

MAINTENANCE OF TRANSCRIPTION FACTOR
NETWORKS IN MATURE NEURONS

by

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ABSTRACT

The lifelong identity and function of a neuron is dictated by the set of effector genes it expresses in its terminally differentiated state. Neuron-specific effector gene expression is established and maintained by networks of transcription factors. How elaborate cascades of transcription factors establish neuronal identities during development has been studied extensively; however, how transcription factor networks are maintained in mature neurons to maintain effector gene expression remains poorly understood. I used the well-characterized transcription factor networks in *Drosophila* Tv1 and Tv4 neurons to further understand how transcription factor networks are maintained in mature neurons. I focused on the transcription factors Apterous and Dimmed, and investigated the *cis*- and *trans*- regulatory transcriptional mechanisms that govern initiation and maintenance of their expression.

I previously identified a 756 bp genomic region (*dimm.K1*) upstream of *dimmed* that is sufficient to initiate and maintain reporter expression in Tv1 and Tv4 neurons. Here I characterized the *trans*-regulatory inputs of *dimm.K1* reporter expression. I found that different transcriptional inputs are required for initiation vs. maintenance of *dimm.K1* reporter expression. Further, inputs required for maintenance differ not only between cell subtypes (Tv1 vs. Tv4) but also between individual cells of the same subtype (Tv1 neurons in thoracic segments 1 and 3).

I also compared *apterous* initiation and maintenance. My previous work had identified a 3.845 kb genomic region (*ap.K1*) upstream of *apterous* that is sufficient to initiate, but not maintain, reporter expression in Tv1 and Tv4 neurons. In this thesis I show that the addition of a region with putative TRE activity to the *ap.K1* initiation element is sufficient to add maintenance of reporter expression in Tv1 and Tv4 neurons.

Overall, I show that a variety of mechanisms can maintain the expression of transcription factors in mature neurons, and that these are different and more varied than the mechanisms of

initiation. Furthermore, for any single transcription factor, the initiation mechanisms that are shared by different cell subtypes can diverge into diverse cell-specific maintenance mechanisms.

PREFACE

All work was performed in Dr. Douglas Allan's lab at UBC.

Dr. Douglas Allan and I conceived all experiments. Dr. Tianshun Lian cloned the putative *ap* enhancer transgene for Figure 18. Kate MacDonald, an undergraduate research assistant, established the transgenic line used in Figure 18. She also performed the dissections and assisted with the immunocytochemistry for Figure 18. I performed all other experiments and all data analysis.

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LIST OF ABBREVIATIONS

A1, A2, A5, A20, A45	Adult day 1, 2, 5, 20, 45
Ap	Apterous
Antp	Antennapedia
BMP	Bone Morphogenetic Protein
bp	base pairs
Cas	Castor
Col	Collier
CNS	central nervous system
CRM	<i>cis</i> -regulatory module
ctrl	control
Dac	Dacshsund
Dimm	Dimmed
Eya	Eyes Absent
GMC	Ganglion Mother Cell
Grh	Grainyhead
Hth	Homothorax
In	initiation
KD	knockdown
M	maintenance
nDsRed	nuclear-localized <i>Drosophila</i> sp. red fluorescent protein
nEGFP	nuclear-localized enhanced green fluorescent protein
Nplp1	Neuropeptide-like precursor 1
OE	over-expression
SEM	standard error of the mean
Stg17	stage 17 embryo
Sqz	Squeeze
TF	transcription factor
Th1, Th2, Th3	thoracic segment 1,2,3
TRE/PRE	Trithorax/Polycomb response element
TrxG/PcG	Trithorax/Polycomb-group
UCSC	University of California Santa Cruz
VNC	ventral nerve cord

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DEDICATION

For mom.

1. INTRODUCTION

1.1 Neuronal diversity

Mature neurons display a high degree of morphological and functional diversity, which can ultimately be attributed to the differential expression of terminal differentiation genes between neuronal subtypes. Terminal differentiation genes, also referred to in this thesis as ‘effector’ genes, are genes expressed throughout the lifetime of a neuron that define its functionality. Thus effector genes include those that encode neuropeptides, ion channels, neurotransmitter biosynthetic enzymes, and synaptic adhesion molecules [1]. Effector gene expression is established and maintained by networks of transcription factors [2,3]. How elaborate cascades of transcription factors direct neuronal differentiation and establish effector gene expression profiles during development has been the subject of many studies [4-9]; however, the mechanisms by which transcription factor networks are maintained over the lifetime of mature neurons to maintain effector gene expression remain poorly understood. The work presented in this thesis uses the well-characterized transcription factor networks of *Drosophila* Tv1 and Tv4 neurons to address this problem.

1.2 Transcription factors and *cis*-regulatory modules

In accordance with the central dogma of molecular biology, genes encoded by DNA are transcribed into messenger RNAs, which are then translated into proteins. This flow of genetic information comes full circle with the production of transcription factors. Transcription factors are proteins that influence the rates at which genes are transcribed, by either sequence-specific DNA binding or by interacting with DNA-bound factors. Transcription factors that bind DNA associate with sequence motifs of 6-12 base pairs (bp) (*cis*-elements) that can be clustered into areas spanning tens to thousands of nucleotide base pairs within non-coding regions of the

genome. Clusters of *cis*-elements are termed ‘*cis*-regulatory modules’ (CRMs). CRMs act as platforms for the recruitment of specific transcription factors that act combinatorially to regulate the spatiotemporal transcription of specific genes. CRMs are considered to be modular, in that genes typically have multiple CRMs, and each CRM recruits different combinations of transcription factors in order to generate specific spatial and temporal domains of gene expression. The effect of transcription factor complexes at CRMs can be to promote transcription (the transcription factor is considered an activator and the CRM is considered an enhancer), or to reduce transcription (the transcription factor is considered a repressor and the CRM is considered a silencer). Furthermore, whether *cis*- or *trans*- regulatory elements/factors act as enhancers/activators or silencers/repressors can be context-dependent; activity can be modulated by the presence of different interacting partners in different cell types and at different stages of development [10-14].

CRM-bound transcription factor complexes accomplish regulation of transcription initiation and/or elongation by either direct physical association with the basal transcription machinery (RNA Polymerase II and associated factors), and/or indirect association with the basal transcription machinery through the Mediator complex and/or through the recruitment of chromatin remodeling factors that govern DNA accessibility at the locus. Unlike promoters, CRMs can be located large distances from the transcription start sites of the genes they regulate and can function irrespective of orientation, and irrespective of their location 5’ or 3’ of the gene. Interactions between CRM-bound transcription factor complexes and promoters that are separated by large spans of the genome are facilitated by chromosome looping [15-17].

It should also be appreciated that non-coding RNAs (ncRNAs), including those transcribed from enhancer regions (eRNAs), have been shown to regulate transcription. There is evidence to support their involvement both in the formation of chromosome loops that facilitate enhancer-promoter interactions and in the recruitment of transcription factors and chromatin

modifying/remodeling complexes, however these mechanisms are not considered further in this study [18-23].

1.3 Neuronal specification and differentiation

Through the process of development, complex multicellular organisms are generated from a single cell. Essentially all cells in an organism have the same genome, with few exceptions; therefore it is the specific set of genes expressed in a cell that provides its unique identity and function within an organism. The terminally differentiated identity of a cell is established by the iterative refinement of gene expression profiles in the cells of its developmental lineage. How transcription factor cascades drive this process is described below, using the development of the *Drosophila* Ventral Nerve Cord (VNC) as an example.

A sheet of neuroectodermal cells located in the ventrolateral region of the *Drosophila* embryo gives rise to the VNC. These cells are patterned by the region-specific expression of ‘spatial selector’ transcription factors along the anterior-posterior and dorsal-ventral axes. As neuronal progenitor cells, called neuroblasts, emerge from this neuroectodermal sheet, the specific combination of spatial selectors expressed by each neuroblast combinatorially defines its identity and begins to establish its competence to generate specific types of neurons and glia. Neuroblasts then undergo a series of asymmetric divisions. At each division, another neuroblast and a second cell known as a ganglion mother cell (GMC) are generated. The GMC then divides once to generate two cells that can be either neuronal or glial. Throughout neuroblast lineage progression over multiple divisions, a second set of transcription factors known as ‘temporal selectors’ function to alter the competence of the neuroblast to generate different neuronal subtypes over time. These temporal selectors are expressed briefly (1-4 divisions) and sequentially. Temporal selectors integrate with spatial selectors, and together they lead to the generation of a great diversity of specific neuronal subtypes, by establishing diverse transcription

factor profiles in young post-mitotic neurons. In the post-mitotic neurons, the activities of specific combinations of these transcription factors ultimately leads to the expression of sets of transcription factors known as ‘terminal selectors’. Terminal selectors initiate the expression of effector genes and thereby complete the establishment of a neuron’s terminally differentiated state [8,24].

1.4 Neuronal maintenance

Neurons are particularly interesting from the perspective of long-term maintenance, because they are non-dividing cells that only exhibit a low rate of replacement in some adult nervous systems that is mostly restricted to particular regions [25-27]. Also, they are highly diverse, and the unique identity and function of a given neuron must be maintained throughout the life of an organism, notwithstanding the homeostatic changes in gene expression that many neurons undergo throughout life to tune gene expression to specific physiological demands [28]. It had historically been considered that, following the generation of neuronal subtypes and the initiation of effector gene expression, mature neurons become passively ‘locked-in’ to their differentiated states, a concept described by Blau and Baltimore (1991) [29]. More recent work has demonstrated, however, that the differentiated states of neurons must be actively maintained. More specifically, several examples across multiple model organisms illustrate the persistent requirement of developmental transcription factors for the maintenance of effector gene expression and thus neuronal identity and function [2,3]. In *C. elegans*, certain developmental transcription factors are required to maintain the cellular identities of their respective neurons: Unc-42 in ASH sensory neurons, Unc-3 in cholinergic motor neurons, Ast-1 and Ceh-43 in dopaminergic neurons [30-33]. In *Drosophila*, a combination of seven developmental transcription factors is required to initiate and maintain olfactory receptor expression in olfactory neurons; developmental transcription factors also ensure that the expression of distinct types of

rhodopsin genes remains restricted to specific photoreceptor neurons and that neuropeptide expression is maintained in Tv1 and Tv4 neurons [34-36]. In mice, developmental transcription factors are required to maintain neurotransmitter identities in serotonergic (Pet-1, Lmx1b), dopaminergic (Nurr1), and noradrenergic (Hand2, Phox2) neurons [37-41]. This concept has proven applicable to other cell types too as loss of the developmental transcription factors Pax5 or Foxa1 and Foxa2, for example, results in the de-differentiation of B cells and β -cells respectively [42-44].

1.5 Models for transcription factor maintenance

It is generally the terminal selectors that persist in mature neurons. Importantly, several lines of evidence have found that upstream transcription factors in the developmental cascade are often not expressed beyond the developmental period. In *C. elegans* AWA olfactory neurons, the Odr-7 maintains cell identity in the absence of its initiating input Lin-11 [45]. In *Drosophila* Tv1 and Tv4 neurons terminal selector networks maintain effector gene expression in the absence of their numerous initiating inputs [36]. In adult mouse serotonergic neurons the terminal selector Pet-1 maintains cell identity in the absence of its initiating input Gata-2 [46]. Thus, in order to achieve a better understanding of how neuronal identity is maintained, we must examine how the transcription factors, that persist in adulthood, are maintained in the absence of the developmental inputs that initiated their expression. Current models for how transcription factors and transcription factor networks are maintained in mature neurons include (1) auto-regulation, (2) cross-regulation, (3) regulation by maintenance-dedicated transcription factors and (4) maintenance by Trithorax Group (TrxG) proteins (**Figure 1**).

1.5.1 Auto-regulation

Auto-regulation (**Figure 1B**), whereby a transcription factor takes over the regulation of its own expression once initiated, is a simple and well-supported mechanism for transcription factor maintenance in the absence of developmental inputs. In *C. elegans*, the transcription factors that maintain the identities of various neuronal subtypes, such as Mec-3 in touch sensory neurons, Che-1 in ASE neurons, Odr-7 in AWA sensory neurons and Ttx-3 and Ceh-10 in cholinergic AIY interneurons, are all maintained through auto-regulation [45,47-49]. In mice the transcription factor Pet-1 auto-regulates in serotonergic neurons [38].

1.5.2 Cross-regulation

Cross-regulatory models (**Figure 1C**) involve transcription factors of a network taking over the regulation of each other's expression after development. Maintenance of Ttx-3 and Ceh-10 expression in the cholinergic AIY interneurons of *C. elegans* illustrates a simple example of cross-regulation and a situation in which auto-regulatory and cross-regulatory models intersect; auto-regulation of Ttx-3 and Ceh-10 is mediated by the cooperative binding of these two transcription factors at each locus [49]. An assessment of transcription factor occupancy at various transcription factor loci and developmental stages in mouse hepatocytes suggests that intricate cross-regulatory interactions are established after development. This extensive cross-regulation provides redundancy and helps explain why certain transcription factors, which are pertinent to the regulation of other transcription factors in their networks during development, are less important for maintaining expression of these transcription factors in mature cells [50]. Since certain transcription factors are not as imperative to the regulation of cell identity features in mature dopaminergic and serotonergic neurons as they are during development, the acquisition of complex cross-regulatory relationships within the transcription factor networks of mature neurons has been proposed as one explanation for such findings [2].

1.5.3 Maintenance-dedicated transcription factors

In models supporting a role for maintenance-dedicated transcription factors, transcription factors that were not present during the initiation of gene expression function thereafter in order to maintain gene expression (**Figure 1D**). In *C. elegans* ASE chemosensory neurons, Che-1, which is important for initiation and maintenance of effector gene expression, also initiates expression of the maintenance-dedicated transcription factor Ceh-36. Che-1 and Ceh-36 then collaborate to maintain the expression of effector genes [48]. Although this example provides evidence of a role for maintenance-dedicated transcription factors in sustaining effector gene expression, the concept could certainly be applied to the maintenance of transcription factor networks.

1.5.4 Maintenance by Trithorax-group proteins

Roles for Trithorax- and Polycomb-group (TrxG/PcG) proteins were first described with regard to *Drosophila* homeobox-family homeotic genes, which encode transcription factors that establish the *Drosophila* body plan along the anterior-posterior axis in the developing embryo. The transcription factors that establish the expression patterns of these homeobox genes are only expressed very transiently and TrxG/PcG proteins function to organize the chromatin environments of homeobox genes into “on” or “off” states, such that the appropriate expression patterns are maintained after the transcription factors that established the expression patterns are no longer present. TrxG complexes function to open local chromatin environments and thereby facilitate gene transcription. Conversely, PcG complexes facilitate tight packing of chromatin structures and thus serve to repress gene transcription. In *Drosophila*, multiple transcription factors have been implicated in the recruitment of TrxG or PcG members to CRMs known as Trithorax/Polycomb response elements (TRE/PREs). Importantly, TRE/PRE activities are dependent upon the spatiotemporal activities of the initiating CRMs with which they are paired,

and thus whether they participate in the maintenance of an active or repressed state is dependent upon the context-specific transcriptional states of the genes with which they are associated [51-57]. The roles of TrxG/PcG proteins are well-conserved to mammals; however, mammalian TRE/PREs are not well-defined [58]. Important in the context of this thesis is the fact that a role for TrxG/PcG proteins in maintaining transcription factor expression in long-lived post-mitotic cells such as neurons has not been established (**Figure 1E**).

Although section 3.9 of this thesis work investigates the requirement of a *cis*-regulatory region with putative TRE activity in the maintenance of transcription factor expression in mature neurons, the biochemical mechanisms of TrxG/PcG recruitment and function will not be addressed. These mechanisms are an active area of study and the reader is directed to recent reviews in order to appreciate the progress that has been made so far in elucidating their complexities [55,56,59,60].

Although each of the transcription factor maintenance models under consideration here have been discussed separately above, it is important to consider that various combinations of these strategies may be employed to maintain any single transcription factor network.

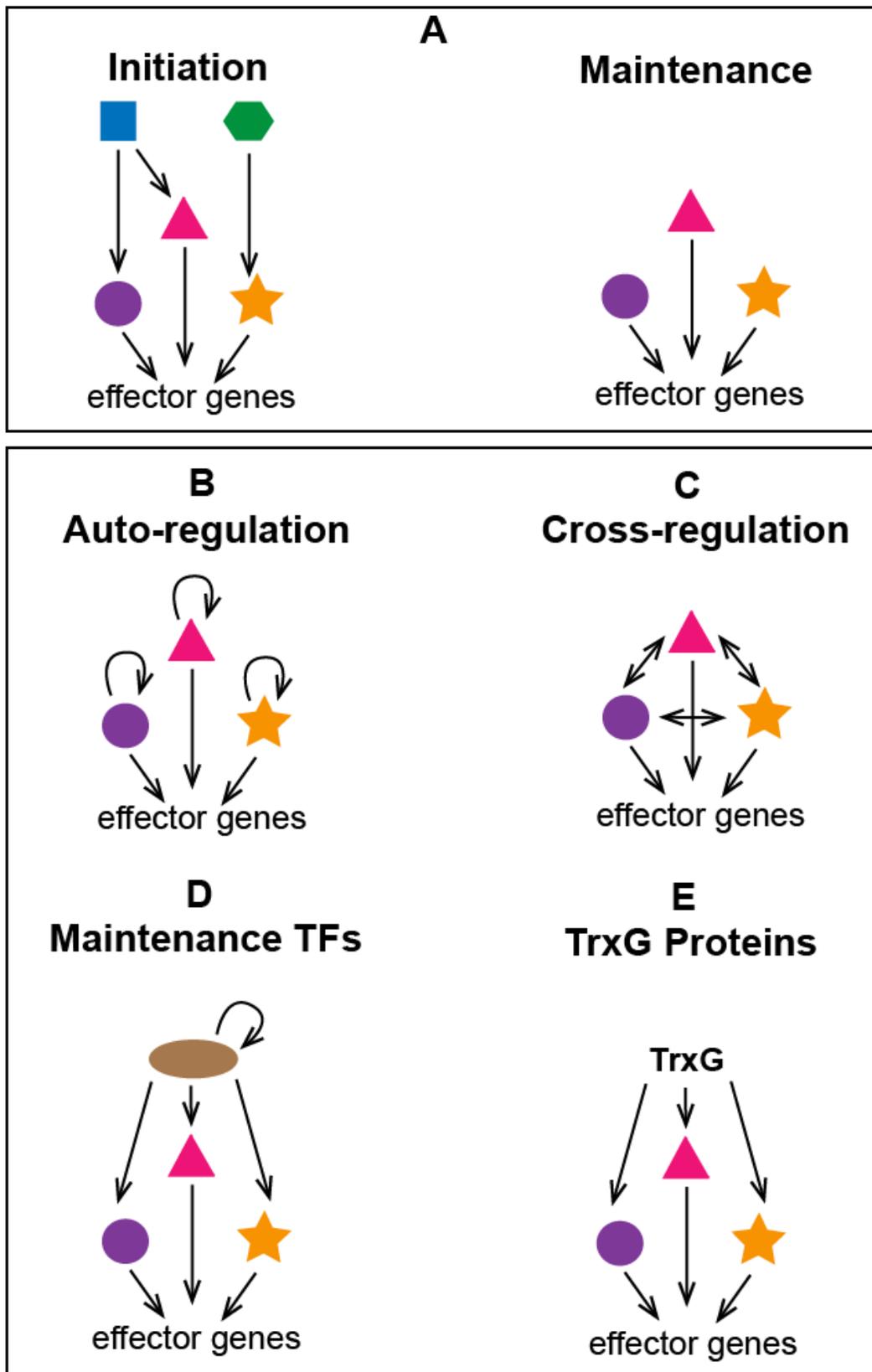


Figure 1.

