 E-F, 3G). Within each pair, there was no significant difference in the average number of Edu+ progeny (Fig 3G). These results indicate that *EyRNAi* clones and control MBNBs proliferate at similar rates, meaning that delays in Syp expression are not due to disrupted cell cycle. We therefore conclude that Syp expression is delayed at 48h APF in *EyRNAi* clones but is expressed by 72hAPF.

Late temporal patterning is controlled by Ey

The latest acting temporal factor, Ecdysone-induced protein 93 (E93), increases steadily through pupal phases in response to ecdysone steroid hormone signaling through the ecdysone receptor (EcR). E93 is key for initiating autophagy in MBNBs to begin termination pathways (Pahl et al., 2019). I hypothesized that if Ey were positively regulating E93, this could be why we see a phenotype of persistence into adulthood for some *EyRNAi* MBNBs. *EyRNAi* clones were assessed at 48h APF and I found that compared to controls, E93 was reduced which is consistent with previous work in the Siegrist lab (Pahl, 2018) (Fig 4A-B). At 72h APF, controls had high levels of E93 in the NB and progeny, but clones still had reduced expression overall (Fig 4C-D). Upon calculating the percentage of E93 positive MBNBs at 48h APF, I found that only 12.5% of *EyRNAi* clone MBNBs expressed E93 compared to 100% of control MBNBs (Fig 4E). At 72hAPF there was an even greater difference with only 8% of *EyRNAi* clone MBNBs positive for

E93 while 100% of control MBNBs were positive (Fig 4F). This significant reduction in E93 expression when Ey is knocked down could be the cause of delayed termination of MBNBs at the end of the neurogenic period as E93 is necessary to reduce PI3K levels leading to elimination.

E93 expression is dependent on stabilization by the late acting temporal factor Syp as well as expression of the EcR (Pahl et al., 2019). Since I had observed that Syp was decreased in *EyRNAi* and could be contributing to the decrease in E93, I then analyzed how *EyRNAi* affects expression of the EcR to further explain why we see reductions in E93. Again, using *EyRNAi* clones, at 48h APF, EcR expression was reduced in clones compared to controls (Fig 5A-B). The fluorescence intensity of EcR was quantified in the controls and clones, and a significant decrease in clone EcR fluorescence was found (Fig 6E). At 72h APF, *EyRNAi* clones showed a slight reduction in EcR expression but to a lesser extent than at 48h APF (Fig 6C-D). EcR fluorescence was quantified at 72h APF and no significant difference was found between *EyRNAi* clone and control MBNBs (*F*ig 6F). Overall, I found that loss of Ey signaling greatly impacts the expression of late acting temporal factor E93. I hypothesized this could be due to a decrease in EcR expression and I noted a significant decrease in EcR expression at 48h APF in *EyRNAi* clone MBNBs. Disrupted Syp, E93, and EcR expression could be the cause of persistence of MBNBs in *EyRNAi* animals. I next assess how *EyRNAi* affects cell death mechanisms in MBNBs.

Loss of Ey leads to defects in autophagy

Preliminary work has shown that loss of Ey results in defects in autophagy, quantified by a reporter for autophagic flux (Pahl, 2018). E93 expression is key to

initiating autophagy by reducing levels of PI3K in late pupal stages. Ey regulation of E93 could be the source of disrupted autophagy in *EyRNAi* conditions, but the identity of other genes Ey regulates that could play a role in termination remain unclear. Eyeless has been shown to regulate a master autophagy gene in the eye called microphthalmia inducing transcription factor (Mitf) in the eye (Bouche et al., 2016; Hallson et al., 2004). Mitf has been shown to induce autophagy in *Drosophila* and when knocked down in the brain, autophagy is impaired (Bouche et al., 2016). I verified Mitf's expression in MBNBs using an anti-Mitf antibody (Supplemental Figure 2). To assess the expression of Mitf through development as MBNBs approach termination, a well vetted fluorescent reporter for Mitf, 4MBox GFP was utilized (Zhang et al., 2015). This reporter makes use of Mitf's affinity to bind M-Box motifs by combining four tandem M-boxes with GFP.

Mitf activity was assessed through pupal phases starting at 48h APF where on average only 0.5 ± 0.2 MBNBs are GFP+ (Fig 6A). Mitf activity starts to increase at 60h APF with an average of 2.8 ± 0.2 MBNBs GFP+ (Fig 6B). This trend continues through 72h APF where 2.9 ± 0.46 MBNBs on average are GFP+, and peaks at 78h APF with an average of 3.5 ± .14 MBNBs positive for 4MBoxGFP (Fig 6C-D). The number of 4MBoxGFP positive MBNBs was quantified, and we found that there was a significant increase from 48h APF at each subsequent time point (Fig 6E). Mitf was also assessed in early development, at freshly hatched (FH) stages and 24h ALH. On average 1.9 ± .28 MBNBs are 4MBoxGFP positive at FH while only 0.13 ± 0.125 MBNBS are 4MBox GFP positive by 24h ALH (Supplemental Figure 3). Overall, I found that Mitf activity steadily increases through pupal phases as the MBNBs prepare for elimination prior to eclosion as expected.

I next assessed how Mitf activity changes under *EyRNAi* conditions. Using the flp-frt clonal system, I created a fly lineage that upon heat shock would produce clones that express *EyRNAi*, 4MBoxGFP, and RFP. 72h APF was chosen to be assessed as it is a pupal timepoint where 4MBox activity is high in control MBNBs. 4MBoxGFP was reduced in *EyRNAi* clones compared to controls at 72h APF (Fig 6F-G). 50% of control MBNBs were 4MBoxGFP positive at 72h APF compared to 33% of *EyRNAi* clones (Fig 6H). This result shows that Mitf activity is slightly affected by the loss of Ey signaling and could be another key contributor as to why *EyRNAi* animals experience defects in autophagy and persist into adulthood.

## Discussion

### Eyeless regulates MBNB late temporal patterning

Through this work, I have identified a novel role of the gene Eyeless in regulating the neurogenic period of MBNBs through both temporal factors and autophagy inducing genes. Eyeless is important throughout development starting in embryogenesis, being one of the factors that determines a MBNB's identity (Kurusu et al., 2000; Noveen et al., 2000). Eyeless is a factor ubiquitously expressed in MBNBs and their progeny through the neurogenic period but playing different roles at different points in development. In larval phases, Ey uncouples MBNBs from requiring nutritional cues to continue proliferation unlike other CBNBs (Sipe et al., 2017). Here, I've shown that Ey also functions during pupal phases to control late temporal patterning. When Ey is knocked down the temporal window is shifted and Syp expression is delayed. In addition, the late acting temporal factor E93 is not expressed as it typically is in control MBNBs. This is in

part due to Ey also regulating upregulation of EcR in late stages of development. In *EyRNAi* animals, EcR expression is greatly reduced at 48h APF but increases towards 72hAPF. EcR and Syp delays at 48h APF are likely why E93 expression is delayed, as they are both required factors for E93 in late pupal stages. This finding is of great interest as bioinformatic analyses have shown that Pax6 in mammals may regulate Syp as well to control neural development.

Eyeless regulates autophagy in MBNBs

I also assessed how Ey may contribute to cell death mechanisms through autophagy. When Ey is knocked down, there are defects in autophagy (Pahl, 2018). In addition, I have seen a reduction in the activity of master autophagy regulator Mitf without Ey, which I found steadily increases its activity through pupal phases in control MBNBs. Without Ey regulating Syp, EcR, and E93 the temporal cassette is extended, leading to delays in activation of autophagy machinery. Loss of eyeless also leads to decreased Mitf activity, further delaying autophagy and allowing some MBNBs to persist into adulthood (Fig 7). This work has provided deeper insight into how *Drosophila* NSCs are regulated through development by the interplay of master regulators, temporal factors, extrinsic signaling, and cell death mechanisms.

## Methods

### Fly Stocks:

Fly stocks utilized, and their source are listed in key resources. Stocks generated were verified by PCR, antibody staining, and dominant markers.

### Animal Husbandry:

All animals were reared in uncrowded conditions at 25°C. Animals were staged from freshly hatched for larval time points and white pre-pupae for pupal time points.

### Clonal Induction:

See Supplemental figure 4 for clone mechanism. Frt-flp clones were heat shocked at 37°C for 10-30 minutes during the first larval instar after staging at freshly hatched. Pupae were screened under a fluorescence microscope to assess if clones were produced.

### Brain Explant Cultures:

Brain explants were prepared according to Keliinui et al., 2022. In brief, supplemented Schneider's media (SSM) was made by adding fetal bovine serum (FBS), insulin, glutathione, glutamine, penicillin, and streptomycin. 48h APF and 72h APF animals were dissected in SSM. Explants were cultured for 4hrs and 8hrs in SSM with edu at 25°C then fixed with 4% PFA. Primary antibodies were applied for 24 hours and washed followed by Alexa-Fluor conjugated secondary antibodies for another 24 hours (Supplemental Table 2). Secondary antibodies were washed and the edu reaction was completed using the Invitrogen Click-iT Edu Proliferation Assay kit, followed by more washes. Brains were stored overnight in glycerol solution and then imaged. *EyRNAi*

clones and controls were quantified for edu+ progeny. These values were compared using a one-way ANOVA followed by a test of multiple comparisons.