Figure 1. Transcription factor maintenance models.

(A) Representations of hypothetical transcription factor networks directing initiation and maintenance of cell identity. The purple circle, pink triangle and orange star represent terminal selectors that regulate initiation and maintenance of effector gene expression. The blue square and green hexagon represent upstream transcription factors that initiate terminal selector expression but are not present after development. (B-E) Proposed models for how the transcription factors that persist in mature neurons are maintained in the absence of their initiating inputs. (B) Auto-regulation, whereby each transcription factor maintains its own expression. (C) Cross-regulation, whereby the transcription factors maintain each other's expression. (D) Stabilization of the network by maintenance-dedicated transcription factors (TFs) (brown oval). For simplicity the maintenance-dedicated transcription factor is shown to auto-regulate here, although other mechanisms could certainly be proposed. (E) Maintenance by TrxG proteins. TrxG proteins maintain open chromatin environments at each transcription factor gene locus.

1.6 Genomic organization of initiator and maintenance CRMs

The models discussed above address possible transcription factor maintenance mechanisms at the *trans*-regulatory level. In order to further our understanding of these mechanisms, however, we must also consider how the corresponding CRMs are organized within the genome.

If we are to assume that different sets of transcription factors are involved in initiating and maintaining gene expression, then this evokes a mechanism whereby distinct CRMs may be postulated. Indeed, identification of differences in the CRMs that are necessary and sufficient for initiation and maintenance provides a useful experimental tool to demonstrate that differences between initiation and maintenance mechanisms actually exist. More specifically, for a given transcription factor gene, we may consider whether the genomic regions of initiation and maintenance are contained within a single experimentally-definable CRM region (either at the exact same motifs or at closely spaced but separable motifs that may be difficult to discriminate without identifying distinct *trans*-acting mechanisms), or whether initiation and maintenance are mediated through experimentally-separable CRMs (and thus a distinct mechanism is evident). All *trans*-regulatory maintenance models discussed above could be compatible with each of these scenarios. Let us consider, for example, two cases of transcription factor maintenance by auto-regulation: the transcription factor BMP-2 auto-regulates through one of the CRMs involved in the initiation of its expression in mouse osteoblasts, whereas the transcription factor Pit-1 auto-regulates through a distinct maintenance-dedicated CRM in mouse pituitary cells [61,62].

1.7 *Drosophila* Tv neurons as a model system

This thesis uses Tv1 and Tv4 neurons as a model. These neurons are members of bilateral clusters of four neurons (the Tv neuron clusters) in each thoracic segment (Th1-3) of the *D.*

melanogaster ventral nerve cord (VNC). Tv1 and Tv4 neurons are of particular interest due to their highly specific expression of the effector neuropeptide genes Neuropeptide-like precursor 1 (Nplp1) and FMRF-amide (FMRFa), respectively. Expression of these neuropeptides is initiated during late embryonic stages and thereafter maintained throughout larval, pupal and adult life (**Figure 2**) [36]. Many groups have used expression of these neuropeptides as markers of the terminally differentiated identities of Tv1 and Tv4 neurons [63,64].

With these neuropeptide genes as terminal fate markers, the transcription factor cascades that specify Tv1 and Tv4 neurons and initiate the expression of these neuropeptides have become well-defined. In Tv1 neurons, the spatial selectors Antennapedia (Antp) and Homothorax (Hth) and the temporal selector Castor (Cas) initiate expression of the terminal selector Collier (Col) in post-mitotic neurons. Col, in turn, initiates the expression of the terminal selectors Apterous (Ap), Eyes absent (Eya) and Dimmed (Dimm). Ap and Eya also contribute to Dimm initiation. Col, Ap, Eya and Dimm initiate expression of Nplp1 (**Figure 3A**). In Tv4 neurons, the spatial selectors Antp and Hth, and the temporal selector Cas, initiate the expression of Col. Antp and Hth also initiate the expression of Dachshund (Dac), and Cas also initiates the expression of the temporal selector Grh, and the expression of Nab and Squeeze (Sqz). A series of regulatory interactions establishes the terminal selector network, which consists of Ap, Eya, Dimm, Dac and Sqz. Ap, Eya, Dimm, Dac and Sqz all initiate the expression of FMRFa. A target derived Bone Morphogenetic Protein (BMP) signal is also required to initiate the expression of FMRFa (**Figure 3B**) [63,65-69].

Work in our lab by Eade *et al.* (2012) contributed to the body of literature describing the requirement of developmental transcription factors for active maintenance of mature neuronal identities. Using immunocytochemistry and adult-specific RNAi knock-down, Eade *et al.* demonstrated that the Tv1 and Tv4 terminal selector networks persist in mature neurons and are

required to actively maintain neuropeptide expression. Most of the transcription factors that initiated expression of the terminal selectors, however, are not expressed after development. In adult Tv1 neurons, Antp and Cas are no longer expressed. Col, Ap, Dimm and Eya expression are maintained and all are required to maintain Nplp1. However, although Col is necessary to initiate Ap, Eya and Dimm, it is not required to maintain them (**Figure 3C**). In adult Tv4 neurons, Antp, Hth, Cas, Nab, Grh and Col are no longer expressed, but Ap, Eya, Dimm, Dac and Sqz are maintained, and in combination with BMP signaling, all maintain FMRFa (**Figure 3D**). It is not resolved how the transcription factors that persist in adult Tv1 and Tv4 neurons are maintained, as only Dimm has been shown to have persistent input from Ap (in Tv1 and Tv4) and Eya (in Tv1) from initiation through to maintenance (**Figure 3C,D**) [36,70].

The work performed for this thesis aimed to further our understanding of how the Tv1 and Tv4 terminal selector networks are maintained in the absence of their initiating inputs.

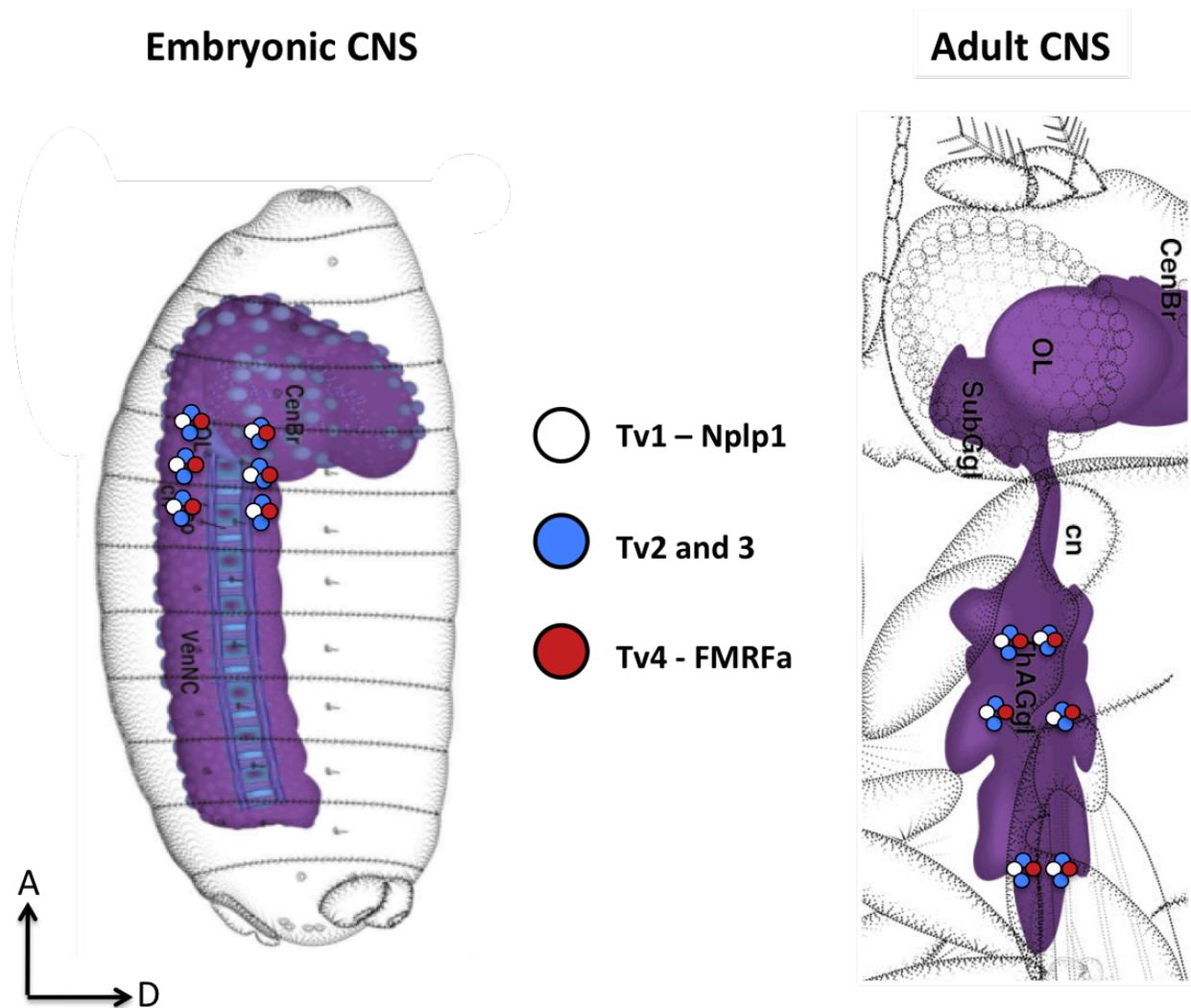


Figure 2. *D. melanogaster* Tv clusters.

The *D. melanogaster* central nervous system (CNS) contains six clusters of Tv neurons situated with bilateral symmetry in the three thoracic segments (Th1-3) of the VNC. These clusters consist of Tv1, Tv2, Tv3 and Tv4 neurons. The Tv1 and Tv4 neurons express the neuropeptides Nplp1 and FMRFa, respectively. Expression of these neuropeptides is initiated during late embryonic stages and is maintained throughout life.

(Adapted from the “Atlas of *Drosophila* Development” by Volker Hartenstein)

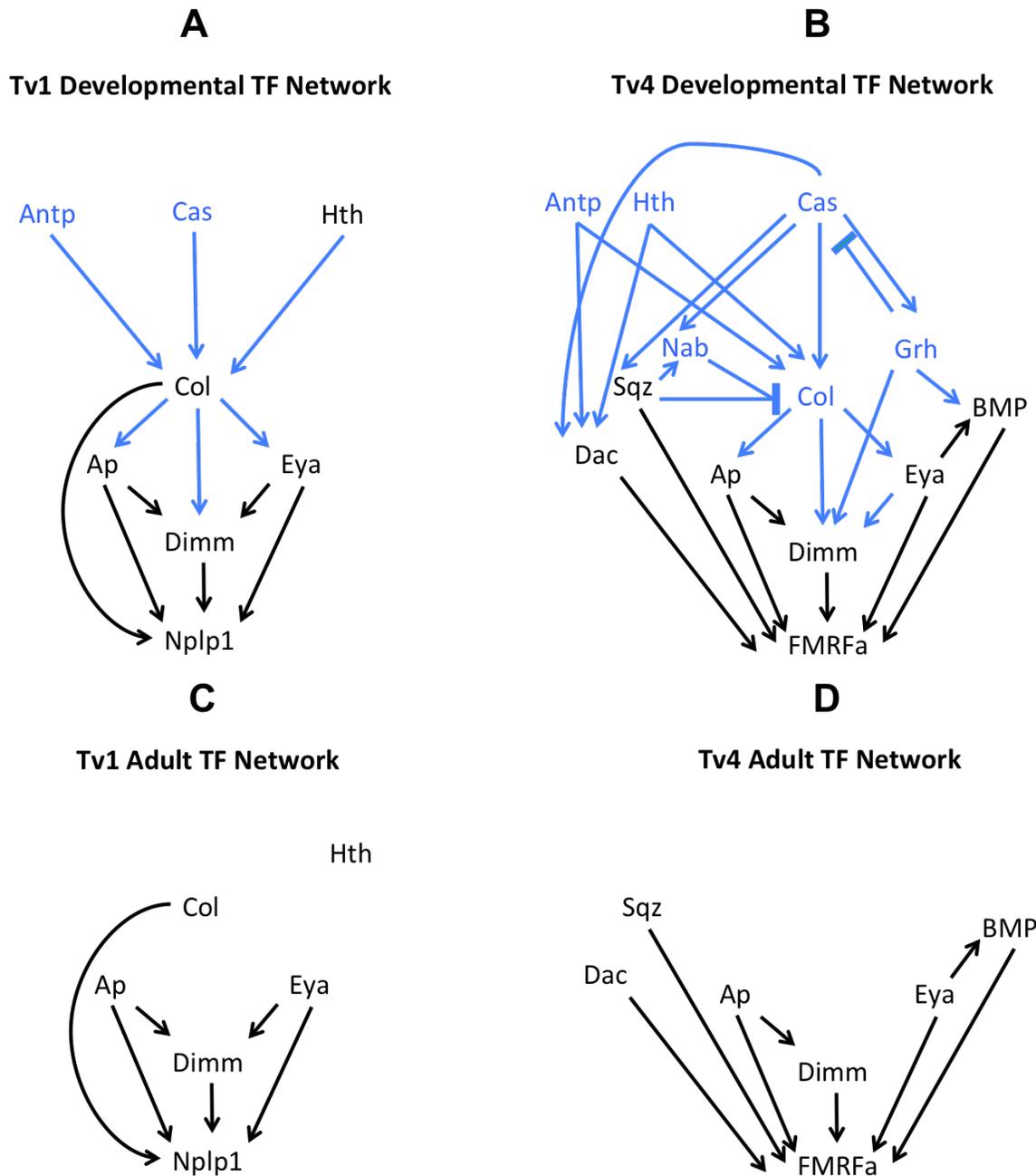


Figure 3. Tv1 and Tv4 transcription factor networks.

Transcription factors and regulatory relationships specific to development are shown in blue; those that exist both during development and in adult neurons are shown in black.

1.8 Identification of CRMs that regulate expression of *ap* and *dimm*

During my undergraduate research in the Allan lab, I took the first step towards elucidating the mechanisms that govern maintenance of transcription factor networks in mature Tv1 and Tv4 neurons. The aim was to identify CRMs responsible for initiation and/or maintenance of each transcription factor in the Tv1 and Tv4 transcription factor networks. To accomplish this, we performed a screen using *D. melanogaster* GAL4 lines generated by the Rubin group at Janelia Farms Research Institute [71]. These GAL4 lines report on the activity of 2-4 kb genomic fragments taken from intergenic and intronic regions (putative enhancer regions) of many *D. melanogaster* genes, including the transcription factor genes we are interested in. We screened all GAL4 lines driven from candidate CRM regions around each transcription factor by crossing them to *UAS-nEGFP* and testing for expression in Tv1 and Tv4 neurons. We dissected VNCs from late stage 17 embryos (Stg17) to test initiation activity and from day five adults (A5) to test maintenance activity. For *ap* we identified a 3.845 kb genomic fragment upstream of *ap* (*ap*^{Gal4-GMR42B11}) that drove *UAS-nEGFP* expression in Tv1 and Tv4 neurons at Stg17, but not at A5 (**Figure 4**). This fragment will be named *ap.K1* hereafter. For *dimm* we identified a 2.908 kb genomic fragment upstream of *dimm* (*dimm*^{Gal4-GMR10G07}) that drove *UAS-nEGFP* expression in Tv1 and Tv4 neurons at both Stg17 and A5 (**Figure 5**). These data suggested that we had identified a CRM region sufficient for initiation of *ap* in Tv1 and Tv4 neurons, but not maintenance, and also a CRM region sufficient for both initiation and maintenance of *dimm* expression in Tv1 and Tv4 neurons. **Appendix B** summarizes progress that has been made on *eya* CRMs.

To more precisely define the location of the CRM(s) within the *dimm*^{Gal4-GMR10G07} genomic fragment that is responsible for Tv1 and Tv4 expression, we used evolutionary conservation data available on the University of California Santa Cruz (UCSC) Genome Browser as a guide [72]. We reasoned that genomic regions that exhibit high evolutionary conservation

are more likely to be functionally relevant [72]. The region encompassed by *dimm*^{Gal4-GMR10G07} contains three prominent conservation islands. The most downstream conservation island (CI.3) is also encompassed by the Janelia Gal4 line *dimm*^{Gal4-GMR10D10}, which we knew from our screen does not drive reporter expression in Tv1 or Tv4 neurons at Stg17 or A5; thus, we focused our attention on the two upstream conservation islands (CI.1 and CI.2) (**Figure 6A**). The regions encompassed by each of these two conservation islands were placed upstream of a DsRed fluorescent reporter gene. Each of these constructs was then integrated into the attP40 site on the second chromosome of the *D. melanogaster* genome by integrase-based transgenesis [73] (**Appendix C**). Only *dimm*^{DsRed-CI.2} exhibited reporter expression in Tv1 and Tv4 neurons at Stg17 and A5, suggesting that it encompasses the CRM(s) responsible for the expression pattern driven by *dimm*^{Gal4-GMR10G07} (**Figure 6B-C**). Hereafter, *dimm*^{DsRed-CI.2} is referred to as *dimm.K1-nDsRed*.

Guided by our identification of ap.K1 and dimm.K1, the overall objective of this thesis work was to further our understanding of the cis- and trans-regulatory architecture that regulate initiation and maintenance of ap and dimm expression in Drosophila Tv1 and Tv4 neurons.