**Immunofluorescence and Confocal Imaging:**

Larval, pupal, and adult brains were dissected according to Pahl et al., 2019. Dissected brains were fixed in a solution of 4% paraformaldehyde in PEM buffer for 20 mins (larvae) or 30 mins (pupae), followed by a series of washes in PBST (1X PBS + 0.1% Triton X-100). Blocking solution of 10% normal goat serum (NGS) in PBST was applied and tissues were stored at 4°C overnight. Primary antibodies were applied and washed followed by Alexa-Fluor conjugated secondary antibodies (Supplemental Table 2). Secondary antibodies were washed, and brains were placed in anti-fade glycerol solution overnight prior to imaging. Z-stacks were taken of each central brain hemisphere by a Leica SP8 laser scanning confocal microscope using a 63x/1.4 oil immersion objective.

**Bioinformatics:**

Data from the sources mentioned in Supplemental Figure 2 were downloaded and uploaded as a data frame in R. The library tidyverse was used for data transformation and analysis.

**Software and Data Analysis:**

Images were analyzed using Fiji ImageJ and processed using Adobe Photoshop. Figures were assembled using Adobe Illustrator. MBNBs were identified by nuclear dpn staining and superficial location along the dorsal surface of the brain.

Fluorescence intensity was measured in ImageJ using the freehand draw tool to circle the NB using the membrane marker scribble. The appropriate channel to be

analyzed was selected and measured using the measure tool. Total cell area, total intensity, nuclear area, and nuclear intensity for control and clone NBs were recorded in Graphpad Prism 9. Control and clone NB values from the same brain hemisphere were combined and averaged. The cell sizes across clones and controls were compared and found to be non-significant. The average nuclear intensity was divided by the nuclear area to create a value of average pixel intensity per micron. Microscope settings (laser intensity, gain, pinhole size) were kept consistent across images.

To identify differences in temporal factor expression, MBNB clones and controls were compared and binned as being positive or negative for the factor assayed in both the NB itself and in the NB progeny.

Sample sizes are listed within the bars of all charts. All data is represented as mean ± standard error of the mean and statistical significance was determined using unpaired two-tailed student's t-tests, paired two-tailed Student's t-tests, or one-way ANOVAs followed by tests for multiple comparisons in Graphpad Prism 9.

## Figures

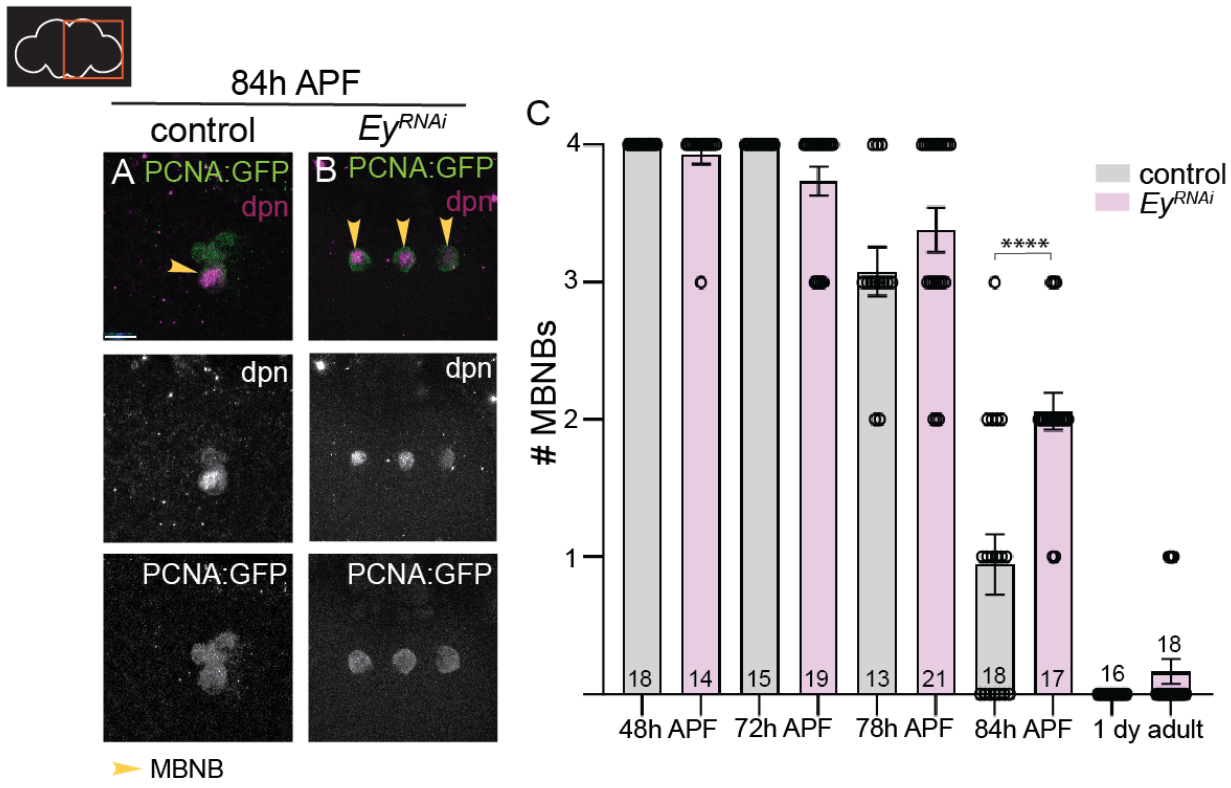



**Figure 1. Eyeless knockdown causes persistence of MBNBs.** Cartoon in top left indicates brain hemisphere imaged for this and all subsequent figures. (**A-B**) Maximum intensity projections of the right hemisphere of an 84h APF pupal brain. Scale bar = 10μm. Yellow arrowheads indicate MBNBs. Antibodies used are indicated in top right of each panel. (**A**) Control brain at 84hAPF show one dpn and PCNA:GFP positive MBNB. (**B**) *EyRNAi* brain at 84h APF shows three dpn and PCNA:GFP positive MBNBs. (**C**) Histogram represents the mean number of MBNBs present at the stated time points for each genotype. Each data point represents a separate brain hemisphere with the N represented within the bar. Error bars represent the SEM, alpha = 0.05, analyzed with a one-way ANOVA followed by a test for multiple comparisons. Significance is indicated with asterisks.