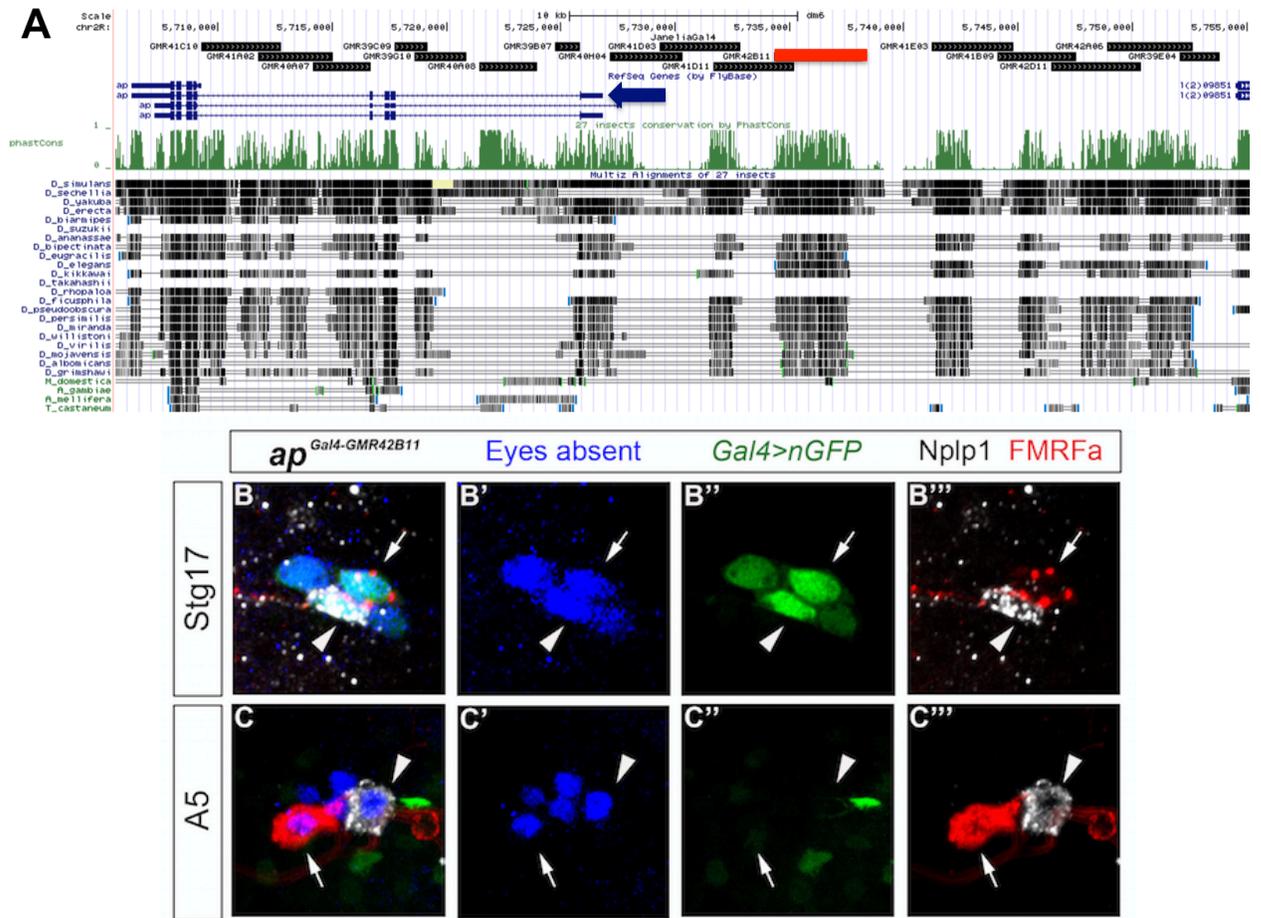


Figure 4. *ap^{Gal4-GMR42B11}* drives *UAS-nGFP* expression in Tv1 and Tv4 neurons at Stg17, but not A5.

(A) A UCSC genome browser view of the *ap* locus. The *ap* gene is indicated with a blue arrow. The tiling black rectangles represent the 2-4 kb genomic regions we tested for reporter expression at Stg17 and A5. The red rectangle is *ap^{Gal4-GMR42B11}*. (B-C) VNCs of *ap^{Gal4-GMR42B11}/UAS-nEGFP* animals were dissected at Stg17 to test initiation activity and A5 to test maintenance activity. Dissected tissue was immunocytochemically stained with anti-Eya to visualize the Tv clusters and anti-Nplp1 and anti-FMRFa to visualize the Tv1 and Tv4 neurons respectively. *ap^{Gal4-GMR42B11}* drove expression of *UAS-nEGFP* in all Tv1 (arrow heads) and Tv4 (arrows) neurons at Stg17 (B-B'''), but did not drive expression in any Tv1 or Tv4 neurons at A5 (C-C''').

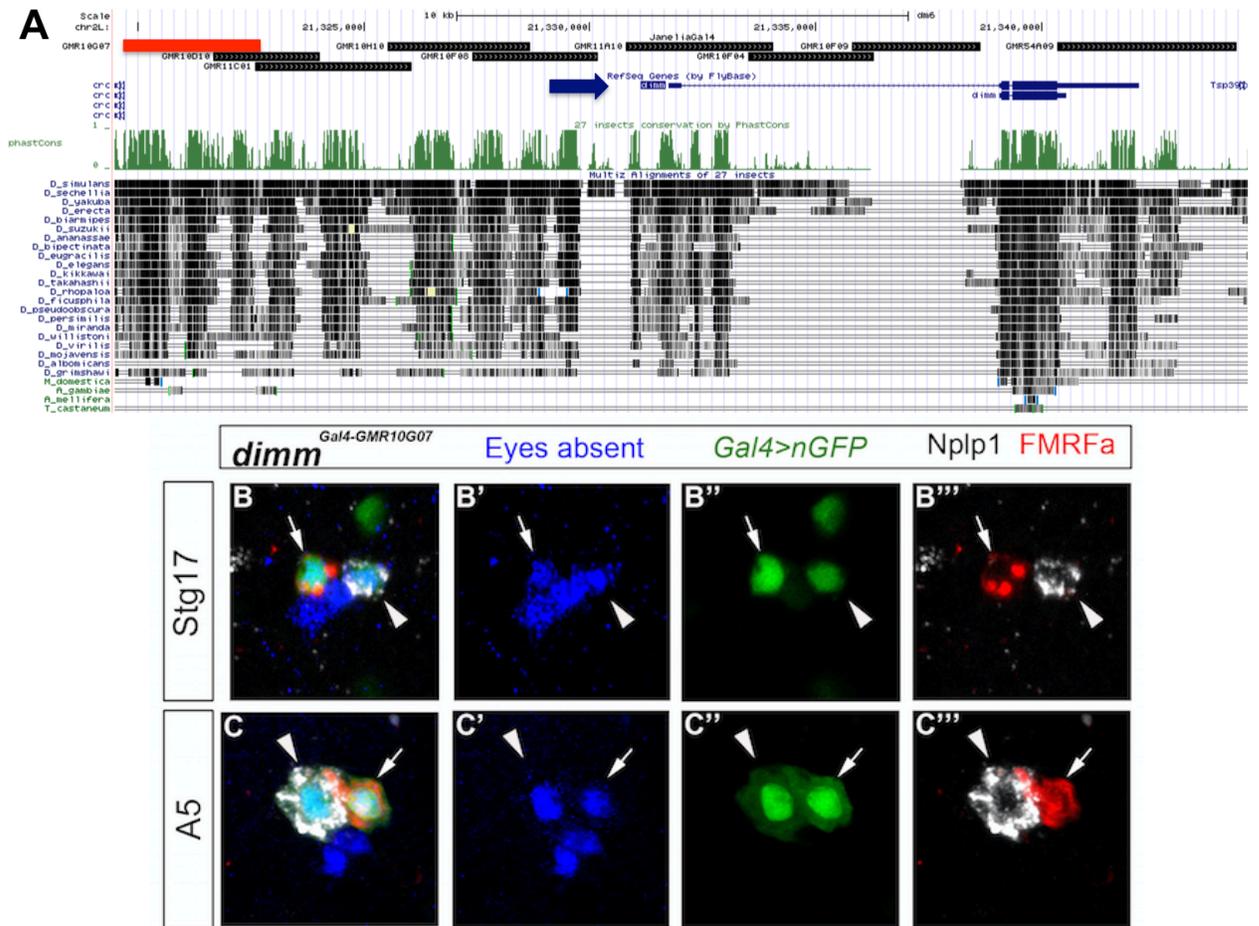


Figure 5. *dimm^{Gal4-GMR10G07}* drives *UAS-nEGFP* expression in Tv1 and Tv4 neurons at Stg17 and A5.

(A) A UCSC genome browser view of the *dimm* locus. The *dimm* gene is indicated with a blue arrow. The tiling black rectangles represent the 2-4 kb genomic regions we tested for reporter expression at Stg17 and A5. The red rectangle is *dimm^{Gal4-GMR10G07}*. (B-C) VNCs of *dimm^{Gal4-GMR10G07}/UAS-nEGFP* animals were dissected at Stg17 to test initiation activity and A5 to test maintenance activity. *dimm^{Gal4-GMR10G07}* drove expression of *UAS-nEGFP* in all Tv1 (arrow heads) and Tv4 (arrows) neurons at both Stg17 (B-B'') and A5 (C-C''). Since *dimm* is only expressed in the neuropeptidergic Tv1 and Tv4 neurons of each Tv cluster, the specificity of reporter gene expression to Tv1 and Tv4 advocates for the fidelity of the regulatory region.

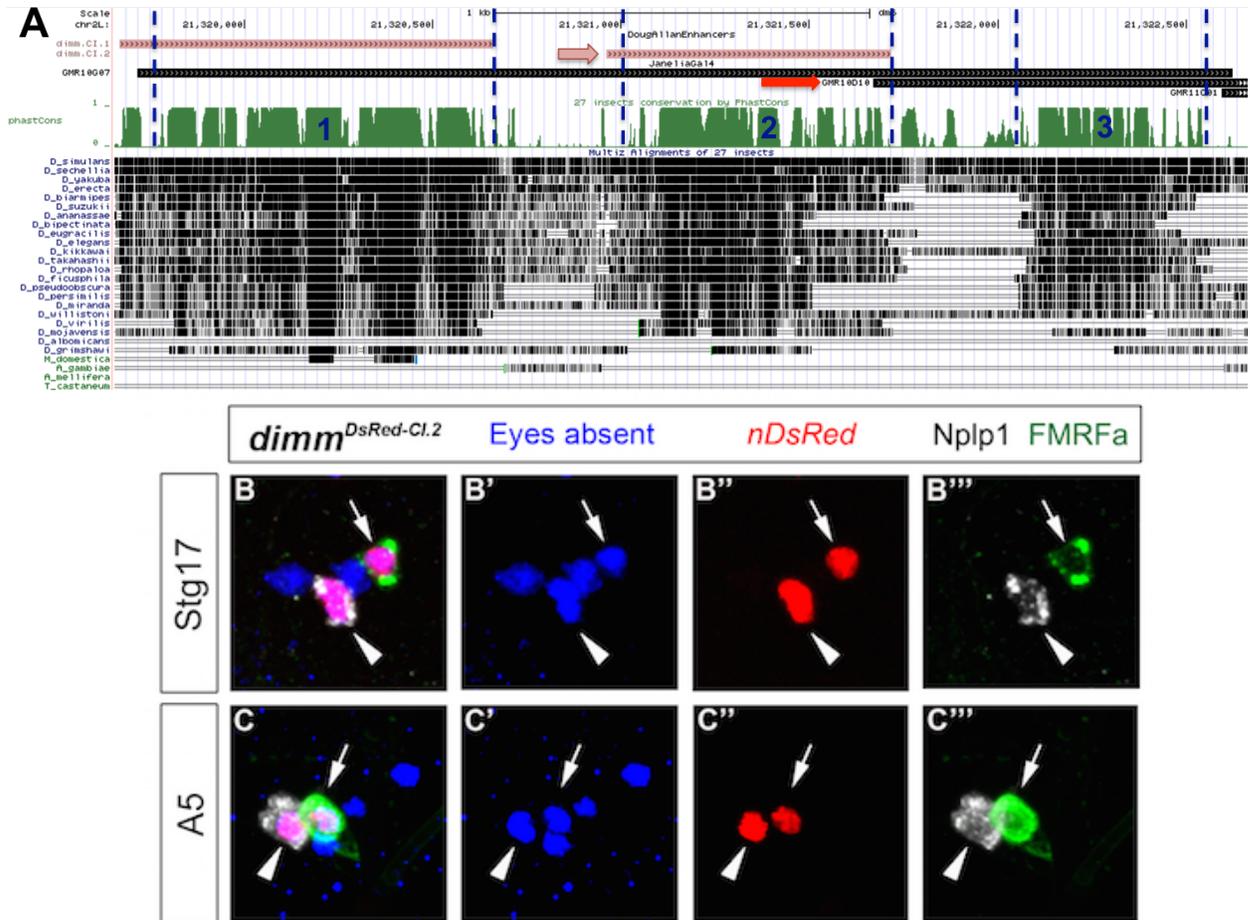


Figure 6. *dimm*^{DsRed-CI.2} drives reporter expression in Tv1 and Tv4 neurons at Stg17 and A5.

(A) A UCSC genome browser view of the genomic region encompassed by *dimm*^{Gal4-GMR10G07}.

The limits of the three prominent conservation islands in this region are shown with blue dashed lines. The most downstream conservation island (3) is also encompassed by the *Janelia* GAL4 line *dimm*^{Gal4-GMR10D10} (red arrow) which we knew from our screen does not drive reporter expression in Tv1 or Tv4 neurons at Stg17 or A5; thus, we focused our attention on the two upstream conservation islands (CI.1 and CI.2, pink rectangles). Only CI.2 (pink arrow) drove reporter expression in Tv1 and Tv4 neurons at Stg17 (B-B''') and A5 (C-C'''). This suggests that CI.2 encompasses the CRM(s) responsible for the expression pattern driven by *dimm*^{Gal4-GMR10G07}.

1.9 Thesis objectives

1.9.1 Characterizing the role of *dimm.K1* in the initiation and maintenance of *dimm* expression in *Drosophila* Tv1 and Tv4 neurons

We aimed to test the hypothesis that *dimm.K1* encompasses the CRM(s) regulating initiation and maintenance of *dimm* expression. More specifically we aimed to:

- (1) Confirm the fidelity of *dimm.K1* as a true *dimm* enhancer that is sufficient to drive reporter activity in a Dimm-specific expression pattern throughout the VNC.
- (2) Assess the *necessity* of *dimm.K1* for *dimm* expression in Tv1 and Tv4 neurons.
- (3) Compare regulation of *dimm.K1-nDsRed* and Dimm protein by known Tv1 and Tv4 terminal selectors at initiation and maintenance stages.

1.9.2 Characterizing the *cis*-regulatory architecture that regulates initiation and maintenance of *ap* expression in *Drosophila* Tv1 and Tv4 neurons

To further characterize the *cis*-regulatory architecture that regulates initiation and maintenance of *ap* expression in *Drosophila* Tv1 and Tv4 neurons we aimed to:

- (1) Assess the *necessity* of *ap.K1* for *ap* expression in Tv1 and Tv4 neurons.
- (2) Identify the CRM(s) required to maintain *ap* expression.

2. MATERIALS AND METHODS

2.1 Fly strains

apterous^{md544} (ap^{Gal4}) and *ap^{P44}* [74]; *eya^{Cli-III}* [75]; *eya^{D1}* [76]; *dac³* [77]; *Df(2L)Exel7066 (Dac^{Df})* (Bloomington, IN); *sqz^{IE}* [65]; *wit^{A12}* and *wit^{B11}* [78]; *tubP>Gal80^{TS}* (temperature sensitive GAL80 under control of the *Drosophila* tubulin 84B promoter) [79]; *UAS-nEGFP* (nuclear localized EGFP); *UAS-dicer2* [80]; *UAS-dimm* [81]; *UAS-tkv^{AGSK}* [GS-box and kinase domain deletion, referred to here as *UAS-tkv^{DN}*] [82]; *UAS-wit^{AI}* [intracellular domain deletion, referred to here as *UAS-wit^{DN}*] [83]; *col^{dsRNAi} #24E* [63]; *ap^{dsRNAi} 8376R-2* and *dac^{dsRNAi} 4952R-2* and *sqz^{dsRNAi} 5557R-2* (NIG-FLY); *dimm^{dsRNAi} GD44470* (VDRC); *eya^{dsRNAi} JF03160* and *hth^{dsRNAi} JF02733* (TRiP); *w¹¹¹⁸*. Alleles were maintained over fluorescently tagged (GFP) balancer chromosomes and GFP was selected against as necessary to acquire animals of the appropriate genotypes.

2.2 Transgene construction

Empty pThunderbird EGFP vector was generated from Tv-nEYFP and from *EGFP* sequence within pHstinger [84,85]. Putative *ap* enhancer regions were amplified from genomic DNA using the primers listed in **Table 1**. Assembled genomic regions consisting of the putative *ap* initiation and maintenance elements were joined by SOE PCR. Assembled regions were digested with BglII and Acc65I and ligated into BglII and Acc65I digested pThunderbird EGFP empty vector.

2.3 Fly transgenesis

All transgenesis was performed by Rainbow Transgenic Flies Inc. (Camarillo, CA, USA). Single copies of each transgene were individually and unidirectionally integrated into the

attPVK00033 site on the third chromosome of the *D. melanogaster* genome using phiC31 integrase-based transgenesis [73].

2.4 Fly husbandry

Flies were maintained on standard cornmeal food preserved with methylparaben, antibiotics (tetracycline and streptomycin OR tetracycline and ampicillin) and sodium potassium tartrate. All experiments were performed in stable environment rooms with temperatures of 18°C, 25°C or 29°C and 70% humidity.

Flies were grown and maintained at 25°C (unless used for temporal induction protocols; see below). Embryos were grown on grape plates supplemented with yeast paste and dechorionated using 50% bleach. Late stage 17 embryos with mouth hooks and air filled trachea were selected for dissection. For adults, vials were cleared as progeny began to eclose. A1s were collected 24 hours later and maintained until the ages indicated in sections 3.1-3.3 and 3.8-3.9.

For temporal genetic manipulations (transcription factor over-expression or *dsRNAi*-mediated transcription factor knockdown), we used the TARGET system [79]. We crossed a screener fly (*ap^{GAL4}, dimm.K1-nDsRed/[CyOAG] ; tubP>Gal80^{TS}, UAS-nEGFP/[TM3SerAG]*) to either a control fly (*w¹¹¹⁸* or *UAS-dicer2*) or an experimental fly genotype (*UAS-transgene* or *UAS-dicer* {;;} *UAS-dsRNAi*). Progeny were grown from embryonic stages at 18°C to prevent GAL4 activation. Vials were cleared as progeny began to eclose. A1 adults were collected 24 hours later and placed at 29°C for induction times optimized by Eade *et al.* [36].

2.5 Antibodies

Primary Antibodies: guinea pig anti-Col (1:1000, gift from Adrian Moore, RIKEN, Japan); mouse anti-Dac (1:10; Mab 2 – 3) and mouse anti-Eya (1:10; clone 10H6) (Developmental Studies Hybridoma Bank, Iowa U, Iowa); guinea pig anti-Dimm (1:1000) and rabbit anti-FMRFa (1:4000) and chicken anti-Nplp1 (1:1000) (gifts from S. Thor, Linkoping U,

Sweden). Secondary Antibodies: donkey anti-chicken Alexa 647, Alexa 488, Alexa 405 and donkey anti-guinea pig Alexa 647 and donkey anti-mouse Alexa 488, Alexa 647 and donkey anti-rabbit Alexa 405, Alexa 488, Alexa 647 (all 1:100, Invitrogen, Carlsbad, USA); donkey anti-mouse Cy3 (1:100, Jackson ImmunoResearch, West Grove, USA).

2.6 Immunocytochemistry

VNCs were dissected in ice-cold phosphate-buffered saline (PBS) and then fixed in ice-cold 4% formaldehyde diluted in 0.1% PBT (PBS containing 0.1% Triton X-100) for 30 minutes. Tissue was washed with 0.3% PBT (PB3T) and then incubated in PB3T containing 5% Donkey Serum (PB3T-DS). Transcription factor primary antibodies were incubated overnight at 4°C. Neuropeptide primary antibodies were incubated for two hours at room temperature. Tissue was washed with PB3T and then incubated in PB3T-DS. Secondary antibodies were incubated for two hours at room temperature. Tissue was washed with PB3T and then mounted in Vectashield (Vector). All antibodies were diluted in PB3T-DS. Control and experimental genotypes were dissected at the same time and mounted in the same well in order to ensure that time to fixation was normalized between genotypes and that the treatment of all tissue was identical.

2.7 Image and statistical analysis

All images were acquired with Olympus FV1000 confocal microscopes. In embryos, Tv neurons from all thoracic segments were analyzed. In adults, only Tv neurons from Th1 and Th3 were analyzed; a second Tv neuron begins expressing FMRFa at pupal stages in Th2, which makes it difficult to unambiguously distinguish the Tv4 neuron. Images of control and experimental samples for any given experiment were acquired with identical settings and within the same imaging session. To confirm any instances of undetectable expression in experimental

animals, images were also captured at settings of maximum sensitivity. For comparison of fluorescence intensities, mean pixel intensity for each neuron was measured from summed Z-projection in Image J (US National Institutes of Health). Each measurement was corrected for background fluorescence and expressed as the percentage of the mean of control for each experiment. All statistical analyses and graphs were generated using Prism 5 software (Graphpad). Data is presented as Mean +/- SEM. Two-tailed t-tests were used to determine statistical significance. Differences between groups were considered to be statistically significant when $p < 0.05$. Body segment specific responses of *dimm.K1* reporter to genetic perturbations were observed, thus all quantified reporter intensities have been reported separately for Th1, Th2 and Th3 neurons.

For the construction of <i>ap.K1.M</i>	
<i>ap.K1</i> Forward	ATACTT AGATCT GAAGTTCCTATTCTCTAGAAAGTATAGGAACT TCCTAAAGCGGTCCTAGTATACCCGGATACC
<i>ap.K1</i> Reverse	<u>TTCCTATACTTTCTAGAGAATAGGAACTTCGGTCGGAACCTTTT</u> GTTCTATAACCC
<i>ap.M</i> Forward	<u>GACCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCATGACA</u> GTTCTTATTGGAAGCAGGC
<i>ap.M</i> Reverse	TCGCGG GGTACC CCTGTTTCTGTGTCGATTCTCCTTC
SOE PCR Forward	<i>ap.K1</i> Forward
SOE PCR Reverse	<i>ap.M</i> Reverse

Table 1. Primers used to generate putative *ap* enhancer transgene.

Restriction digestion sites are highlighted in red, FRT sites are italicized and over-lapping regions engineered for SOE PCRs are underlined.

3. RESULTS

3.1 All neurons of the VNC that express *dimm.K1-nDsRed* also express Dimm.

In addition to initiating and maintaining reporter expression in Tv1 and Tv4 neurons (**Figure 6**), *dimm.K1* also drives reporter expression in other cells of the VNC at Stg17 and A5. This is consistent with the fact that Dimm is expressed in many additional VNC neuropeptidergic cells [86]. We tested whether *dimm.K1* reporter activity is restricted to Dimm-expressing neurons, in order to assess the fidelity of *dimm.K1* as a true *dimm* enhancer. Stg17 and A5 *dimm.K1-nDsRed* VNCs were stained with anti-Dimm and co-localization between *dimm.K1* reporter and Dimm was assessed. We found that in all Stg17 (n=5) and A5 (n=5) VNCs observed, every neuron that expressed *dimm.K1-nDsRed* also expressed Dimm (**Figure 7**). Thus, we conclude that *dimm.K1* encompasses a true *dimm* enhancer that does not generate ectopic activity when isolated into a reporter transgene.

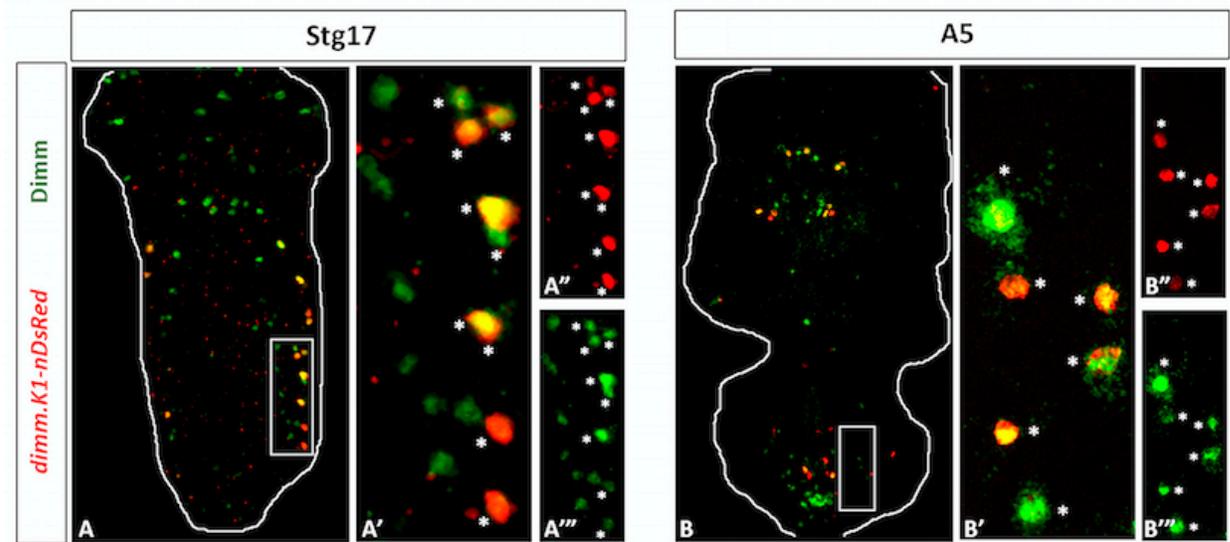


Figure 7. Co-localization of *dimm.K1-nDsRed* and anti-Dimm in the VNC.

In all Stg17 and A5 VNCs observed, every neuron that expressed *dimm.K1-nDsRed* also expressed Dimm. Notably, not all Dimm-expressing neurons co-express *dimm.K1-nDsRed*. This is likely due to the fact that other *dimm* enhancer regions control *dimm* expression in those neurons. **(A)** A single Z-slice through the Stg17 CNS (outlined in white) that is representative of *dimm.K1-nDsRed* and anti-Dimm overlap. The area within the white rectangular box is shown at higher magnification in **A'-A'''**. **(B)** A full Z-projection of the entire A5 VNC (outlined in white). A single Z-slice in the dorsal region of the VNC within the white rectangular box is shown at higher magnification in **B'-B'''**.

3.2 *dimm.K1* drives long-term reporter maintenance in Tv1 and Tv4 neurons.

We assessed the sufficiency of *dimm.K1* to drive life-long maintenance of reporter expression in adult Tv1 and Tv4 neurons. *dimm.K1* reporter intensities were assessed in A20 and A45 animals using A1 intensities as a reference point. The intensities of anti-Dimm were assessed simultaneously in order to distinguish between potential decreases in *dimm.K1* reporter intensities that could suggest a decrease in the sufficiency of *dimm.K1* to drive gene expression, and those that correlate with changes in the expression of Dimm itself. Both Dimm and *dimm.K1* reporter were expressed robustly at A1, A20 and A45 (**Figure 8**) and *dimm.K1* reporter intensity was maintained in all neurons (**Figure 9**).

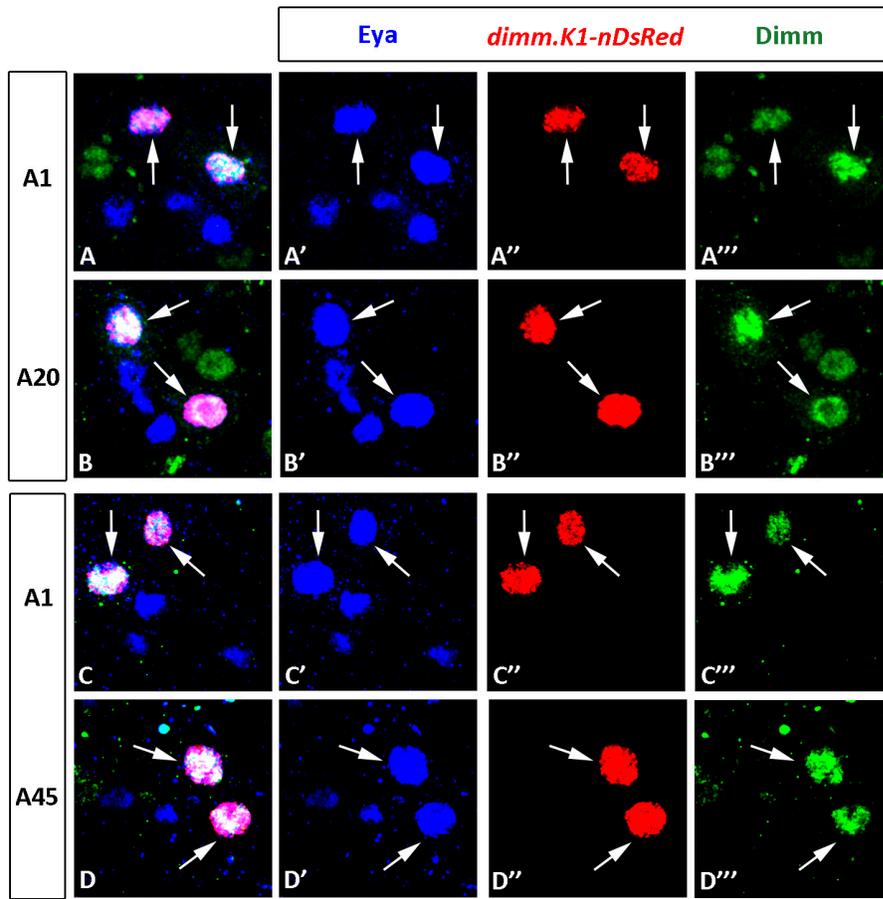


Figure 8. *dimm.K1-nDsRed* and anti-Dimm at A1, A20 and A45.

Intensities of anti-Dimm and *dimm.K1* reporter were assessed in A20 and A45 animals, using A1 as a reference point. Tissue was stained with anti-Eya and anti-Dimm. Both *dimm.K1-nDsRed* and Dimm continue to be expressed robustly in Tv1 and Tv4 neurons (white arrows) at A20 (**B-B'''**) and A45 (**D-D'''**).

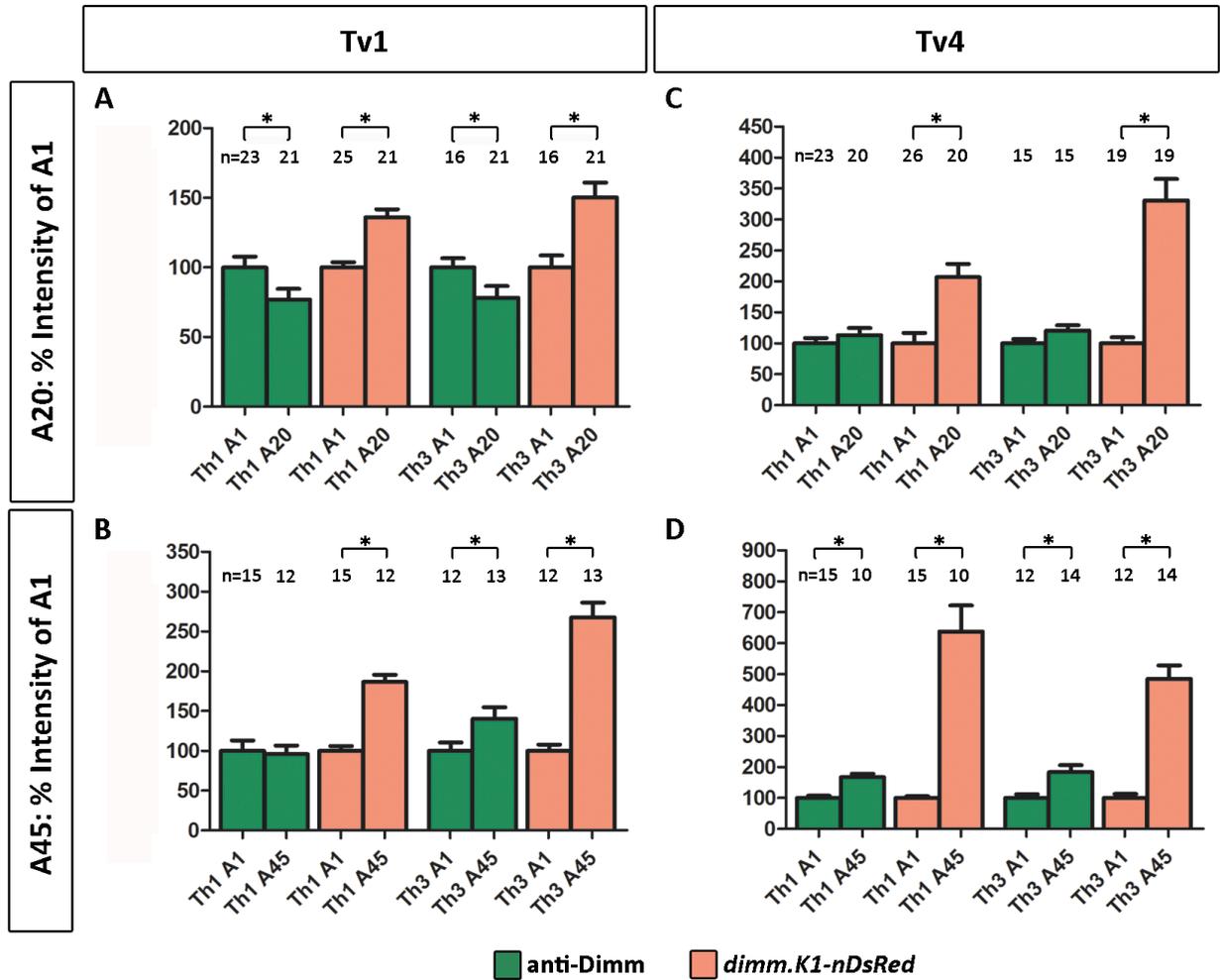


Figure 9. Quantification of *dimm.K1-nDsRed* and anti-Dimm intensities at A20 and A45, relative to A1.

The intensities of *dimm.K1-nDsRed* and anti-Dimm at A20 and A45, relative to A1. Relative intensities are presented as the % mean of A1 expression levels +/- SEM. Student's two-tailed t-tests were used to compare each type of neuron (Tv1-Th1, Tv1-Th3, Tv4-Th1 and Tv4-Th3) between the two ages indicated (*= $p < 0.05$). Intensities of anti-Dimm are shown in green and *dimm.K1-nDsRed* intensities are shown in pink. Detailed statistics are provided in **Table 2** (**Appendix A**). The intensity of *dimm.K1* reporter is maintained in all Tv1 and Tv4 neurons.

3.3 *dimm.K1* is necessary for *dimm* expression in Tv1 and Tv4 neurons.

The Thor lab generated a line (*dimm Δ 1141*) in which the genomic region encompassing endogenous *dimm.K1* is deleted (**Figure 10A**), and also a transgenic line (*dimm.1141-nEGFP*) that reports expression driven by the deleted fragment. They found that embryonic Tv1 neurons fail to express Dimm protein in the deletion mutant and that the reporter generates expression in embryonic Tv1 neurons (unpublished personal communication). In order to verify that their reporter also initiates expression in Tv4 neurons, and maintains expression in Tv1 and Tv4 neurons, we generated a fly line combining both *dimm* reporters (*dimm.K1-nDsRed/+ ; dimm.1141-nEGFP/+*) and dissected these animals at Stg17 to test initiation and at A5 to test maintenance. Reporter expression was driven by both *dimm.K1* and *dimm.1141* in all Tv1 and Tv4 neurons at Stg17 (n=27) (**Figure 10B**) and A5 (n=31) (**Figure 10C**). We then tested whether *dimm.K1/dimm.1141* is necessary for *dimm* initiation in Tv4 neurons; *w¹¹¹⁸* (control) and *dimm Δ 1141* VNCs were dissected at Stg17 and we found that Dimm was robustly expressed in all *w¹¹¹⁸* Tv4 neurons (n=44) (**Figure 11A**) but could not be detected in any *dimm Δ 1141* Tv4 neurons (n=54) (**Figure 11B**). Thus, *dimm.1141* is indeed necessary to initiate *dimm* expression in Tv4 neurons.

We also dissected *w¹¹¹⁸* (control) and *dimm Δ 1141* VNCs at A5. All *w¹¹¹⁸* Tv1 (n=31) and Tv4 (n=34) neurons exhibited robust Dimm expression (**Figure 11C**), but Dimm was not detected in any *dimm Δ 1141* Tv1 (n=59) or Tv4 (n=62) neurons (**Figure 11D**).

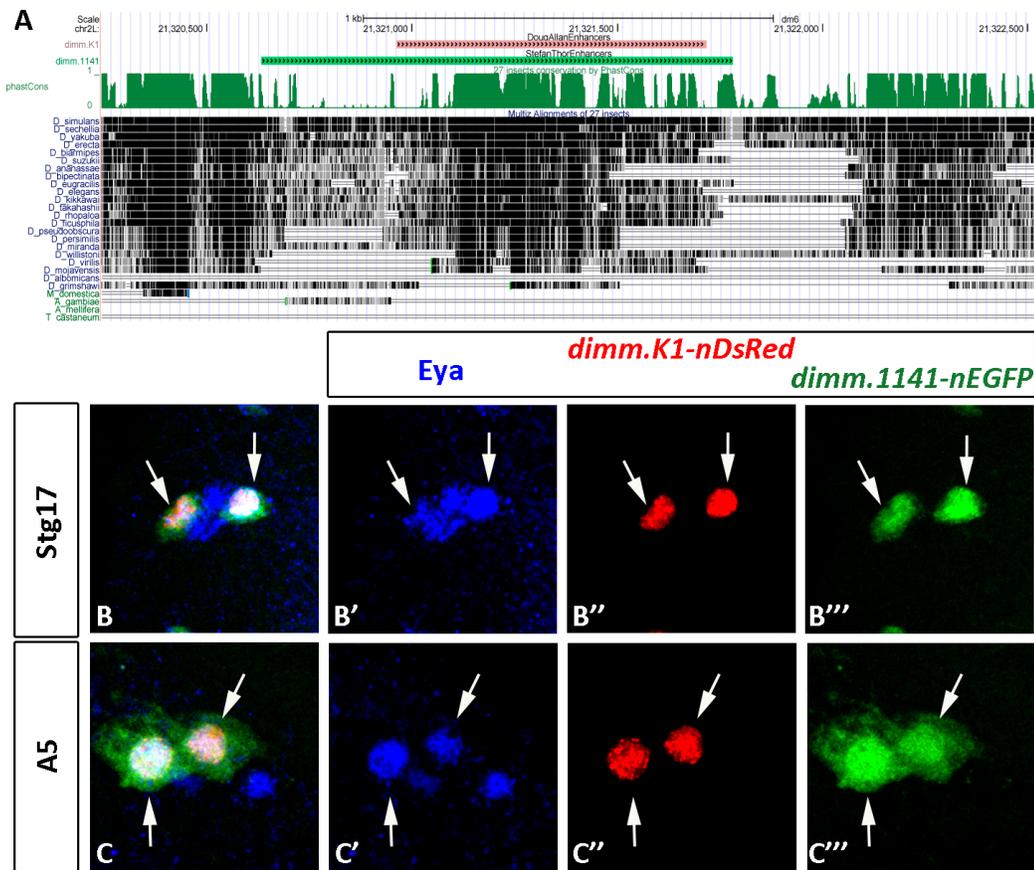


Figure 10. Co-expression of *dimm.1141* and *dimm.K1* reporters verifies that these overlapping regions both report initiation and maintenance in Tv1 and Tv4 neurons.