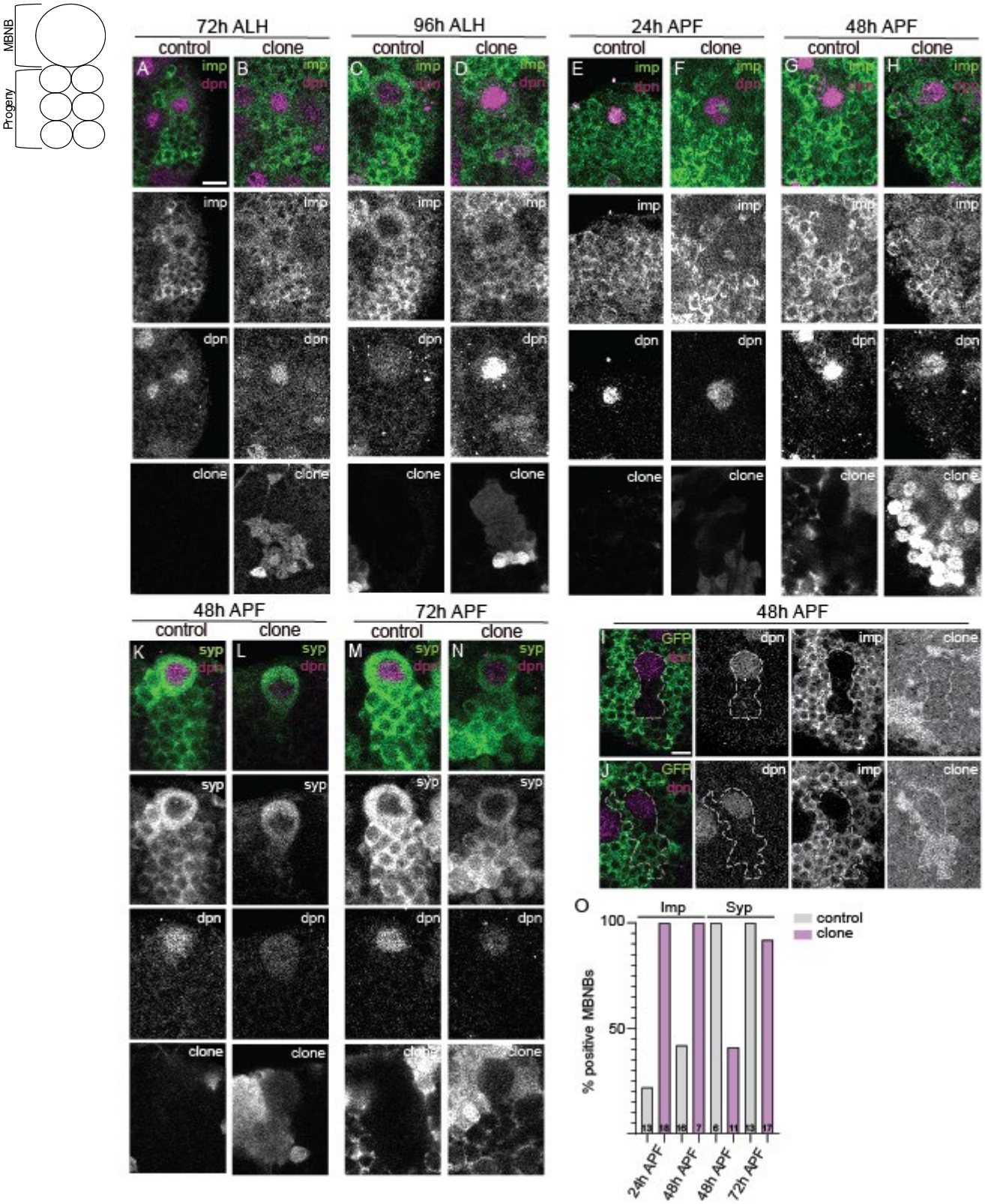

MBNB
Progeny
72h ALH
96h ALH
24h APF
48h APF
control
clone
imp
dpn
syp
GFP
clone
48h APF
72h APF
O
Imp
Syp
control
clone
% positive MBNBs
100
50
24h APF
48h APF
48h APF
72h APF

**Figure 2. Imp expression is not disrupted in *EyRNAi* clones, but Syncrip expression is diminished.** (**A-H**) Single z-stack images of isolated control MBNBs and *EyRNAi* clone MBNBs at various timepoints through larval and pupal development. Graphic in top left represents MBNB and its lineage. Scale bar = 10μm. Time points and genotype indicated above each column of panels. Antibodies used indicated at the top right of each panel. (**I-J**) Imp GFP expression across control and clone MBNBs at 48h APF. Antibodies indicated in top right of each panel. Scale bar = 10μm. MBNB and progeny outlined with white dashed line. (**K-N**) Single z-stack images of isolated control MBNBs and *EyRNAi* clone MBNBs at various pupal timepoints. Scale bar = 10μm. Time points and genotype indicated above each column of panels. Antibodies used indicated at the top right of each panel. (**O**) Percent of control and clone MBNBs and their progeny positive for either Imp or Syp (indicated at top of histogram). Number of MBNBs quantified represented by N within bars. Timepoints represented underneath histogram. Number of animals measured: (**A-B**) 10 animals, (**C-D**) 7 animals, (**E-F**) 8 animals, (**G-H**) 6 animals, (**I-J**) 5 animals, (**K-L**) 4 animals, (**M-N**) 9 animals.

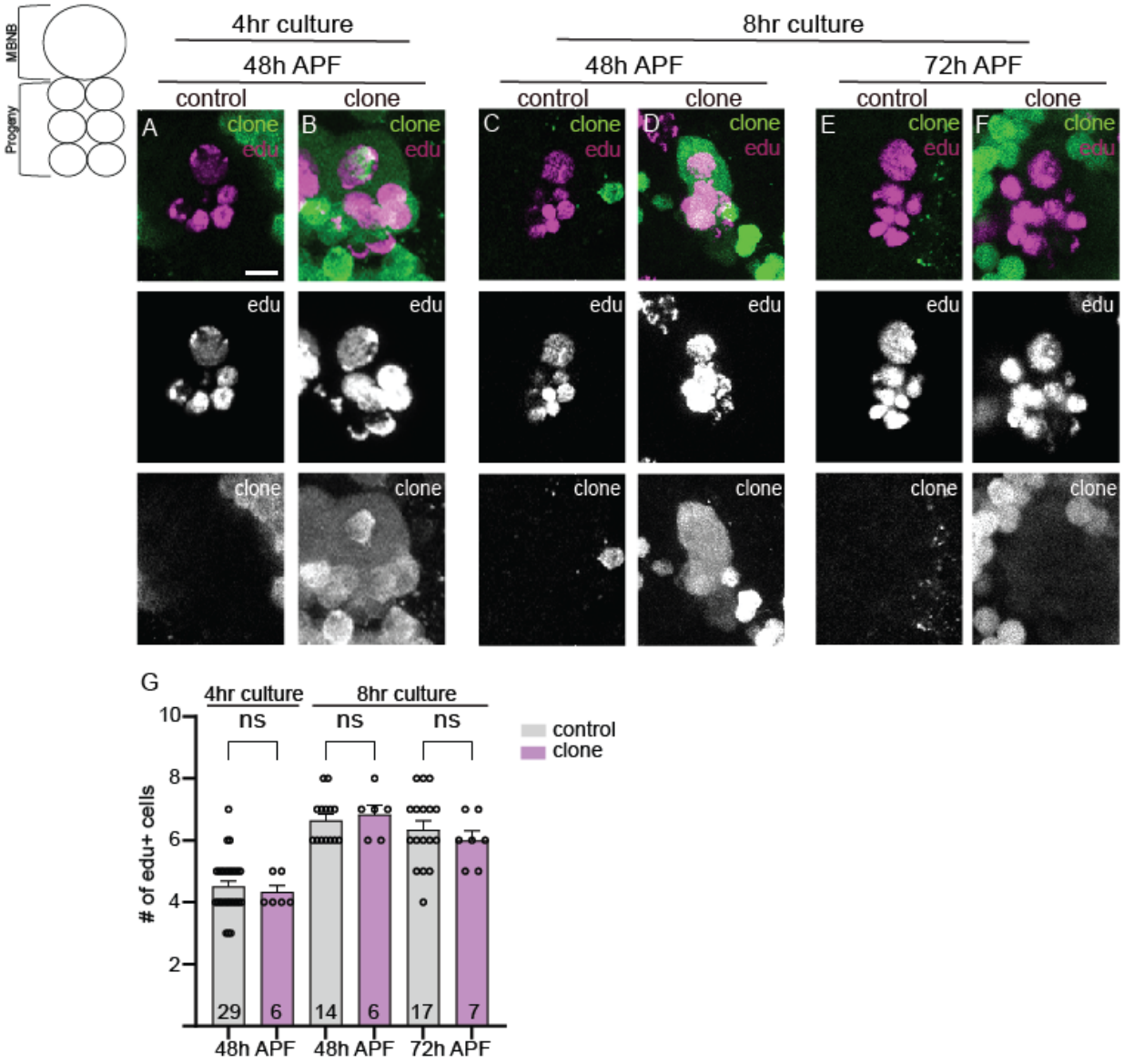



**Figure 3. *EyRNAi* clones and control MBNBs proliferate at the same rate.** (**A-F**) Single z-stack images of isolated control MBNBs and *EyRNAi* clone MBNBs cultured for 4hr or 8hrs at 48h APF and 72hAPF timepoints. Graphic in top left represents MBNB and its lineage. Scale bar = 10μm. Time points, length of culture, and genotype indicated above each column of panels. Antibodies used indicated at the top right of each panel. (**G**) Histogram of the number of edu+ cells of *EyRNAi* clones and controls cultured for 4 or 8 hours at 48h APF or 72h APF. Number of control MBNBs and clone

MBNBs shown as N represented within bars. Analyzed with one way ANOVA with test for multiple comparisons. Alpha = 0.05. Animals measured (**A-B**) 5 animals, (**C-D**) 7 animals, (**E-F**) 8 animals.