(A) A UCSC genome browser comparison of the genomic regions encompassed by *dimm.K1* (pink) and *dimm.1141* (light green). Both encompass the same well-conserved island (dark green peaks) likely to contain all regulatory information pertinent to initiation and maintenance of reporter expression in Tv1 and Tv4 neurons. (B-C) To confirm that *dimm.1141* is sufficient to initiate and maintain reporter expression in Tv1 and Tv4 neurons, VNCs of *dimm.K1-nDsRed/+*; *dimm.1141-nEGFP/+* animals were dissected at Stg17 (initiation) and A5 (maintenance). The Tv clusters were identified using anti-*Eya*, and we found that *dimm.K1* and *dimm.1141* reporters consistently co-localize in two neurons of each Tv cluster (white arrows) at both Stg17 (B-B''') and A5 (C-C'''), indicating that, like *dimm.K1*, *dimm.1141* is sufficient to initiate and maintain reporter expression in Tv1 and Tv4 neurons.

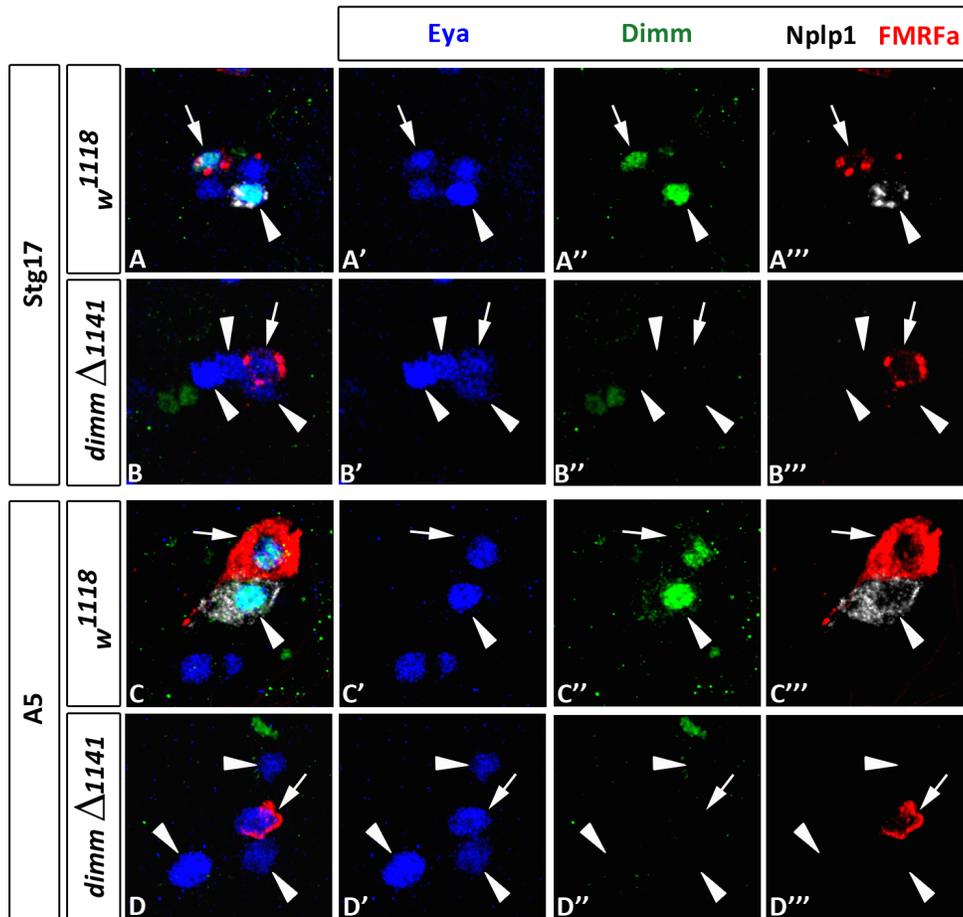


Figure 11. *dimm.1141* is necessary for *dimm* expression in Tv1 and Tv4 neurons.

(A-B) Stg17 *w¹¹¹⁸* (control) and *dimmΔ1141* VNCs were stained with anti-Eya to visualize the Tv clusters, anti-Nplp1 to visualize the Tv1 neurons and anti-FMRFa to visualize the Tv4 neurons, and anti-Dimm. Dimm expression was robust in all control Tv1 (arrow heads) and Tv4 (arrows) neurons (A-A'''), but could not be detected in any *dimmΔ1141* Tv clusters (B-B''').

(C-D) At A5 Dimm expression was robust in all control Tv1 (arrow heads) and Tv4 (arrows) neurons (C-C'''), but could not be detected in any *dimmΔ1141* Tv clusters (D-D'''). Thus, *dimm.K1/dimm1141* is necessary for *dimm* expression in Tv1 and Tv4 neurons.

3.4 Initiation of *dimm.K1-nDsRed* expression in Tv1 and Tv4 neurons is dependent upon the same transcription factors as initiation of Dimm.

In order to test the utility of the *dimm.K1-nDsRed* reporter as a valid *dimm* reporter and also as a primary CRM for *dimm*, we tested whether its expression in Stg17 Tv1 and Tv4 neurons is dependent upon input from the same known transcription factors as Dimm itself. The Thor lab is studying *dimm* initiation in Tv1, so we restricted our quantitative analysis to Tv4 neurons. We did, however, make qualitative observations regarding Tv1 that facilitate the interpretation of results presented in upcoming sections.

In addition to Dimm, the Tv4 terminal selector network consists of Ap, Eya, Dac, Sqz and pMad (BMP signalling). Whereas all of these transcription factors contribute to FMRFa initiation, only Ap and Eya contribute to Dimm initiation (**Figure 12A**) [63, 65-67, 69]. To test inputs required for *dimm.K1-nDsRed* expression, the reporter was placed in mutant backgrounds for all Tv4 terminal selectors, and the intensity of *dimm.K1* reporter relative to controls was quantified at Stg17. In *ap* mutants, Tv4 *dimm.K1* reporter intensity was severely reduced to 10% (Th1), 22% (Th2) and 29% (Th3) of controls; $p < 0.0001$ for all (**Figure 12B**). In *eya* mutants *dimm.K1* reporter was not detectable in any thoracic segments of the VNC (**Figure 12F**). No significant decrease in *dimm.K1* reporter intensity was observed in the Tv4 neurons of *dac*, *sqz* or *wit* (BMP) mutants relative to controls (**Figure 12C-E**). The only unexpected finding was the significant up-regulation (to 134%; $p = 0.02$) of *dimm.K1* reporter intensity in Th3 of *dac* mutants (**Figure 12C**).

Overall these data are consistent with previous data showing that Dimm protein expression in Tv4 neurons is Ap and Eya dependent (summarized in **Figure 12A**). We also observed a total loss of *dimm.K1-nDsRed* expression in Stg17 Tv1 neurons of *ap* and *eya* mutants (**Figure 24, Figure 12F**), equivalent to data showing that Dimm expression in Tv1 neurons is Ap and Eya dependent [63]. Thus Ap and Eya are required to initiate expression of

dimm.K1-nDsRed in both Tv1 and Tv4 neurons (summarized in **Figure 12G**). We conclude that initiation of *dimm* expression in Tv1 and Tv4 neurons occurs through the activity of Ap and Eya at the *dimm.K1* CRM.

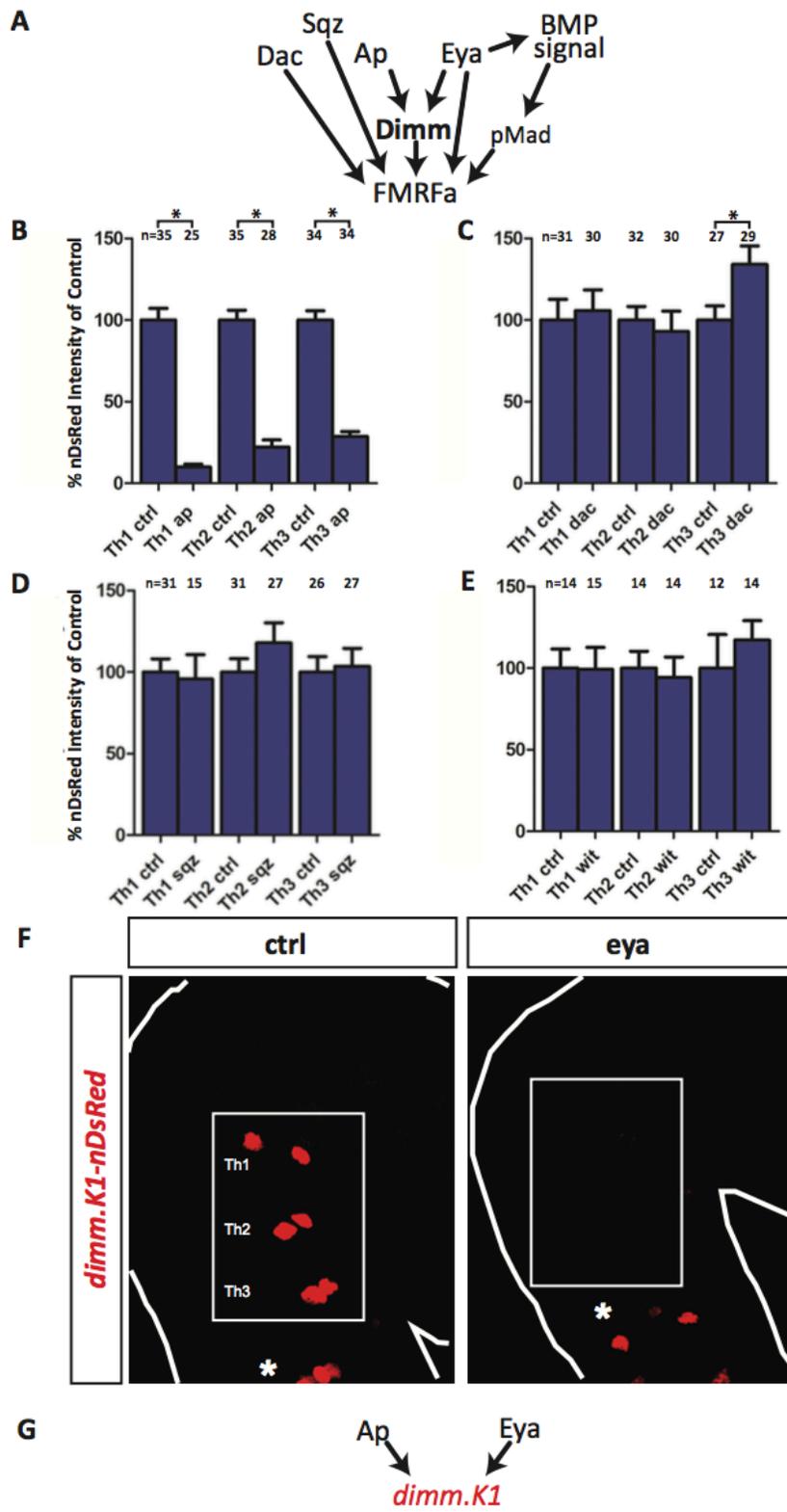


Figure 12.

Figure 12. Initiation of *dimm.K1-nDsRed* expression in Tv4 neurons.

(A) The Tv4 terminal selector initiation network defined previously. Only Ap and Eya contribute to Dimm initiation. (B-F) Tv4 *dimm.K1-nDsRed* activity was examined in all transcription factor mutant backgrounds. (B-E) The intensity of *dimm.K1* reporter relative to controls (ctrl) was quantified in Tv4 neurons and the relative intensities are presented as the % mean of control expression levels +/- SEM. Student's two-tailed t-tests were used to compare control and experimental populations (*=p<0.05). Ap and Eya are required for *dimm.K1* reporter activity in Tv4 neurons, whereas Dac, Sqz and BMP signalling are not. The asterisks in F mark the posterior limit of the VNC's thoracic segments. (G) A summary of our findings confirming that initiation of *dimm.K1-nDsRed* expression requires Ap and Eya, just as previously found for Dimm protein. **Genotypes.** Control: *dimm.K1-nDsRed/dimm.K1-nDsRed*. Experimentals: (B) *ap^{Gal4}*, *dimm.K1-nDsRed/ap^{P44}*, *dimm.K1-nDsRed* (C) *dac³*, *dimm.K1-nDsRed/Df(2L)Exel7066(Dac^{Df})*, *dimm.K1-nDsRed* (D) *dimm.K1-nDsRed/dimm.K1-nDsRed*; *sqz^{IE}/sqz^{IE}* (E) *dimm.K1-nDsRed/dimm.K1-nDsRed*; *wit^{A12}/wit^{B11}* (F) *eya^{ClI-IID}*, *dimm.K1-nDsRed/eya^{D1}*, *dimm.K1-nDsRed*.

3.5 Dimm auto-regulates its own maintenance through *dimm.K1*, in Tv4 only

Eade *et al.* demonstrated differences in the transcription factors that initiate and maintain Dimm in Tv1 and Tv4 neurons. Ap, Eya and Col all initiate Dimm. In Tv1 neurons, only Ap and Eya maintain Dimm. In Tv4 neurons, only Ap regulates Dimm maintenance (**Figure 3**) [36]. We undertook an analysis of the transcription factors that maintain *dimm.K1* reporter activity in Tv1 and Tv4 neurons. First, we explored the mechanism of auto-regulation.

The TARGET system was used to knockdown (*dimm^{dsRNAi}*) Dimm from A1 onwards in Tv1 and Tv4 neurons, after normal development [79]. Successful knockdown of Dimm was confirmed by anti-Dimm immunocytochemistry (**Figure 25**). Induction of *dimm^{dsRNAi}* at A1 had distinct effects in Tv1 and Tv4 neurons by A10. In Tv1, no significant effect on *dimm-K1* reporter intensity was observed (**Figure 13A**). However, in Tv4, *dimm-K1* reporter intensity was reduced to 40% (p<0.0001) in Th1 and 71% (p=0.0006) in Th3 (**Figure 13B**). We wished to test the sufficiency of Dimm for auto-maintenance, to verify this apparent Tv4-specific effect. Using the TARGET system we overexpressed Dimm (*UAS-Dimm*) in Tv1 and Tv4 neurons from A1 onwards; successful over-expression of Dimm was confirmed by immunocytochemistry (**Figure 26**). We observed increased *dimm-K1* reporter intensity in Tv4 neurons to 150% (p<0.0005) of control levels, but no effect on *dimm-K1* reporter intensity in Tv1 neurons (**Figure 13C,D**). Thus, by loss and gain of function tests, we confirm that *dimm* auto-maintenance through *dimm.K1* only occurs in Tv4 neurons.

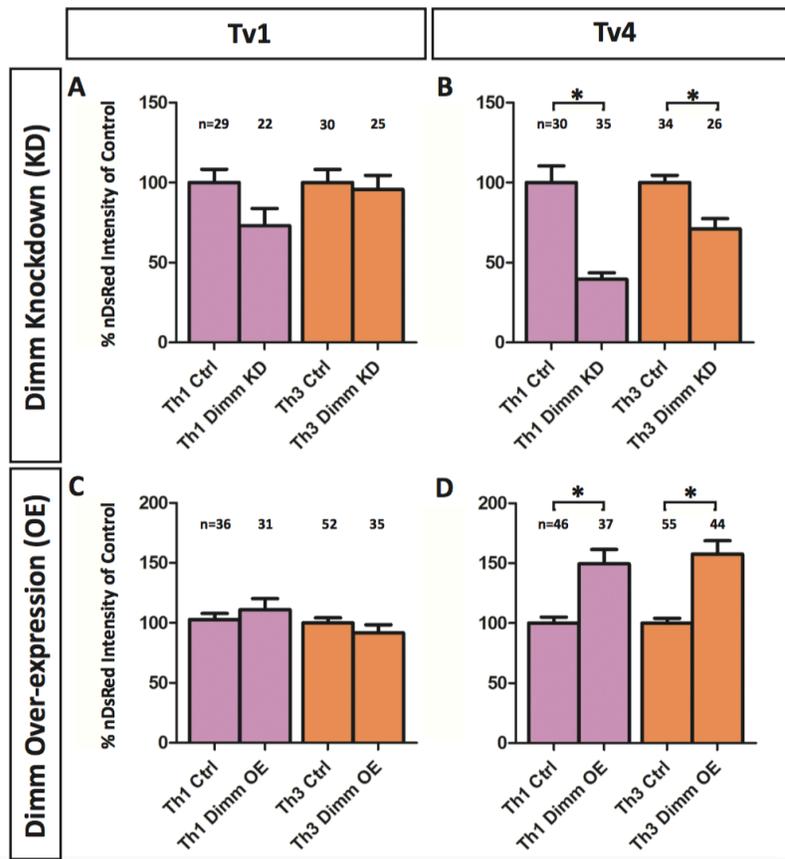


Figure 13. Dimm regulates maintenance of *dimm.K1-nDsRed* in Tv4, but not Tv1, neurons.

The TARGET system was used to knockdown (*dimm^{dsRNAi}*) or over-express (*UAS-dimm*) Dimm in Tv1 and Tv4 neurons, at A1. Intensity of *dimm.K1* reporter relative to controls was quantified. Relative intensities are presented as the % mean of control expression levels +/- SEM. Student's two-tailed t-tests were used to compare control and experimental populations (*= $p < 0.05$). Loss or gain of Dimm expression only affected *dimm.K1* reporter activity in Tv4 neurons; our data indicates that loss of Dimm reduces *dimm.K1* reporter activity and excess Dimm increases *dimm.K1* reporter activity. We conclude that Dimm becomes auto-regulatory in Tv4 neurons.

Genotypes in A and B: Ctrl (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*), Dimm knockdown (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/rev4*, *UAS-dimm^{dsRNAi}*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*) **Genotypes in C and D:** Ctrl (*ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP*), Dimm over-expression (*ap^{Gal4}*, *dimm.K1-nDsRed/UAS-dimm*; *tubP>Gal80^{TS}*, *UAS-nEGFP*).

3.6 Maintenance of *dimm.K1-nDsRed* in Tv1 neurons

Col, Ap, Eya and Dimm all initiate and maintain Nplp1 in Tv1 neurons, but only Ap and Eya (and not Col) continue to regulate expression of Dimm in adult Tv1 neurons (**Figure 14A**) [36]. We assessed whether maintenance of *dimm.K1-nDsRed* in Tv1 neurons is regulated by these same transcription factors. Using the TARGET system we knocked down Ap, Col and Eya in adult Tv1 neurons at A1. Successful knockdown of each transcription factor was confirmed by immunocytochemistry (**Figure 27**). Knockdown of Ap (*ap^{dsRNAi}*) reduced *dimm.K1* reporter intensity to 60% ($p=0.0014$) of control levels in Th1, but had no effect in Th3 neurons by A20 (**Figure 14B**). Knockdown of Col (*col^{dsRNAi}*) had no significant effect on *dimm.K1* reporter intensity by A10 (**Figure 14C**). Knockdown of Eya (*eya^{dsRNAi}*) increased *dimm.K1* reporter intensity to 190% ($p<0.0025$) of control levels by A20 (**Figure 14D**).

Recent work identified a role for Hth in Tv neuron development [68]. Prior to this thesis work, we had found that Hth continues to be expressed in adult Tv1 neurons, but that knockdown of Hth (*hth^{dsRNAi}*) at A1 had no obvious effect on Nplp1 immunoreactivity by A15, suggesting that Hth does not regulate maintenance of Nplp1 or the Tv1 terminal selectors that are required for Nplp1 maintenance (data not shown). We addressed the role of Hth in the maintenance of Tv1 identity and the maintenance of Dimm expression more rigorously by quantifying the effects of its knockdown on Nplp1 immunoreactivity, Dimm immunoreactivity and *dimm.K1* reporter intensity. Hth knockdown had no significant effect on Nplp1 immunoreactivity (**Figure 14E**). Intriguingly, however, anti-Dimm intensity increased to 146% ($p=0.0096$) of control levels in Th1 neurons, and *dimm.K1* reporter intensity increased to 1781% ($p<0.0001$) in Th1 and 330% ($p<0.0001$) in Th3 (**Figures 14F,G**).

A summary of these findings is provided in **Figure 14H**, which illustrates the roles of each Tv1 terminal selector in the maintenance of *dimm.K1* reporter activity and Dimm protein levels.

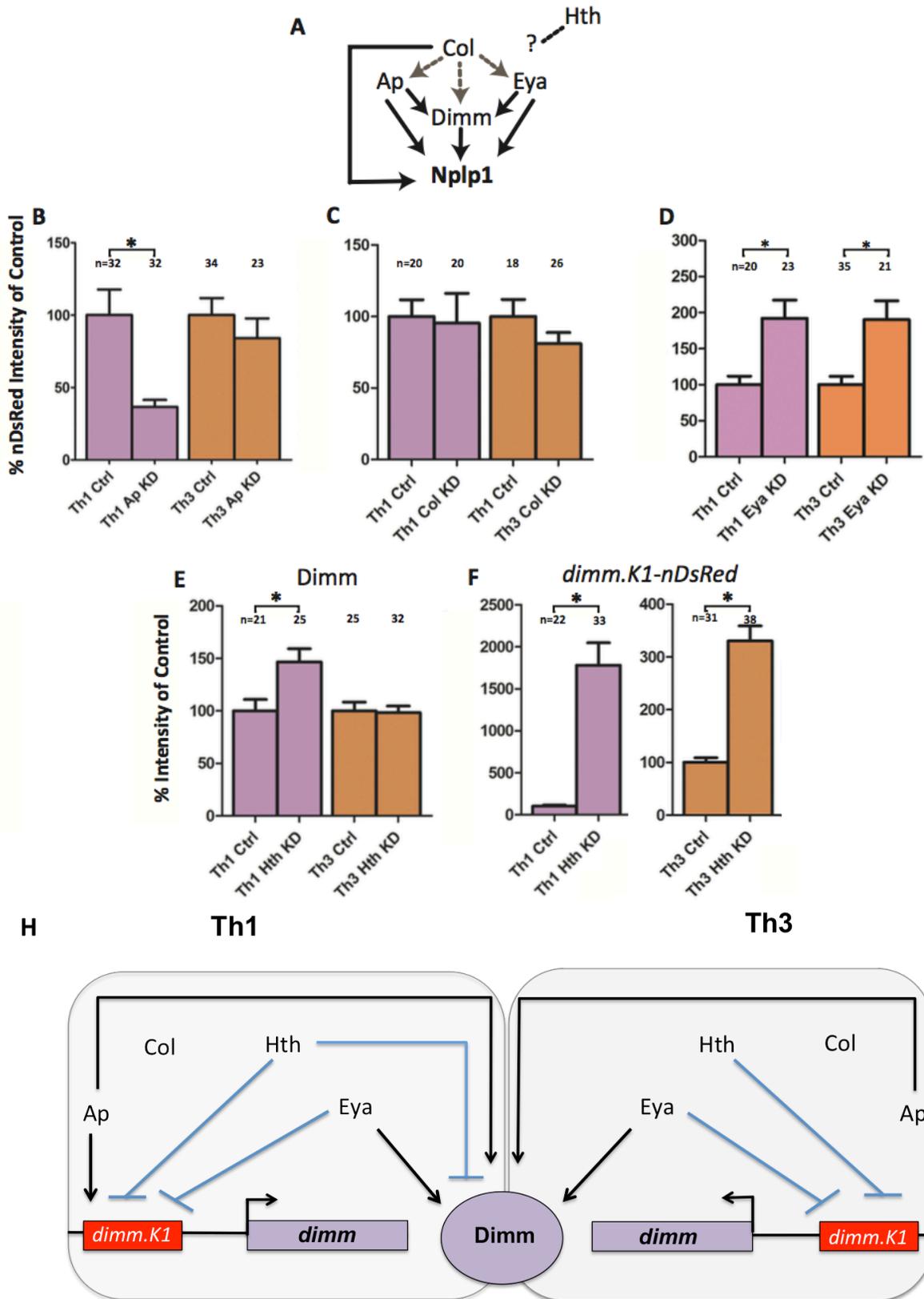


Figure 14.

Figure 14. Maintenance of *dimm.K1-nDsRed* in Tv1 neurons.

(A) The previously defined Tv1 adult terminal selector network. Gray dotted arrows: regulatory relationships specific to initiation. Black arrows: regulatory relationships that exist at initiation and maintenance. (B-D) The TARGET system was used to knockdown Ap, Col and Eya from A1 onwards and the intensity of *dimm.K1* reporter relative to controls was quantified. Ap knockdown only decreased *dimm.K1* reporter activity in Th1, Col knockdown had no effect on *dimm.K1* reporter activity and Eya knockdown up-regulated *dimm.K1* reporter activity in Th1 and Th3. (E-G) The TARGET system was used to knockdown Hth from A1 onwards. Hth knockdown had no effect on Nplp1 immunoreactivity, but up-regulated Dimm immunoreactivity in Th1 and upregulated *dimm.K1* reporter activity in Th1 and Th3. For all data, relative intensities are presented as the % mean of control expression levels +/- SEM. Student's two-tailed t-tests were used to compare control and experimental populations (* = p<0.05). (H) A summary of terminal selector maintenance function, highlighting our findings that a transcription factor's role can be body-segment specific, and can differ for *dimm.K1* and Dimm protein maintenance. Black arrows represent regulatory relationships that exist both during development and in adult neurons. Blue arrows represent maintenance-specific relationships. **Genotypes:** Ctrl (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*), Ap KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-ap^{dsRNAi}*), Col KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-col^{dsRNAi}*), Eya KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-eya^{dsRNAi}*), Hth KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-hth^{dsRNAi}*).

3.7 Maintenance of *dimm.K1-nDsRed* in Tv4 neurons.

Eade *et al.* (2012) demonstrated that Ap, Eya, Dimm, Dac, Sqz and BMP signaling all maintain FMRFa in the adult, and also that although Ap and Eya both initiate Dimm, only Ap maintains Dimm (**Figure 15A**) [36]. Here we assessed how these data on Dimm protein compare to *dimm.K1-nDsRed* maintenance in adults. As above, we knocked down Ap, Eya, Dac, Sqz and BMP signalling and quantified the intensity *dimm.K1* reporter relative to controls. Successful knockdown of each transcription factor was confirmed by immunocytochemistry (**Figure 28**). Ap knockdown reduced *dimm.K1* reporter intensity to 20% ($p < 0.0001$) of controls by A20 (**Figure 15B**). Knockdown of BMP signaling (*tkv^{DN}* ; *wit^{DN}*) reduced *dimm.K1* reporter intensity to 19% in Th1 ($p = 0.0001$) and 15% in Th3 ($p = 0.0096$) by A10. (**Figure 15C**). Dac knockdown (*dac^{dsRNAi}*) reduced *dimm.K1* reporter intensity to 49% in Th1 ($p = 0.0032$) and 65% in Th3 ($p = 0.0030$) by A10. (**Figure 15D**). Eya knockdown reduced *dimm.K1* reporter intensity to 25% ($p < 0.0003$) of control levels by A20 (**Figure 15E**). Sqz knockdown (*sqz^{dsRNAi}*) reduced *dimm.K1* reporter intensity to 54% in Th1 ($p = 0.0001$) and 74% in Th3 ($p = 0.0037$) by A15 (**Figure 15F**). Since knockdown of every terminal selector resulted in a decrease in *dimm.K1* reporter intensity in Tv4 neurons, we wished to help eliminate concerns of non-specific effects. We confirmed that adult-specific expression of *col^{dsRNAi}* had no effect on *dimm.K1* reporter intensity in Tv4 neurons by A10. We also confirmed that knockdown of Tv4 specific regulators, such as BMP signalling, did not decrease reporter expression in Tv1 neurons. Further, we confirmed differences between *dimm.K1-nDsRed* and Dimm responses to genetic perturbation in the same neurons. Following Eya knock-down we simultaneously measured anti-Dimm and *dimm.K1* reporter intensities; the effects on *dimm.K1* reporter intensities are presented in **Figures 14D** and **15E**, and the effects on anti-Dimm intensities were consistent with previously published work (**Figure 29**) [36].

A summary of these findings is provided in **Figure 15G**, which illustrates the roles of each Tv4 terminal selector in the maintenance of *dimm.K1* reporter activity and Dimm protein

levels. In addition, a comprehensive summary of all adult Tv1 and Tv4 findings is provided in **Figure 16.**

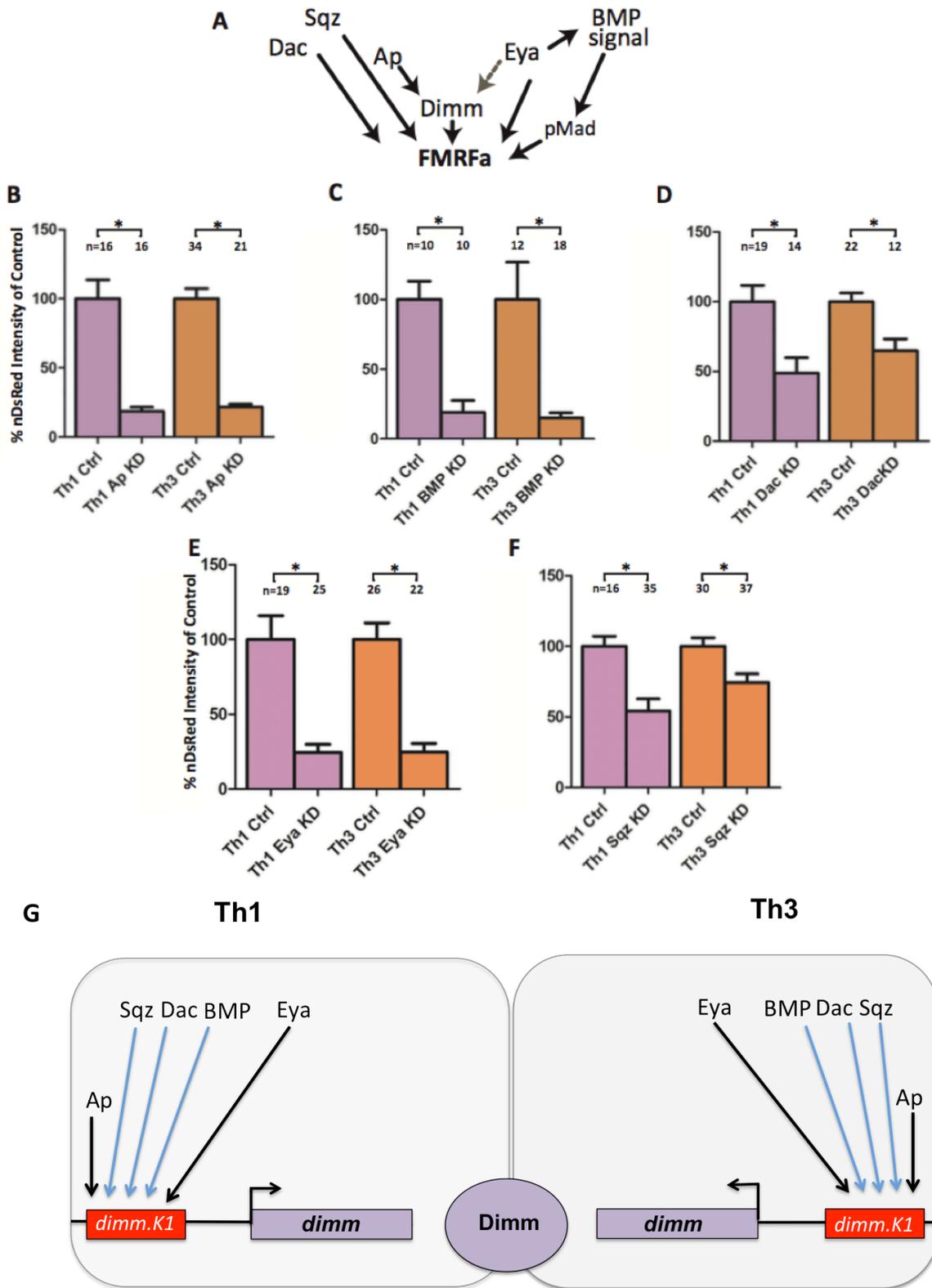


Figure 15.

Figure 15. Maintenance of *dimm.K1-nDsRed* in Tv4 neurons.

(A) The previously identified Tv4 adult terminal selector network. Gray dotted arrows: regulatory relationships specific to initiation. Black arrows: regulatory relationships that exist at initiation and maintenance. (B-F) Knockdown of terminal selectors and quantification of *dimm.K1* reporter intensities relative to controls. Relative intensities are presented as the % mean of control expression levels +/- SEM. Student's two-tailed t-tests were used to compare control and experimental populations (* = p<0.05). (G) A summary of terminal selector maintenance function. Black arrows represent regulatory relationships that exist both during development and in adult neurons. Blue arrows represent maintenance-specific relationships. Adult-specific genetic perturbations can have different consequences for *dimm.K1-nDsRed* than they do for Dimm. **Genotypes:** Ctrl (UAS-dicer2/Y ; ap^{Gal4}, *dimm.K1-nDsRed*/+ ; tubP>Gal80^{TS}, UAS-nEGFP/+ and ap^{Gal4}, *dimm.K1-nDsRed*/+ ; tubP>Gal80^{TS}, UAS-nEGFP/+), Ap KD (UAS-dicer2/Y ; ap^{Gal4}, *dimm.K1-nDsRed*/+ ; tubP>Gal80^{TS}, UAS-nEGFP/UAS-ap^{dsRNAi}), BMP KD (ap^{Gal4}, *dimm.K1-nDsRed*/UAS-*tkv*^{DN} ; tubP>Gal80^{TS}, UAS-nEGFP/UAS-wit^{DN}), Dac KD (UAS-dicer2/Y ; ap^{Gal4}, *dimm.K1-nDsRed*/dac³, UAS-dac^{dsRNAi} ; tubP>Gal80^{TS}, UAS-nEGFP/+), Eya KD (UAS-dicer2/Y ; ap^{Gal4}, *dimm.K1-nDsRed*/+ ; tubP>Gal80^{TS}, UAS-nEGFP/UAS-*eya*^{dsRNAi}), Sqz KD (UAS-dicer2/Y ; ap^{Gal4}, *dimm.K1-nDsRed*/+ ; tubP>Gal80^{TS}, UAS-nEGFP/UAS-*sqz*^{dsRNAi}).

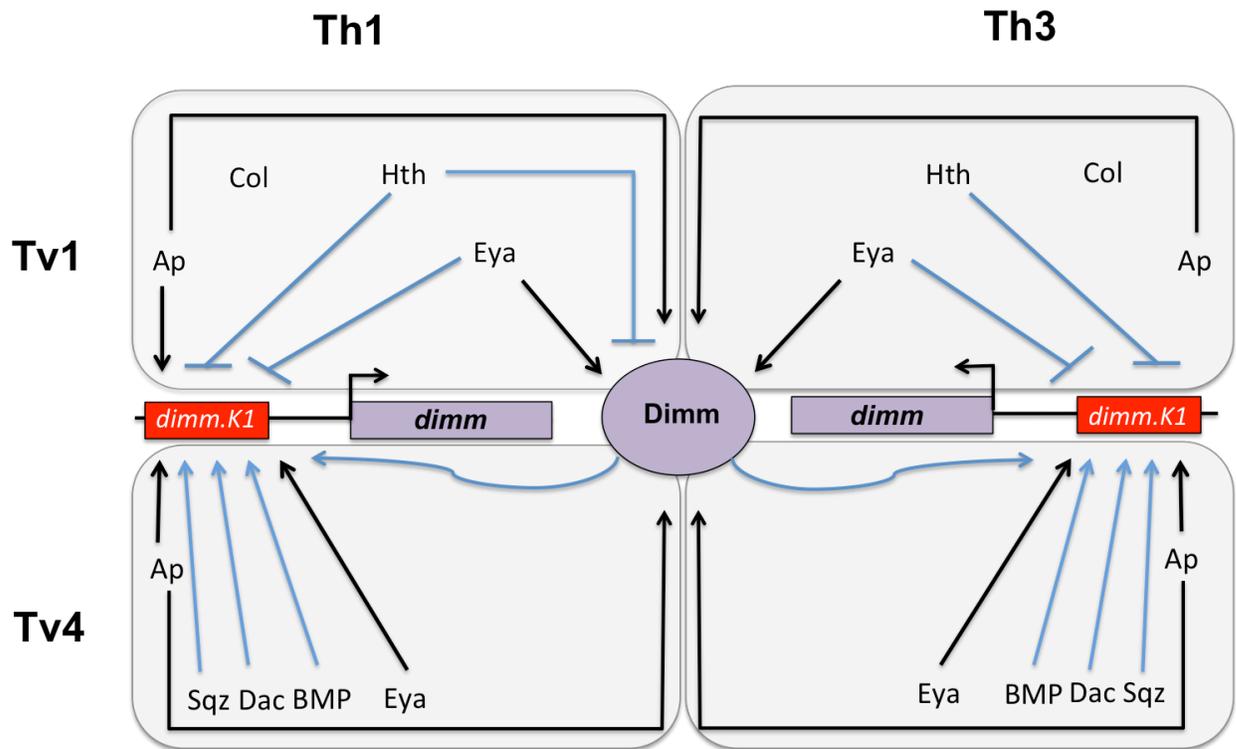


Figure 16. Maintenance of *dimm.K1* and Dimm.

A comprehensive summary of how known terminal selectors regulate maintenance of *dimm.K1-nDsRed* and Dimm protein in Tv1 and Tv4 neurons. Black arrows: regulatory relationships that exist at both initiation and maintenance. Blue arrows: regulatory relationships that exist at maintenance only.

3.8 *ap.K1* is necessary for *ap* expression in Tv1 and Tv4 neurons.

Prior to this thesis work we had identified *ap.K1*, a ~3.8 kb region of genomic DNA upstream of *ap* that is sufficient to initiate, but not maintain, reporter expression in Tv1 and Tv4 neurons. The Thor lab generated a line (*apΔS*) in which a genomic region overlapping with endogenous *ap.K1* is deleted (**Figure 17A**). A reliable Ap antibody was not available, so they demonstrated that this region is necessary to initiate *ap* expression in Tv1 neurons by showing that *Stg17* deletion mutants exhibit a reduction in *Nplp1* expression that phenocopies *ap* mutants (unpublished personal communication). We tested whether the *ap.K1/ap.S* CRM region is necessary for *ap* initiation in Tv4 neurons. We dissected *w¹¹¹⁸* (control) and *apΔS* VNCs at *Stg17* and found that anti-FMRFa intensity in *apΔS* Tv4 neurons (n=35) was reduced to 33% of controls (n=36); p<0.0001. Since this result also phenocopies *ap* mutants, we conclude that *ap.K1/ap.S* is necessary to initiate *ap* expression in Tv4 neurons [85]. We also confirmed the *Nplp1* phenotype, observing that all (n=36) *w¹¹¹⁸* Tv1 neurons exhibited robust anti-*Nplp1*, whereas only 30% (n=36) of *apΔS* Tv1 neurons exhibited anti-*Nplp1* immunoreactivity, and this was severely reduced compared to wild-type (**Figure 17B-D**).