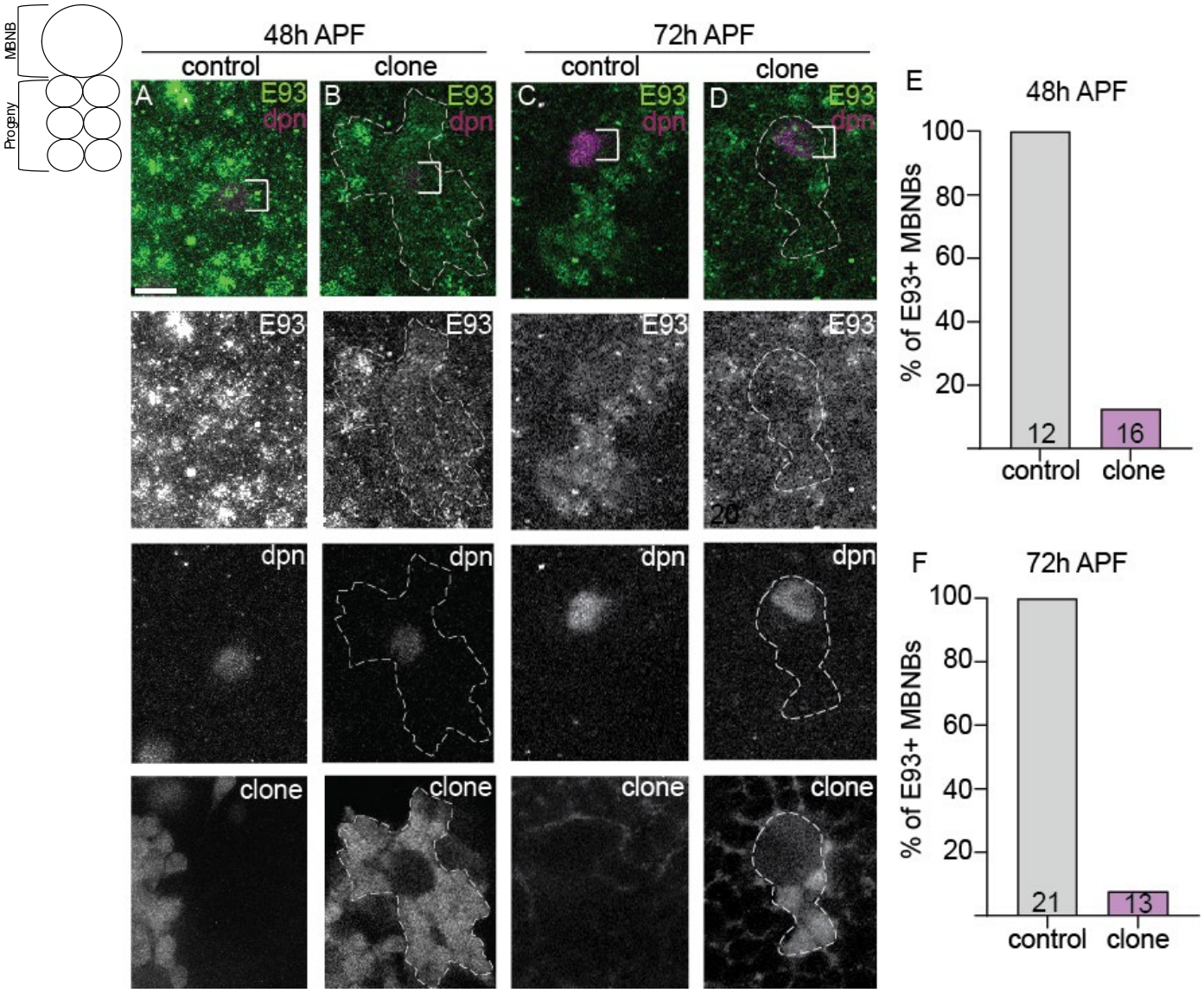



**Figure 4. E93 expression is diminished in late pupal stage *EyRNAi* clones.**

(**A-D**) Single z-stack images of isolated control MBNBs and *EyRNAi* clone MBNBs at 48h APF and 72h APF. Graphic in top left represents MBNB and its lineage. Scale bar = 10μm. Time point and genotype indicated at the top of each column of panels. Antibodies used indicated at the top right of each panel. *EyRNAi* clone MBNB and progeny outlined with white dashed line. MBNB indicated with white brackets. (**E-F**) Percent of MBNBs of each genotype that were E93 positive. The N for each group is

within the bar and represents the number of control or clone MBNBs quantified. Animals measured: (**A-B**) 5 animals, (**C-D**) 13 animals.

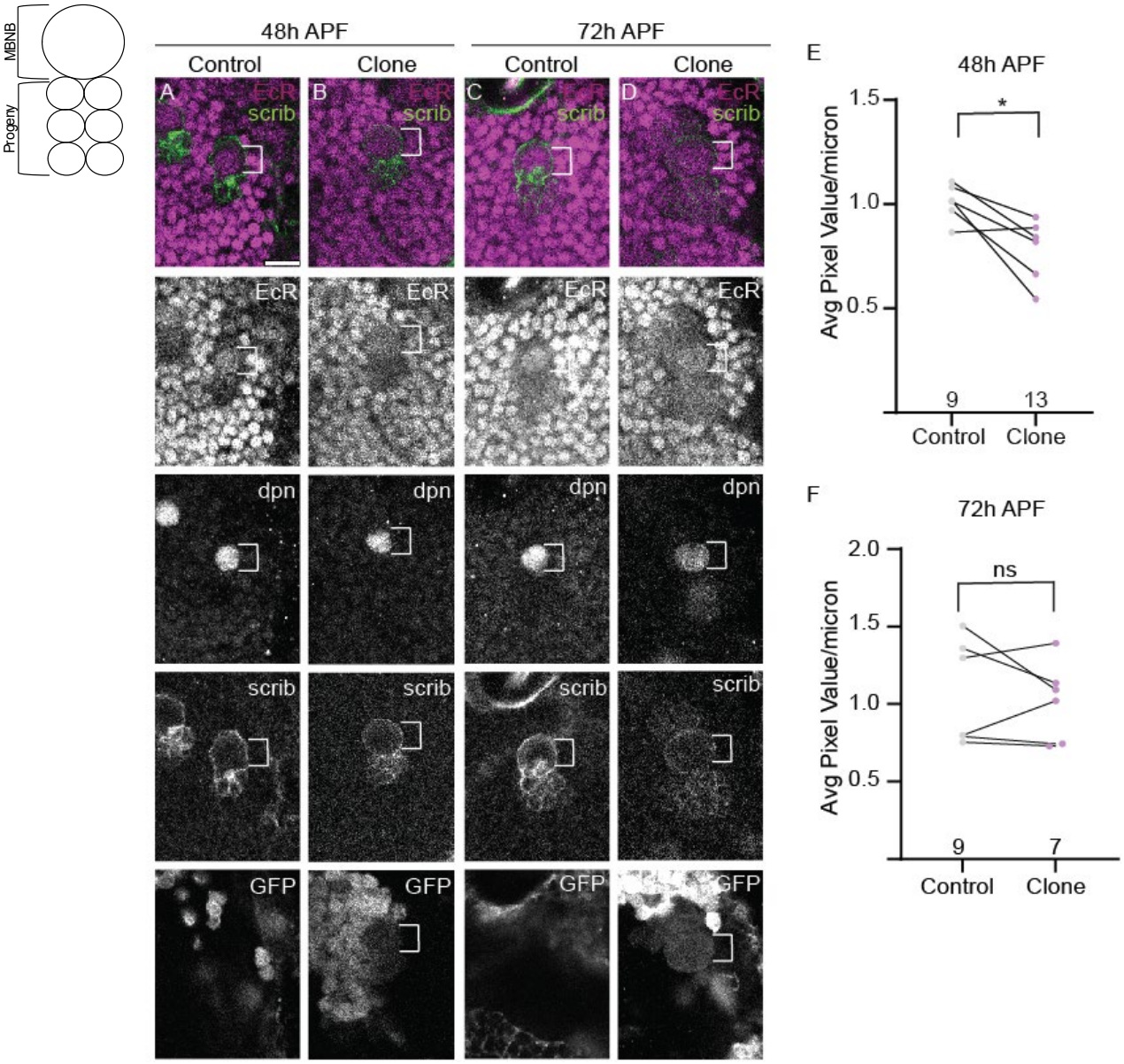



**Figure 5. EcR is reduced in late pupal stage *EyRNAi* clones.** (**A-D**) Single z-stack images of isolated control MBNBs and *EyRNAi* clone MBNBs at 48h APF and 72h APF. Graphic in top left represents MBNB and its lineage. Scale bar = 10μm. Time point and genotype indicated at the top of each column of panels. Antibodies used indicated at the top right of each panel. *EyRNAi* clone MBNB and control MBNB indicated with white bracket. (**E-F**) Average pixel intensity of EcR fluorescence measured in the nucleus of control and *EyRNAi* MBNBs per micron (see methods). Control and clone MBNBs from

the same brain hemisphere were compared and are shown by connecting lines. N = number of control and clone MBNBs measured. Analyzed with a paired student t-test. Alpha = 0.05. Significance indicated with asterisks. Animals measured by timepoint: (**A-B**) 6 animals, (**C-D**) 6 animals.