We also assessed neuropeptide expression in *w¹¹¹⁸* (control) and *apΔS* Tv1 and Tv4 neurons at A2 (*apΔS* animals do not survive to A5). All (n=40) *w¹¹¹⁸* Tv1 neurons exhibited robust anti-*Nplp1*, whereas only 27% (n=40) of *apΔS* Tv1 neurons exhibited *Nplp1* immunoreactivity, and this was severely reduced compared to wild-type. Similarly, but not as dramatically, the intensity of FMRFa immunoreactivity in *apΔS* Tv4 neurons (n=26) was reduced to 56% of controls (n=30); p<0.0001 (**Figure 17E-G**).

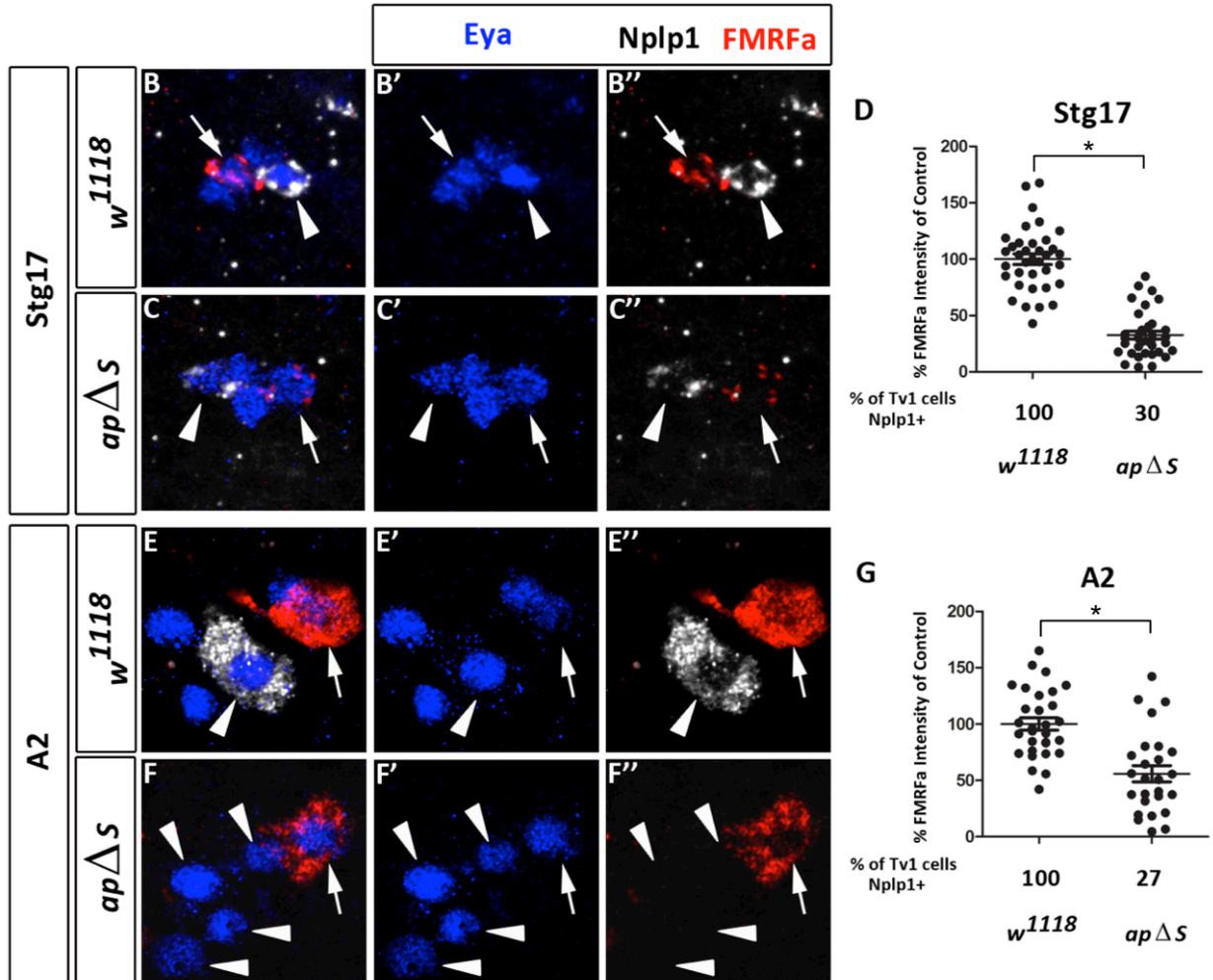
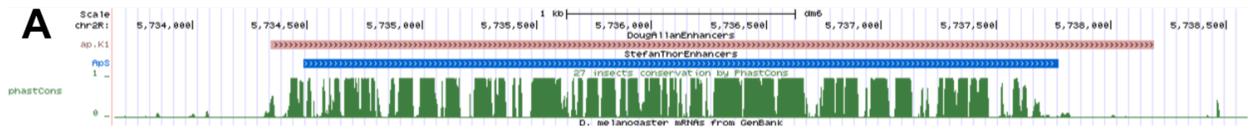


Figure 17.

Figure 17. *ap.K1* is necessary for *ap* expression in Tv1 and Tv4 neurons.

(A) A UCSC genome browser comparison of the genomic regions encompassed by *ap.K1* (pink) and *ap.S* (blue). (B) A representative Stg17 *w¹¹¹⁸* (control) Tv cluster. (C) A representative Stg17 *apΔS* Tv cluster. (D) At Stg17 anti-FMRFa intensity in *apΔS* Tv4 neurons was reduced to 33% of controls and only 30% of *apΔS* Tv1 neurons were positive for anti-Nplp1. (E) A representative A2 *w¹¹¹⁸* Tv cluster. (F) A representative A2 *apΔS* Tv cluster. (G) At Stg17 anti-FMRFa intensity in *apΔS* Tv4 neurons (n=35) was reduced to 56% of controls and only 27% of *apΔS* Tv1 neurons were positive for anti-Nplp1. Relative intensities are presented as the % mean of control expression levels +/- SEM. Student's two-tailed t-tests were used to compare control and experimental populations (*=p<0.05).

3.9 Identification of a putative Tv1 and Tv4 *ap* maintenance CRM

Ap's expression pattern in the *Drosophila* larval imaginal wing disc is crucial to proper development of the adult wing. Importantly, Ap is expressed exclusively in the dorsal compartment of this tissue, and several lines of evidence support a model in which PcG proteins are recruited to a TRE/PRE proximal to the *ap* promoter in order to suppress *ap* expression in the ventral compartment [87-90]. Bieli *et al.* (2015) demonstrated that *ap* expression in the dorsal compartment is initiated and refined by two CRMs located upstream of *ap*, and that the TRE/PRE proximal to the *ap* promoter works synergistically with these two elements to maintain *ap* expression as the wing disc continues to develop [91]. They further demonstrated that the maintenance function of the TRE/PRE is dependent upon TrxG proteins. Thus, ultimately, these data support a model in which the TRE/PRE proximal to the *ap* promoter acts as a PRE in the ventral compartment of the wing disc, where it recruits PcG proteins to keep *ap* expression off, and as a TRE in the dorsal compartment, where it recruits TrxG proteins to keep *ap* expression on. We tested whether the TRE/PRE-encompassing region further characterized by Bieli *et al.* maintains *ap* expression in Tv1 and Tv4 neurons as it does in the dorsal compartment of the imaginal wing disc.

We placed *ap.K1* and the putative maintenance element (M) upstream of a nuclear-localized EGFP encoding reporter gene and integrated this construct into the third chromosome of the *D. melanogaster* genome. *ap.K1.M-nEGFP* VNCs were dissected at Stg17 to test initiation and A5 to test maintenance. We found that *ap.K1.M* drove reporter expression in Tv1 and Tv4 neurons at both Stg17 and A5 (**Figure 18**). Thus the addition of the M element adds a maintenance function to the *ap.K1* CRM, indicating that M is a functional maintenance element.

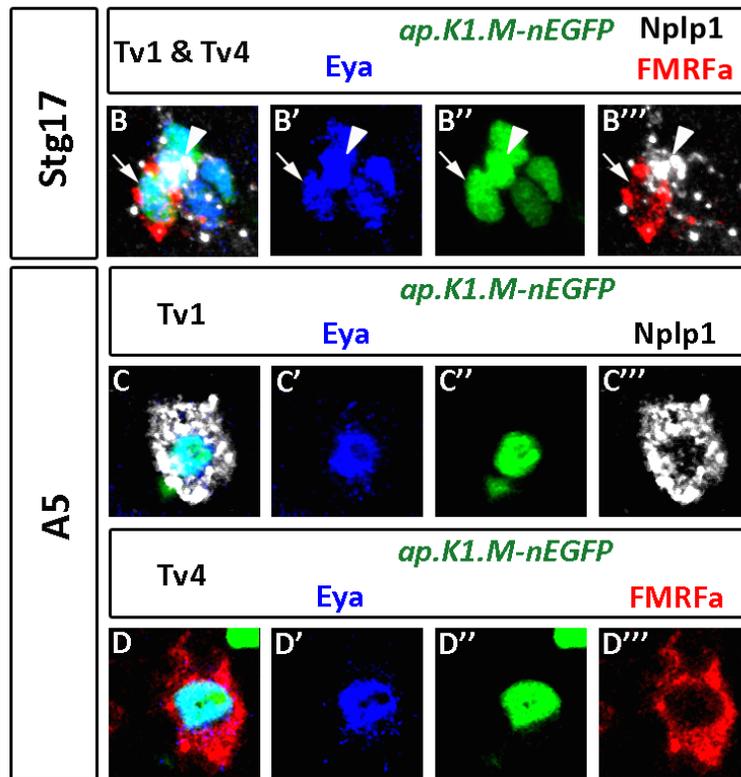
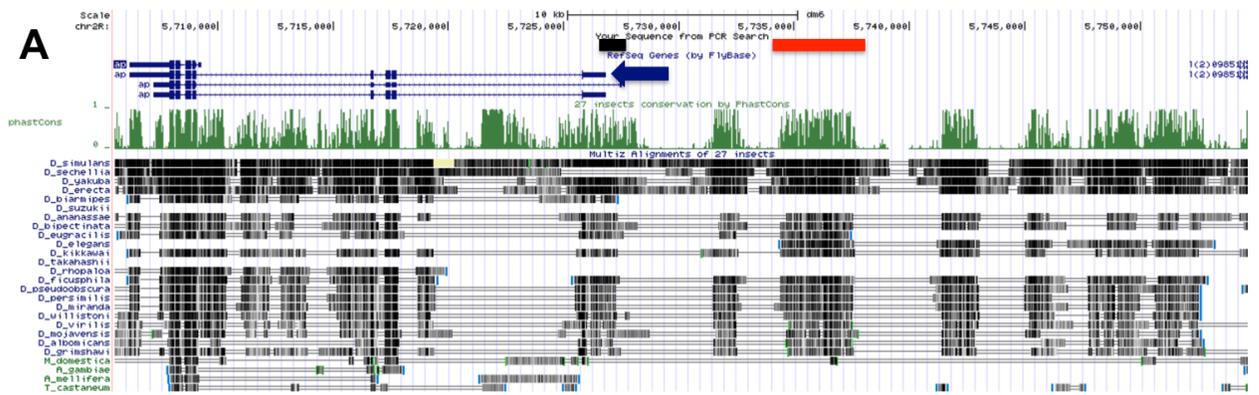


Figure 18. Identification of a putative Tv1 and Tv4 *ap* maintenance CRM.

(A) A UCSC genome browser view of the *ap* locus. The *ap* gene is indicated with a blue arrow. The red rectangle represents *ap.K1* and the black rectangle represents the putative maintenance element. (B-D) We dissected *ap.K1.M-nEGFP* VNCs at Stg17 to test initiation and A5 to test maintenance. Tissue was stained with anti-Eya to visualize the Tv clusters, anti-Nplp1 to visualize the Tv1 neurons, and anti-FMRFa to visualize the Tv4 neurons. Reporter expression was driven in all Tv1 (arrow heads) and Tv4 (arrows) neurons at both Stg17 (B-B''') and A5 (C-C''' and D-D''').

4. DISCUSSION

4.1 Summary of major findings

Prior to this thesis work we had identified *dimm.K1*, a 756 bp region of genomic DNA upstream of *dimm* that is sufficient to initiate and maintain reporter expression in all Tv1 and Tv4 neurons. We had also identified *ap.K1*, a ~3.8 kb region of genomic DNA upstream of *ap* that is sufficient to initiate, but not maintain, reporter expression in Tv1 and Tv4 neurons (see Section 1.8). The major findings of this thesis work include:

- *dimm.K1* is necessary for *dimm* expression in Tv1 and Tv4 neurons (**Figure 11**).
- The transcriptional initiation of *dimm.K1* matches that found for Dimm protein, and is the same for all Tv1 and Tv4 neurons (**Figure 12**).
- The transcriptional regulation of *dimm.K1* maintenance differs between Tv1 and Tv4 neurons. Further, we find that different transcriptional inputs are required for maintenance of *dimm.K1* in Tv1 neurons of different body segments. Furthermore, we find the *dimm.K1* reporter and Dimm protein show differences in their responses to the loss of specific transcriptional inputs in adult neurons (**Figure 16**).
- *ap.K1* is necessary for *ap* expression in Tv1 and Tv4 neurons (**Figure 17**).
- We identified a genomic region that, when added to the *ap.K1* CRM, is sufficient to drive maintenance of reporter activity in Tv1 and Tv4 neurons. This maintenance element region is proximal to the *ap* promoter and has previously been described to have TRE/PRE activity in another *ap*-expressing tissue (**Figure 18**).

These findings lead us to the following major conclusions (**Figure 19**):

- 1) Initiation and maintenance mechanisms can be very distinct for terminal selector transcription factors.
- 2) Various maintenance mechanisms can be employed to maintain the expression of different transcription factors within a single network.
- 3) The maintenance mechanisms for a single transcription factor can differ between cell subtypes, and also exhibit difference between individual neurons of a single subtype.

With reference to **Figure 19**, the specific findings (**A**) through (**D**) show that the common mechanism of *dimm.K1* initiation in Tv1 and Tv4 neurons diverges into a variety of distinct maintenance mechanisms that differ between cell subtypes, and even between neurons of the same subtype. (**E**) shows that mechanisms of *ap* initiation and maintenance are generated from distinct CRMs, emphasizing that initiation and maintenance mechanisms are distinct. Our findings can also be discussed with regard to the existing models of transcription factor maintenance (**Figure 1**). In Tv4 neurons, maintenance of *dimm.K1* is dependent on persistent input from Ap and Eya, auto-regulation (**Figure 1B**) and cross-regulation (**Figure 1C**) by Sqz, Dac and BMP signaling. In Th1 Tv1 neurons, maintenance of *dimm.K1* is dependent on persistent input from Ap. Maintenance of *dimm.K1* in Th3 Tv1 neurons is dependent upon unknown inputs; this could suggest that unidentified maintenance-dedicated transcription factors (**Figure 1D**) (or unidentified developmental transcription factors) maintain *dimm.K1* expression in Th3 Tv1 neurons. The putative *ap* maintenance element that is sufficient to maintain reporter expression in Tv1 and Tv4 neurons when added to *ap.K1* encompasses a region that functions as a TRE in the dorsal compartment of the developing *Drosophila* imaginal wing disc. The putative TRE function of this region has yet to be investigated in Tv1 and Tv4 neurons, but cumulatively the evidence suggests that *ap* expression may be maintained by TrxG proteins in Tv1 and Tv4

neurons (**Figure 1E**). Therefore, various combinations of possible maintenance strategies are employed in the Tv1 and Tv4 transcription factor networks. Furthermore, we have found that the specific configurations of these strategies can exhibit a high degree of cell-specificity.

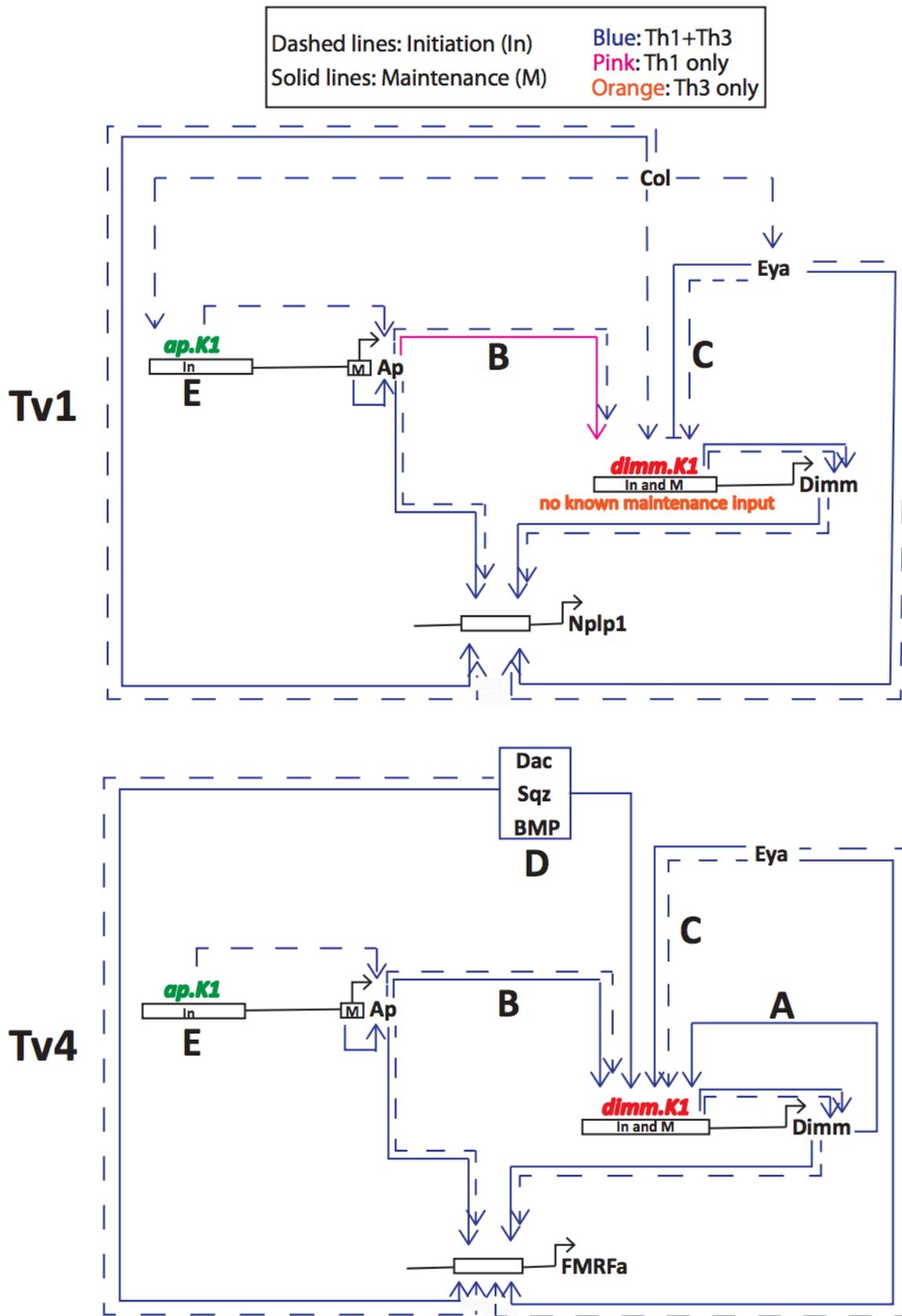


Figure 19.