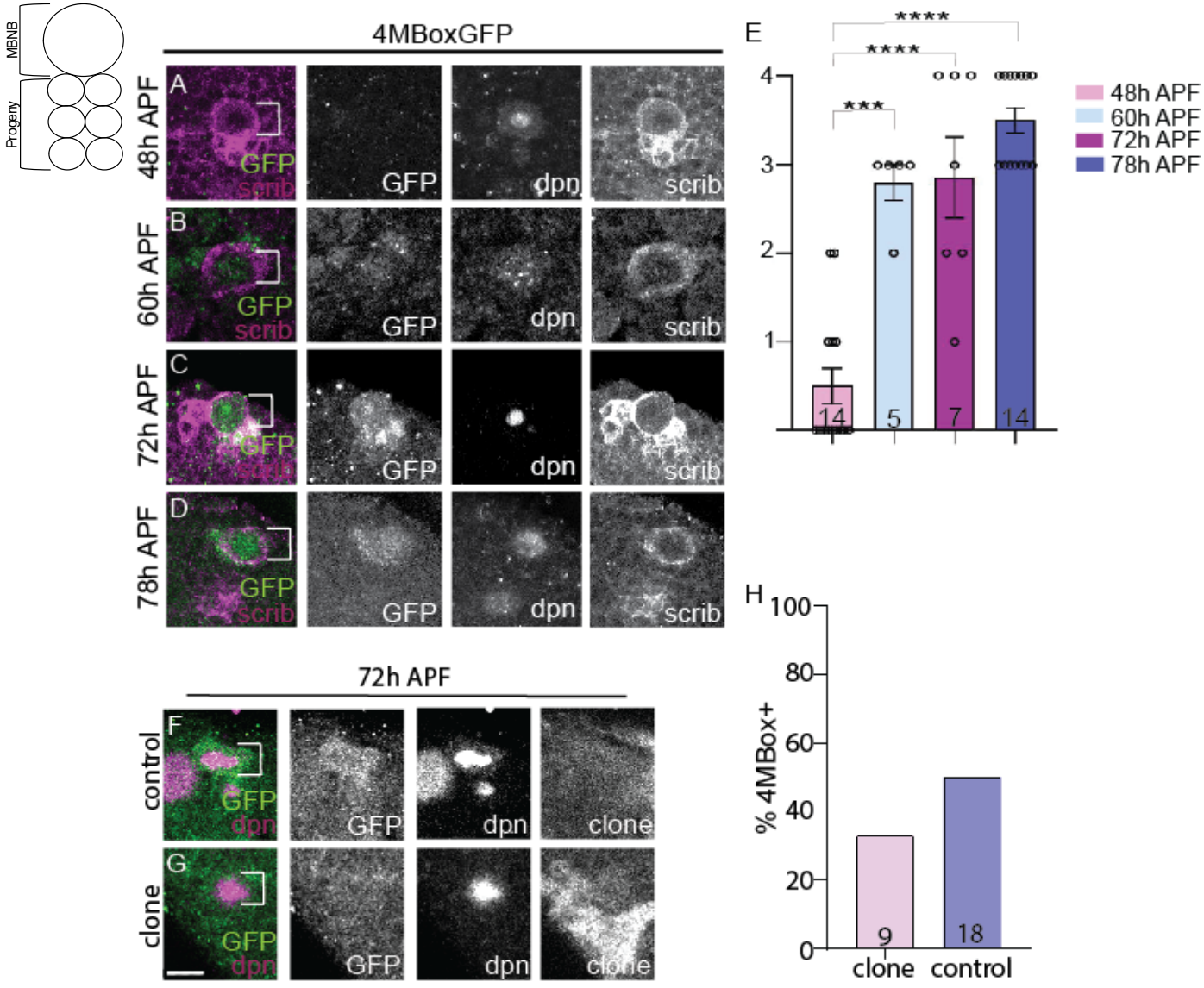



**Figure6. Mitf activity increases through pupal phases and is diminished with *EyRNAi*.** (**A-D**) Single z-stack images of isolated MBNBs at various timepoints through pupal development. Graphic in top left represents MBNB and its lineage. Scale bar = 10μm. Mitf activity was visualized with the activity reporter 4MBoxGFP. Antibodies indicated in bottom right of each panel. Brackets indicate MBNB. (**E**) Histogram represents the mean number of MBNBs present at the stated time points for the *4MBoxGFP* genotype. Each data point represents a separate brain hemisphere with the N represented within the bar. Error bars represent the SEM, alpha = 0.05, analyzed with

a one-way ANOVA followed by a test for multiple comparisons. Significance is indicated with asterisks. (**F-G**) Single z-stack images of isolated clone and control MBNBs at 72h APF. Scale bar = 10μm. Mitf activity was visualized with the activity reporter 4MBoxGFP. Antibodies indicated in bottom right of each panel. Brackets indicate MBNB. (**H**) Percent of 4MBox GFP positive *EyRNAi* clones compared to controls. Number of animals measured: (**A**) 8 animals, (**B**) 3 animals, (**C**) 5 animals, (**D**) 9 animals, (**F-G**) 9 animals.

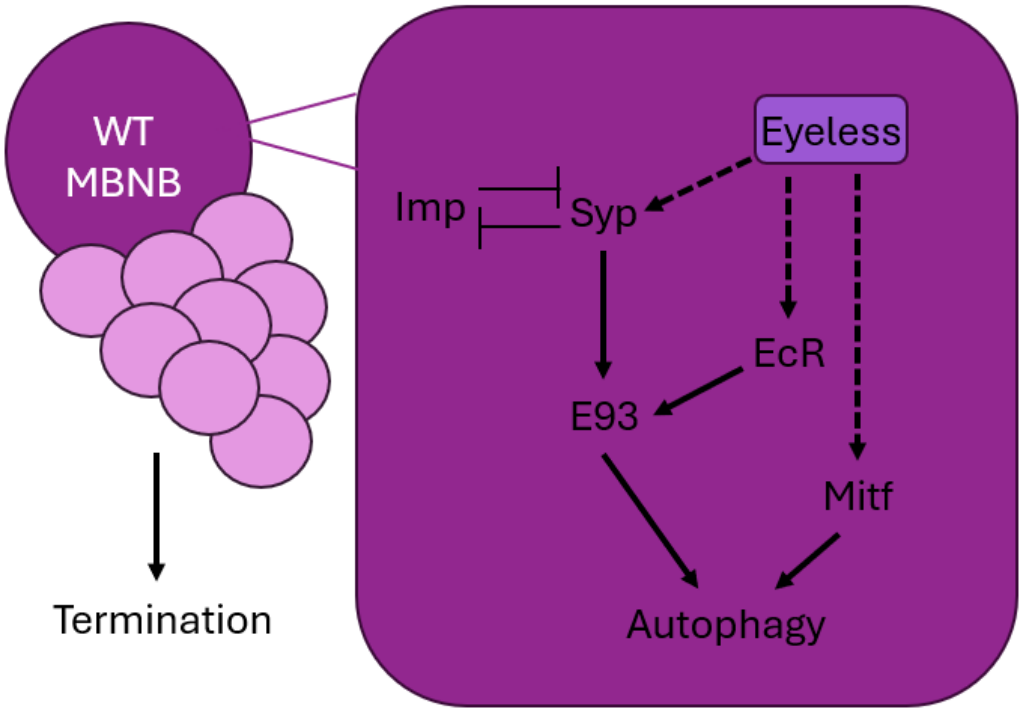
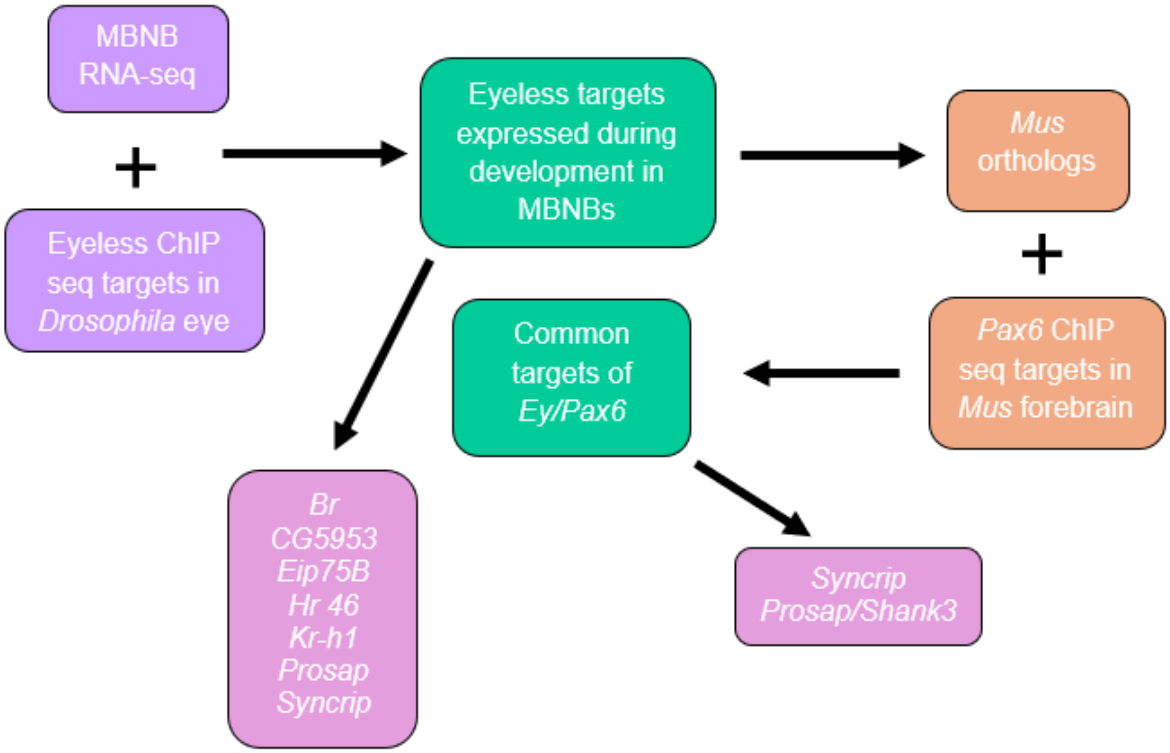
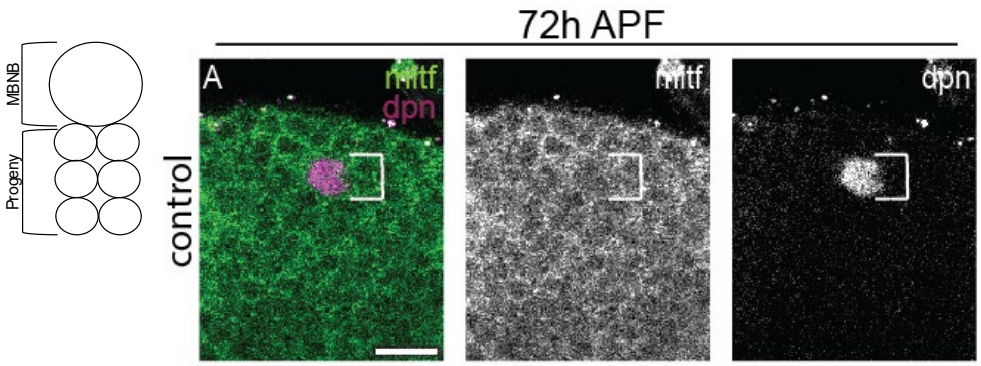



**Figure 7. Eyeless regulates the MBNB neurogenic period through temporal patterning and autophagic cell death.** Eyeless is a master regulator that controls Syp and EcR during pupal phases to progress the temporal cassette forward. Ey also regulates Mitf activity at the end of pupal stages to properly advance the MBNB towards autophagy and subsequent termination. In the absence of Ey, MBNBs do not terminate on time and experience defects in autophagic cell death.

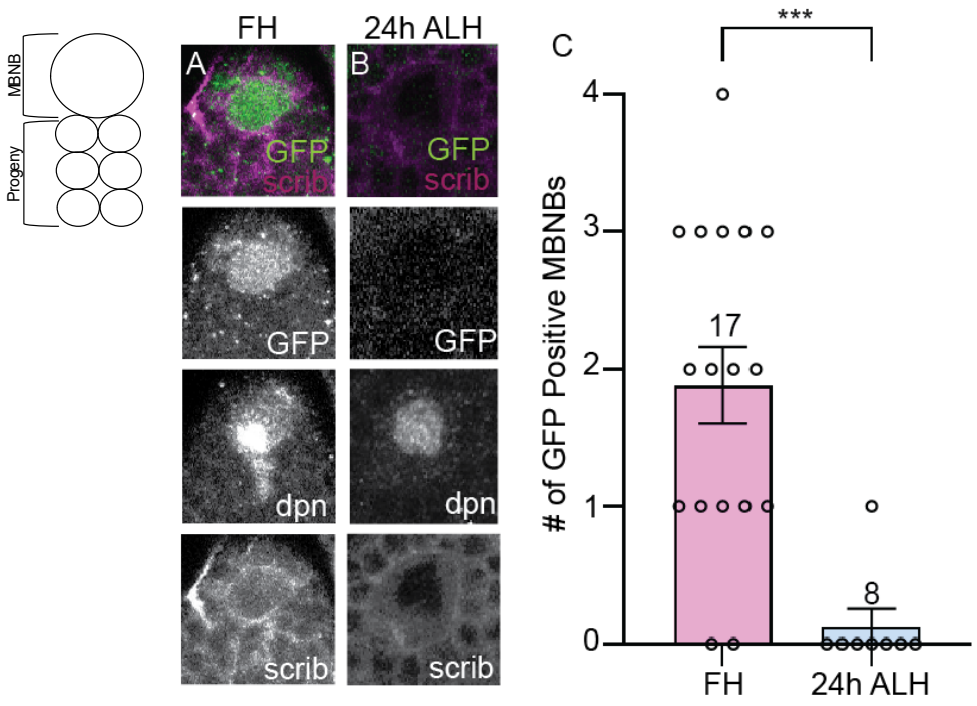
## Supplemental Figures



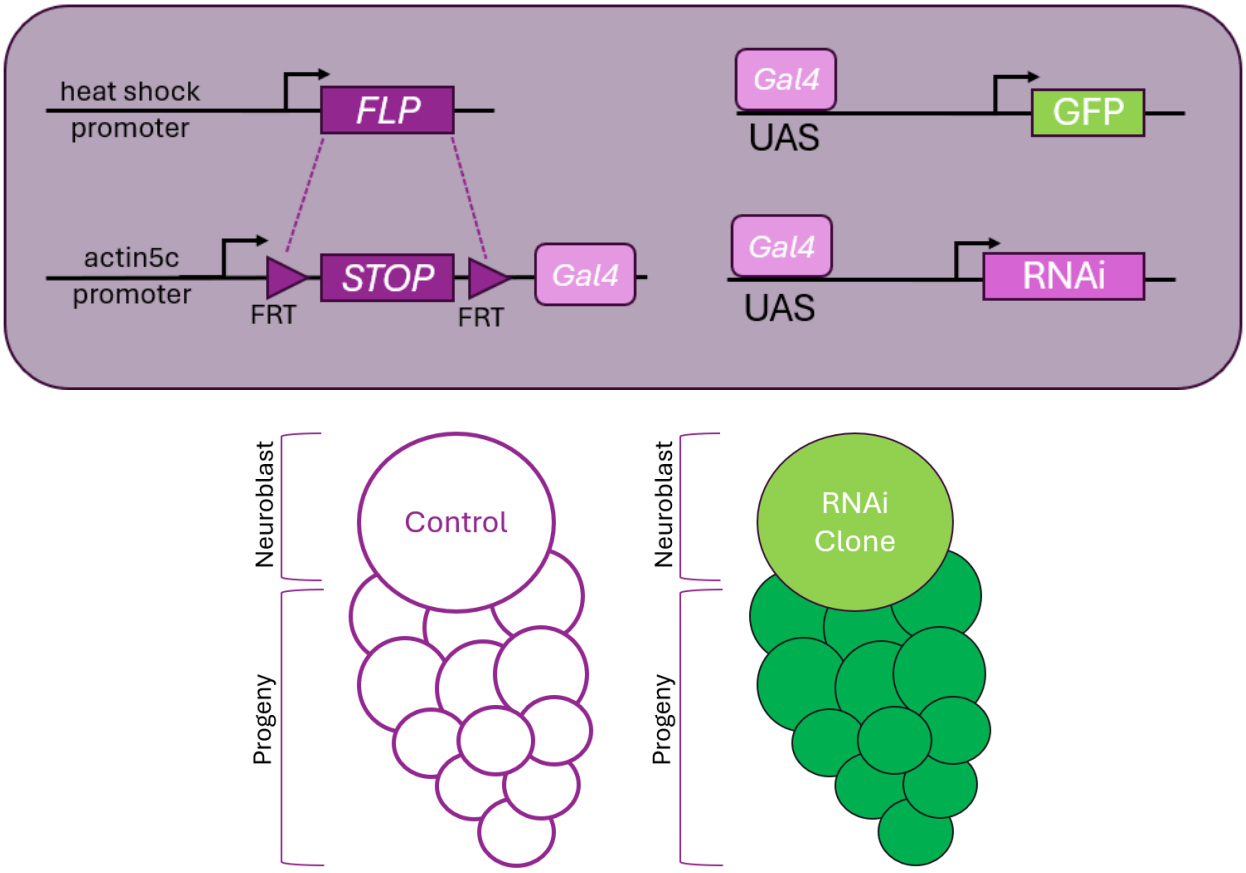

**Supplemental Figure 1. Bioinformatics pipeline to identify candidates regulated by Ey/Pax6.** Eyeless ChIP-seq data from Yeung et al., 2018 was combined with MBNB bulk RNA-seq from Liu et al., 2015 to identify gene targets of *Ey* in the *Drosophila* eye that are highly expressed during pupal phases in MBNBs. This resulted in a list of 7 unique genes. This list of candidates was converted into *Mus musculus* orthologs and compared to *Pax6* target genes found in a ChiP-seq of mouse forebrain by Xie et al., 2013. The resulting genes found as common targets of *Ey* and *Pax6* that are highly expressed in the *Drosophila* MBNBs were *Syncrip* and *Prosap* (*Shank3*). Analysis performed using R and bash.




**Supplemental Figure 2. Mitf antibody staining in MBNBs at 72hAPF.** (A) Single z plane image of a control MBNB at 72hAPF. Antibodies used indicated in top right of panel. MBNB indicated with white brackets. Scale bar = 10μm. Animals measured = 8.




**Supplemental Figure 3. Mitf activity decreases in the first 24 hours in control animals.** (A) Single z plane image of 4MBox GFP positive MBNB in freshly hatched brain. (B) Single z plane image of a 4MBox GFP negative MBNB in a 24h ALH brain. Antibodies indicated in bottom right of each panel. (C) Quantification of the number of 4MBox GFP positive MBNBs in FH compared to 24h ALH Each data point represents a separate brain hemisphere with the N represented above the bar. Error bars represent the SEM, alpha = 0.05, analyzed with a one-way ANOVA followed by a test for multiple comparisons. Significance is indicated with asterisks. Number of animals measured: (A) 9 animals, (B) 5 animals.




**Supplemental Figure 4. Heat-shock flp-frt clone mechanism.** RNAi clones were made by combining a heat-shock inducible flippase to an actin5c promoter containing Gal4 with a frt-site flanked stop codon. This stop codon prevents Gal4 expression. Upon heat shock, the flippase enzyme becomes active, cleaving out the stop codon by binding to its flanked frt sites. This allows Gal4 to activate binding UAS sequences to drive transcription of fluorescent protein and RNAi of interest. This produces a mosaic brain where some NBs remain wild type controls and others express the RNAi and GFP in the NB and its lineage.

**Table 1. Genotype by Figure**

| Figure | Genotype |
|---|---|
| 1A | *WornGAL4/+ (Oregon R); pcnaGFP/ + (Oregon R)* |
| 1B | *WornGAL4/+; pcnaGFP; UAS-EyRNAi* |
| 2A-H, 2K-N, 3A-E, 4A-D, 5A-D, Supp 3, Supp 4 | *hsFlp; UAS-EyRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-GFP* |
| 2I-J | *hsFlp; ImpGFP; UAS--EyRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* |
| 6A-D, Supp 2 | *4MboxGFP/4MBoxGFP (II)* |
| 6F-G | *hsFlp; 4MboxGFP/4MBoxGFP; UAS--EyRNAi/Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* |

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Preliminary data (Fig 1, Fig 6A-B) generated by Dr. Matthew Pahl and Dr. Susan Doyle. Assistance from Carina Karp on generating data (Fig 6C-H, Supp 3). All other data and all figures generated by KRB. Experimental design and conceptualization by KRB and SES. Manuscript written and edited by KRB with edits and input from SES. Thank you to the Francesca Pignoni lab for providing reagents to study Mitf expression and activity. Thank you to Dr. Bharath Sunchu for assistance with bioinformatics analysis in supplemental figure 1. Thank you to Dr. Susan Doyle for guidance with brain-explant cultures performed in Figure 3 and editorial advice on this manuscript. I would like to thank all members of the Siegrist lab for their suggestions and comments on this project and resulting manuscript.

**Key Resources**

| Reagent | Source | Identifier |
|---|---|---|
| **ANTIBODIES** | | |
| rat ant-Dpn (1:100) | Abcam | ab195173 |
| chicken anti-GFP (1:500) | Abcam | ab13970 |
| rabbit anti-dsRed (1:1000) | Clontech | 632496 |
| rabbit anti-Scribble (1:500) | Gift from Chris Q. Doe | |
| mouse anti-Dlg (1:40) | Developmental Studies Hybridoma Bank | 4F3 |
| guinea pig anti-E93 (1:250) | Gift from Chris Q. Doe | |
| mouse anti-EcR (1:50) | Developmental Studies Hybridoma Bank | AB_10683834 |
| mouse anti-Ey (1:100) | Developmental Studies Hybridoma Bank | AB_2253542 |
| rabbit anti-Imp (1:250) | Gift from Paul MacDonald | |
| rat anti-Imp (1:250) | Gift frorm Claude Desplan | |
| rabbit anti-Syncrip (1:250) | Gift from Chris Q. Doe | |
| guinea pig anti-Mitf (1:500) | Gift from Francesca Pignoni | |
| goat anti-chicken Alexa 488 (1:300) | Thermo Fisher Scientific | A32931 |
| goat anti-rat Alexa 555 (1:300) | Thermo Fisher Scientific | A48263 |
| goat anti-rat Alexa 647 (1:300) | Thermo Fisher Scientific | A48265 |

| | | |
|---|---|---|
| goat anti-rabbit Alexa 405 (1:300) | Thermo Fisher Scientific | A48254 |
| goat anti-rabbit Alexa 555 (1:300) | Thermo Fisher Scientific | A21428 |
| goat anti-rabbit Alexa 633 (1:300) | Thermo Fisher Scientific | A21071 |
| goat anti-guinea pig Alexa 488 (1:300) | Thermo Fisher Scientific | A11073 |
| goat anti-guinea pig Alexa 555 (1:300) | Thermo Fisher Scientific | A21435 |
| goat anti-mouse Alexa 405 (1:300) | Thermo Fisher Scientific | A48255 |
| goat anti-mouse Alexa 488 (1:300) | Thermo Fisher Scientific | A11001 |
| goat anti-mouse Alexa 555 (1:300) | Thermo Fisher Scientific | A32727 |
| **CHEMICALS** | | |
| SlowFade™ Diamond antifade reagent | Invitrogen | Catalog # S36963 |
| SlowFade™ Gold antifade reagent | Invitrogen | Catalog # S36937 |
| Normal Goat Serum | Thermo Fisher Scientific | Catalog # 31873 |
| Paraformaldehyde 16% solution EM grade | Electron Microscopy Sciences | Catalog # 15710 |
| Click-iT Edu Proliferation Assay | Invitrogen | Catalog # C10340 |
| Triton X-100 | Sigma | Catalog # T9284 |

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

| **EXPERIMENTAL MODEL: *Drosophila melanogastor*** | | |
|---|---|---|
| Oregon R | Bloomington Drosophila Stock Center | |
| *wor-Gal4* | (Albertson and Doe, 2003) | |
| *4MBoxGFP* | Gift from Francesca Pignoni | |
| *UAS Eyeless RNAi* | Bloomington Drosophila Stock Center | 32486 |
| *Imp-GFP* | Bloomington Drosophila Stock Center | 60237 |

| | | |
|---|---|---|
| *pcna-GFP* | (Thacker et al., 2003) | |
| *Hsflp (on X)* | Gift from Iswar Hariharan | |
| *Act5c-FRT-CD2-FRT-Gal4, UAS-RFP* | Bloomington Drosophila Stock Center | 30558 |
| *Act5c-FRT-CD2-FRT-Gal4, UAS-GFP* | gift from Iswar Hariharan | |

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# Chapter 4: Discussion and Future Directions

## Discussion

### Neural Stem Cell Regulation

Understanding the genetic and molecular underpinnings that regulate neural stem cell division (NSCs) is a key question in developmental biology. In order to properly treat neurodevelopmental diseases that arise either during development or later in life, we must first know how NSC proliferation occurs and what regulates this process. In this thesis, I have investigated two master regulators of neural stem cells, Notch (N) and Eyeless (Ey). Both of these genes and the regulatory networks they interact with are highly conserved throughout the animal kingdom (Austin & Kimble, 1989; Coffman et al., 1990; Stubbs et al., 1990; Xu et al., 1999). In addition, defects in Notch and Eyeless signaling have been shown to cause dysregulation of NSCs in mammals and flies (Clements et al., 2009; Grandbarbe et al., 2003; Hitoshi et al., 2002; Kammermeier et al., 2001; Manuel et al., 2015). While the relationship between Ey and Notch has been characterized in the eye, their relationship in the central brain (CB) and the central brain neuroblasts (CBNBs) had not yet been elucidated (Onuma et al., 2002). The findings I have shown here have provided a greater insight into *Drosophila* NSC regulation that can be translated to other organisms and provides expanded knowledge of the mushroom body neuroblasts (MBNBs).

### MBNBs versus other CBNBs

Of the *Drosophila* central brain neuroblasts, MBNBs experience a unique pattern of proliferation that is in part dictated by their temporal patterning (Ito et al., 1997; Ito &

Hotta, 1992; Liu et al., 2015; Rossi & Desplan, 2020). Notch signaling promotes the early factor Imp to maintain a longer window of proliferation early compared to other central brain neuroblasts (CBNBs) (Branham et al., 2024). A prolonged Imp window allows for the production of more early born MB neurons called gamma kenyon cells (KC) (Liu et al., 2015). As MBNBs continue dividing into pupal stages, shifts in temporal patterning from Imp to Syncrip occur allowing for production of middle-born alpha beta prime KCs (Liu et al., 2015). Finally, in late pupal stages as the levels of late factor Syp increase, the MBNBs produce the latest born alpha/beta prime KCs (Islam & Erclik, 2022). The prolonged period of MBNB proliferation to produce all the KCs required for a complete MB would not be possible if not for positive regulation of early factor Imp by Notch signaling.

MBNBs also differ from other CBNBS in how they end their neurogenic period through termination. Rather than experiencing cell cycle exit and terminal differentiation, MBNBs undergo parallel mechanisms of autophagy and apoptosis to terminate their divisions (Pahl et al., 2019; Siegrist et al., 2010). Again, this requires a precise interaction of extrinsic steroid hormone signaling, steroid hormone receptor expression in the form of EcR, and late acting temporal factor E93 (Pahl et al., 2019). While these factors all exist in both lineages of CBNBs, I have found that Eyeless functions in MBNBs to positively regulate EcR and E93. I also identify that Ey expression induces autophagy through activity of the downstream effector Mitf, allowing for MBNBs to experience a different form of termination from other CBNBs by degrading self-renewal factors.

Notch and Eyeless Interact to Regulate Neurogenesis

While many works have described the interaction of Notch and Eyeless in the development of the *Drosophila* eye, this work documents the genetic interaction between Eyeless and Notch in the central brain of *Drosophila* (Onuma et al., 2002). I found that when Ey is knocked down, there is a reduction in Notch activity as seen through the reporter enhancer of split GFP (E(spl)GFP) suggesting that Eyeless promotes Notch activity. Furthermore, when Eyeless is overexpressed in the CBNB lineages that do not typically have Ey signaling while also knocking down Notch pathway components, the Notch knockdown phenotype of ectopic persistence is partially lost. Thus, Ey overexpression is able to overcome the effects of *DIRNAi* in the non-MBNB CBNBs. Finally, I identified a lethal combination in the Ey null animal with the activated form of Notch (NICD). There is a strong genetic interaction between Ey and Notch that contributes to lineage specific differences between MBNBs and other CBNBs. This work has been expanded upon by temporally restricting the induction of the NICD through a temperature sensitive tubulin-Gal80 construct. This experiment involved rearing animals at 18C until pupariation then shifting animals to 29C. Taking into account the different rates of development at these temperatures, animals were to be dissected in late pupal phases to quantify the number of MBNBs present. Even when induction of NICD is temporally restricted, these animals were dying before reaching the desired late pupal stage. Further work would be required to further unravel the complex genetic interaction between Ey and Notch in late pupal development of MBNBs.

**Future Directions**

There remains potential to uncover how Eyeless regulates MBNB neurogenesis. It is still unknown how Ey controls the initiation of proliferation through temporal patterning. It is probable that ecdysone signaling plays a role but what else could be at play? In addition, while I have shown that Syp expression is dependent on Ey signaling, more evidence is needed to exactly identify the degree to which Syp is delayed in *EyRNAi* animals and in what manner Ey functions to regulate Syp. There are also some biases involved when assessing temporal factor expression as the presence or absence of a temporal factor is determined by qualitatively binning based on the visualization of a certain antibody or not. This protocol could be improved upon in the future by creating a standard metric for what is considered expression or lack of expression in both the MBNB itself and its progeny, allowing for better future reproducibility.

There also remain avenues to investigate rescue experiments to reverse the *EyRNAi* phenotype. Restoring late temporal factor expression of Syp or E93 could provide interesting results but would require intricate temporal regulation to only express these factors in late pupal stages. It could also be insightful to induce EcR expression in *EyRNAi* animals but again the timing of expression of this construct would need to be very tightly controlled.

Through this work, I observed a decrease in Mitf activity under *EyRNAi* conditions. While it is clear Ey plays a role in regulating Mitf, it remains unclear if this is a direct relationship, or potentially through a downstream effector like E93. It would be interesting to assess if Mitf activity is disrupted under *E93RNAi* conditions in late pupal stages. It would also be insightful to rescue the autophagy defects seen in *EyRNAi* by

overexpressing Mitf late in pupal stages. Again, this would require precise temporal regulation to express this construct at the ideal time point. This would provide novel information about the role Mitf plays in autophagy induction and answer the question of whether Mitf is capable of inducing autophagy in the absence of Ey.

Finally, there is clearly a strong interaction between Ey and Notch but it remains unclear at what level Ey regulates Notch. Future experiments to determine if Ey regulates the Notch receptor, its ligand Delta, or another component of this signaling pathway could further elucidate this genetic interaction.

**Conclusions**

This work provides novel insight into how Eyeless functions to regulate *Drosophila* NSCs through temporal patterning and identifies a previously unknown relationship between Ey and EcR. I also establish an interaction between Ey and Mitf activity outside of the *Drosophila* eye. It has previously been unknown what creates lineage specific differences between mushroom body neuroblasts and other central brain neuroblasts. I found that Ey functions to regulate Notch activity in MBNBs and has a very strong genetic interaction with Notch signaling during neural development. This work shows that Eyeless, a MBNB specific factor, regulates Notch signaling, temporal patterning, and autophagy to differentiate MBNBS from other CBNBs (Figure 1). Ultimately, I provide evidence that Ey functions as a MBNB specific factor to control temporal patterning and termination, shaping the neurogenic period of the MBNB.

**Figures**

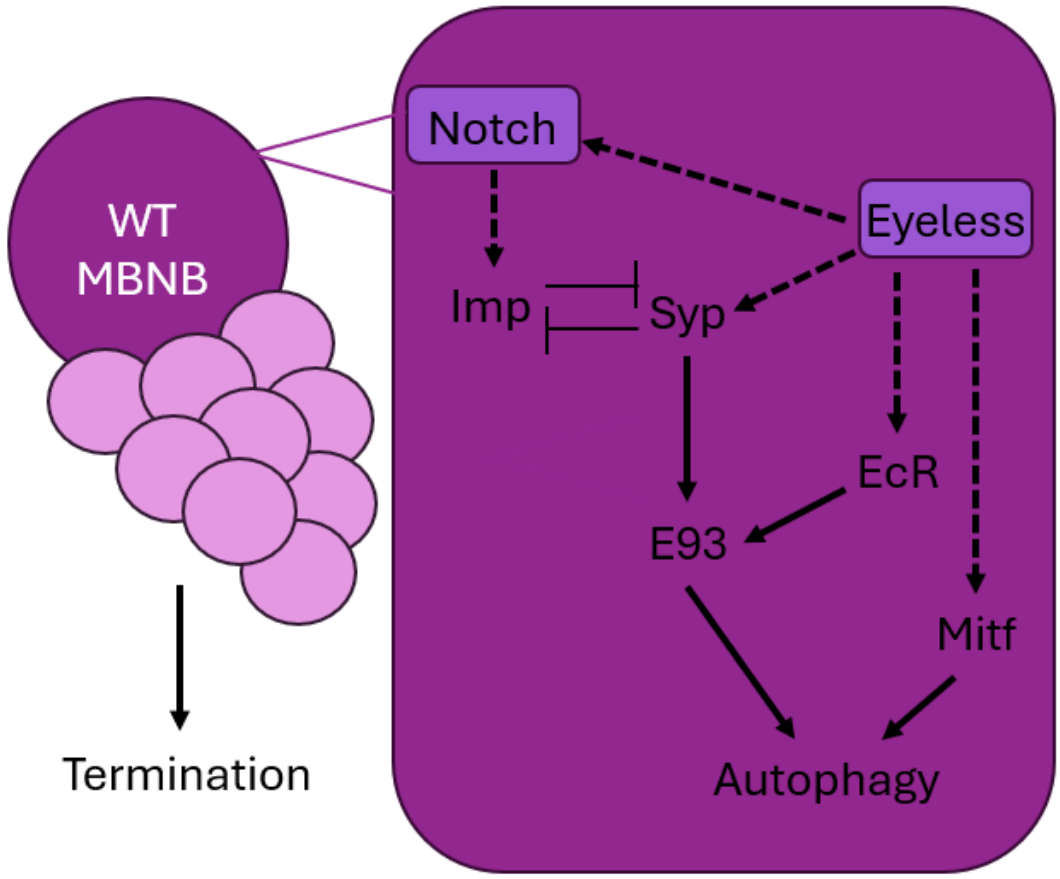


**Figure 1. Eyeless and Notch interact to regulate the MBNB neurogenic period differently from other CBNBs.**

MBNBs experience a longer proliferative window than other CBNBs. Lineage specific differences are established through Eyeless and Notch. Eyeless positively regulates Notch which maintains the early factor Imp window. Longer Imp expression allows for a longer proliferative period for MBNBs. Once the MBNBs have produced the progeny required to form the mushroom body, Ey then upregulates late factors E93 and EcR to begin the process of termination. Ey also positively regulates master autophagy gene Mitf to induce autophagy to end the neurogenic period.

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