Figure 19. Maintenance of Tv1 and Tv4 subtype identities.

Dashed lines represent initiation (In) and solid lines represent maintenance (M). Regulatory relationships common to both Th1 and Th3 neurons are shown in blue. Regulatory relationships specific to Th1 or Th3 neurons are shown in pink or orange respectively. Key findings that contribute to the major conclusions of this thesis work include: **(A)** Auto-regulation maintains *dimm.K1* expression in Tv4 neurons only. **(B)** *Ap* maintains *dimm.K1* expression in Th1 and Th3 Tv4 neurons but only regulates *dimm.K1* maintenance in Th1 Tv1 neurons. **(C)** *Eya* promotes initiation and maintenance of *dimm.K1* in Tv4 neurons; in Tv1 neurons *Eya* promotes *dimm.K1* at initiation but represses *dimm.K1* at maintenance. **(D)** *dimm.K1* maintenance is subject to cross-regulation by other known terminal selectors in Tv4 only. **(E)** The insufficiency of *ap.K1* for maintenance makes it clear that differences between *ap* initiation and maintenance mechanisms exist.

4.2 Biological relevance of our findings

Our results suggest that the strategies used to maintain *ap* expression are different from those used to maintain *dimm* expression. The expression of *ap* may be maintained by TrxG proteins that stably ‘lock-in’ an open chromatin environment at the *ap* locus. In contrast, *dimm.K1* appears to require constant processing of various terminal selector inputs throughout life. Interestingly, *dimm.K1* uses a maintenance strategy similar to those used by Nplp1 and FMRFa, and this observation may provide insight into the biological significance behind the use of different maintenance strategies. In the cases of Nplp1 and FMRFa, persistent dependence on multiple inputs may facilitate dynamic mechanisms of gene maintenance that can efficiently up- or down-regulate neuropeptide expression in response to environmental cues. In addition to its roles in the transcriptional regulation of Nplp1 and FMRFa, Dimm also regulates multiple genes involved in neuropeptide processing and secretory machinery biogenesis [92]. Thus, Dimm’s involvement at multiple levels of neuropeptide production and release may necessitate its use of a similarly dynamic strategy of gene maintenance. Ap, on the other hand, is a higher order transcription factor in the Tv1 and Tv4 networks and functions to maintain both terminal selector and neuropeptide expression. Thus, a stable ‘lock-in’ mechanism of *ap* maintenance by TrxG proteins may be more appropriate.

It is also worth emphasizing that the cell-specific maintenance mechanisms we have found are not stochastic. In other words, we have not found that Tv1 and Tv4 neurons use a common mechanism of *dimm.K1* initiation and then randomly adopt one of many possible maintenance strategies. If this were the case, the differences we have shown would not have been detected. Ultimately, each cell-specific difference is deterministic and reproducible. The cell-specific gene regulatory mechanisms exhibited amongst Tv1 neurons of different body segments (**Figure 14**) is particularly striking, but is consistent with emerging concepts in the literature. There is growing appreciation for the fact that cell populations previously thought to

be homogeneous, due to the fact that they express a common set of effector genes, are actually heterogeneous when their characteristics are observed at higher resolution. High resolution, single-cell gene profiling approaches have been used to demonstrate heterogeneity at the level of transcriptomes and have been successful in defining cell types with more precision [93]. At the level of epigenomes, heterogeneity in cell populations can manifest as differences in the DNase hyperaccessibility of particular loci or as differences in the organization of the genome, which is thought to have implications for cell-to-cell differences in enhancer-promoter communication patterns and thus gene regulatory mechanisms [94-97]. These studies highlight the power of our model system, which allows us to study Tv1 and Tv4 neurons at single-cell resolution *in vivo*, and reproducibly from one animal to the next; we are in an excellent position to contribute to these emerging concepts and to handle any implications that may arise from these studies.

4.3 Considerations and limitations of data interpretation relevant to discussion of our data

The cell-specific mechanisms of *dimm.K1* maintenance is an important finding. However, we must remember that our ultimate goal is to obtain a detailed understanding of how *dimm* is initiated and maintained, and thus an understanding of how *dimm.K1* fits into this objective. There are two major considerations that emerged during this thesis work that do limit our interpretation of the data in this respect:

- 1) The response of *dimm.K1* reporter activity to genetic perturbations was compared to the response of Dimm protein levels. The primary caveat here is that we wish to study how *dimm.K1* regulates *dimm* transcription, and Dimm protein levels are not necessarily an accurate measure of this (due to possible post-transcriptional effects, such as transcript regulation and translational and/or post-translational regulation). Therefore, it is difficult to determine whether the discrepancies in our data regarding how adult-specific genetic perturbations affect *dimm.K1* reporter activity and how they affect Dimm protein levels are due to a discrepancy between *dimm.K1* and *dimm* transcriptional activities, or due to a discrepancy between the outcome of these perturbations on *dimm* transcriptional activity and Dimm protein levels. To resolve this we plan to knock a fluorescent reporter gene into the native *dimm* locus and compare the effects of genetic perturbations on *dimm.K1* and transcription of the native *dimm* gene.
- 2) We are studying the transcriptional regulation of *dimm.K1* outside of its endogenous locus. For decades we have learned a lot about enhancer biology from studying isolated regulatory regions in this way; nonetheless, given advancements in our understanding of chromatin heterogeneity and in how intra- and inter-chromosomal interactions within the three dimensional organization of the nucleus may influence transcription, we must be aware of the fact that these experiments could be limited by lack of appropriate context. However, *dimm.K1* reporter activity is restricted to Dimm expressing neurons in the

VNC and regulation of *dimm.K1* initiation matches regulation of Dimm initiation; these findings suggest that the *dimm.K1* reporter transgene does indeed provide biologically relevant insight into the involvement of *dimm.K1* in initiation and maintenance of *dimm* expression.

4.4 Possible involvement of additional CRMs in *dimm* maintenance

If we knock a fluorescent reporter gene into the native *dimm* locus and find that the response of this reporter gene to adult-specific genetic perturbations is equivalent to the response of Dimm protein levels, we will have to address the discrepancies that exist between *dimm.K1* and *dimm* transcriptional activities in adult neurons. To explain these possible discrepancies it may be reasonable to propose that *dimm.K1* acts in conjunction with other CRMs in order to maintain *dimm* expression (**Figure 20**). Studies making use of chromosome conformation capture technologies have shown that promoters are often involved in complex regulatory interactions with multiple CRMs, and thus there is emerging appreciation for the idea that CRMs coalesce into regulatory ensembles in order to stabilize transcription from gene promoters [16, 98-100]. Given emerging evidence, I now consider the existence of additional CRMs involved in *dimm* maintenance to be a probable scenario; thus, I address below how we could approach such complexities, should it become necessary.

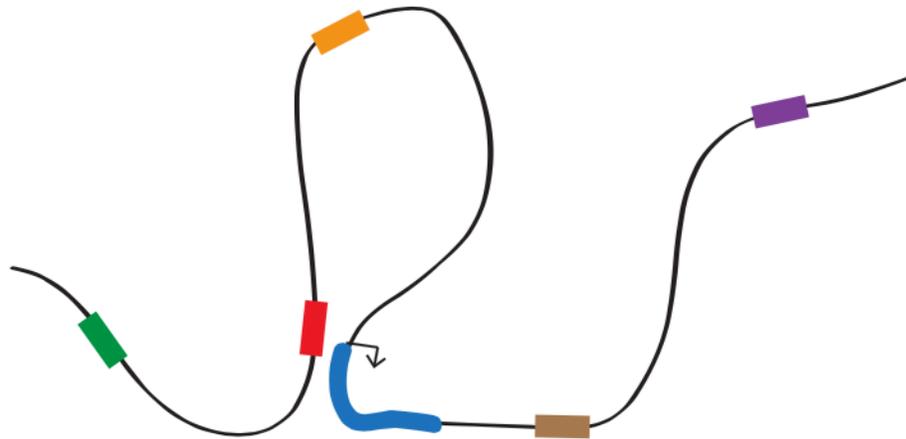
How promoters integrate information from multiple CRMs is not well understood, but we can gain some mechanistic insight from literature on ‘shadow enhancers’. Mike Levine and colleagues initially coined the term ‘shadow enhancer’ to describe redundant secondary enhancers that increase robustness of gene expression under adverse conditions, such as environmental stress and genetic perturbations. Use of the term has since evolved to more generally describe situations in which a gene is regulated by multiple distinct enhancers, each of which drives the same or similar expression patterns. Although examples of multiple separable

enhancers driving apparently redundant expression patterns for a single gene have existed for decades, new evidence suggests a variety of functional roles for such *cis*-regulatory arrangements [101]. It has been proposed, for example, that the temporally-specific dominance of one shadow enhancer over another is crucial to the refinement of gap gene expression patterns in the developing *Drosophila* embryo [102]. In another study particularly relevant to the findings presented in this thesis, computational models suggest that regulation of the *Drosophila* pair-rule gene *even-skipped* (*eve*) in a specific domain of the developing embryo is accomplished by the activities of two shadow enhancers. Importantly, each enhancer in this model uses different regulatory logic to generate the same expression pattern. Thus the effects of genetic perturbations on gene expression driven by each enhancer, and on endogenous gene expression, are different. A single transcription factor, for example, is proposed to have bifunctionality in its regulation of gene expression, acting as an activator through one of the enhancers and as a repressor through the other [11].

To illustrate how concepts from the above examples could explain some of our data, let us consider our observation that, in adult Tv1 neurons, Eya acts as an activator of Dimm expression, but a repressor of *dimm.K1*. Perhaps this is a situation in which more than one CRM is involved in regulating maintenance of *dimm* expression, and Eya has bifunctionality in that it acts as a repressor through *dimm.K1* and an activator through an additional CRM. If this additional CRM is dominant under steady-state conditions, this could explain why knock-down of Eya ultimately results in down-regulation of *dimm* expression (**Figure 21**). Let us also consider our observation in adult Tv4 neurons, that while knock-down of Ap results in reduced expression of both *dimm.K1-nDsRed* and Dimm, knock-down of other regulators such as Sqz, Dac and BMP signaling specifically affect maintenance of *dimm.K1*. Perhaps Ap regulates *dimm* maintenance through both *dimm.K1* and an additional CRM, and this additional CRM can compensate to sustain wild-type levels of *dimm* under the genetic perturbations that specifically

affect maintenance of gene expression through *dimm.K1* (**Figure 22**). These two examples have been highlighted to illustrate how one might go about thinking about such concepts. With so many unknowns, it is difficult to speculate further on what the *cis*-regulatory architecture involved in *dimm* maintenance might look like should these additional CRMs exist.

A. *dimm* Initiation



B. *dimm* Maintenance

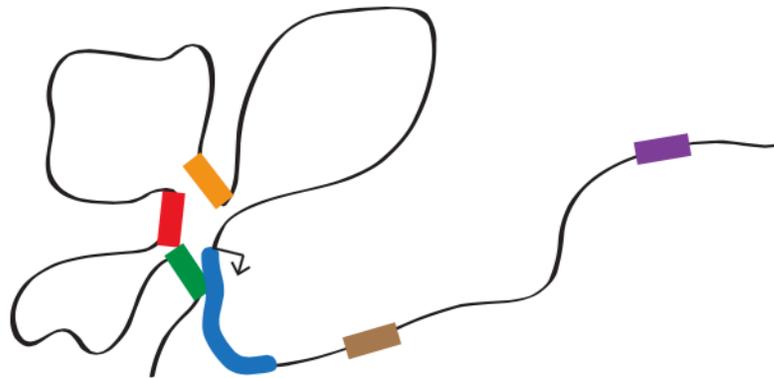


Figure 20. Possible involvement of other CRMs in *dimm* maintenance.

If the response of *dimm* transcription to adult-specific genetic perturbations is equivalent to the response of Dimm protein levels this could suggest that *dimm.K1* encompasses the primary CRM(s) governing initiation of *dimm* expression, but that *dimm.K1* acts in conjunction with other CRMs in a cell-specific manner in order to maintain *dimm* expression. Possible model: (A) Developmental transcription factors including Ap and Eya assemble on *dimm.K1* (red) and interactions between *dimm.K1* and the *dimm* (blue) promoter initiate gene expression in all Tv1 and Tv4 neurons. (B) Other regulatory regions (orange, green) (and corresponding transcription factors) coalesce to confer dynamic stability of gene expression.

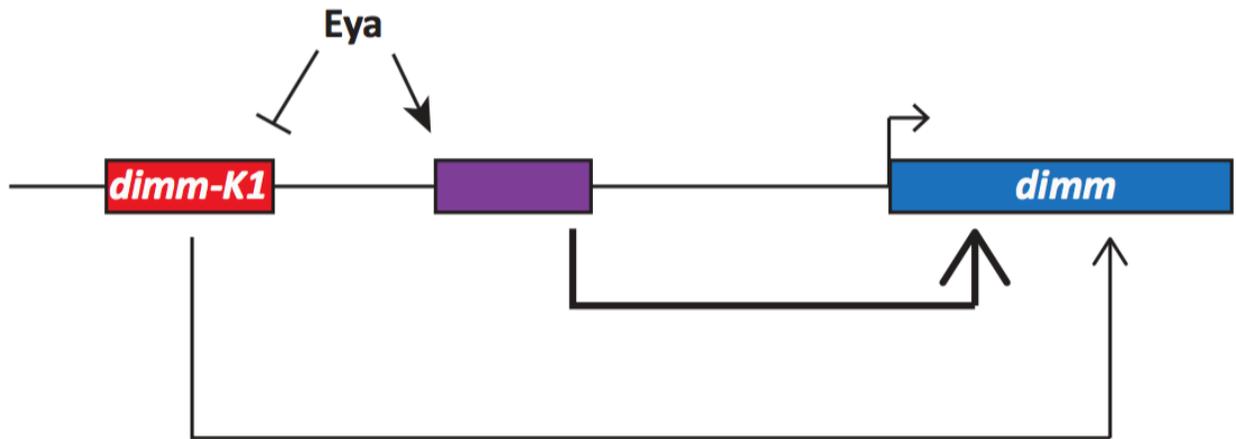
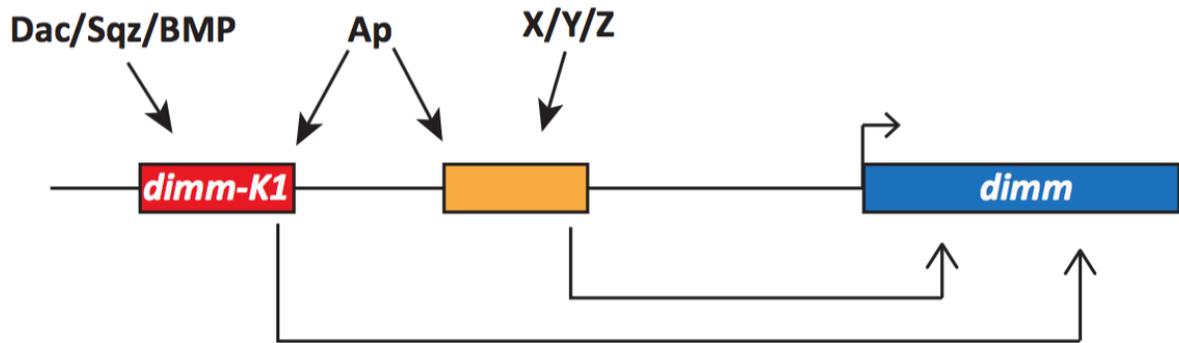


Figure 21. If Eya acts as an activator of *dimm* expression, but a repressor of *dimm.K1*.

Perhaps this is a situation in which more than one CRM is involved in regulating maintenance of *dimm* expression, and Eya has bifunctionality in that it acts as a repressor through *dimm.K1* and an activator through an additional CRM. If this additional CRM is dominant under steady-state conditions, this could explain why knockdown of Eya ultimately results in down-regulation of *dimm* expression.

A



B

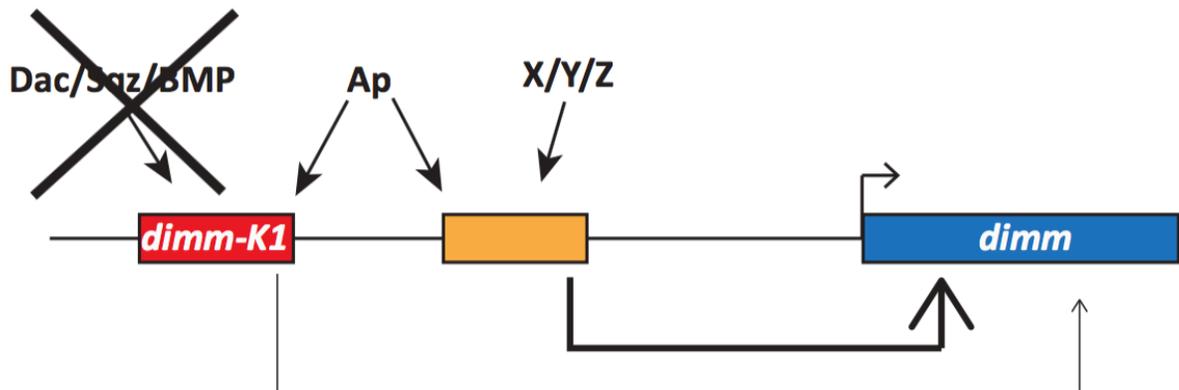


Figure 22. If Sqz, Dac and BMP signaling affect maintenance of *dimm.K1*, but not *dimm*.

(A) Perhaps Ap regulates *dimm* maintenance through both *dimm.K1* and an additional CRM, (B) and this additional CRM can compensate to sustain wild-type levels of *dimm* under the genetic perturbations that specifically affect maintenance of gene expression through *dimm.K1*.

4.5 Future directions

4.5.1 *dim*

4.5.1.1 Immediate goals

As discussed previously it will be important for us to knock a fluorescent reporter gene into the native *dim* locus and compare the effects of genetic perturbations on *dim.K1* and transcription of the native *dim* gene. To completely uncouple regulation of this reporter gene from that of Dimm protein, we plan to replace the endogenous *dim* gene with the fluorescent reporter gene. Although Dimm is auto-regulatory, this should not affect the interpretation of our results, as we can use the fluorescent reporter gene as a heterozygote and the resulting decrease in Dimm dosage will be consistent between control and experimental genotypes. These experiments will resolve whether the discrepancies in our data regarding how adult-specific genetic perturbations affect *dim.K1* reporter activity and how they affect Dimm protein levels are due to a discrepancy between *dim.K1* and *dim* transcriptional activities, or due to a discrepancy between the outcome of these perturbations on *dim* transcription and Dimm protein levels.

Also, to unambiguously determine whether *dim.K1* is necessary for maintenance of *dim* expression, we must assess Dimm expression following deletion of *dim.K1* specifically in adult neurons, after development. We are planning to FRT-flank the *dim.K1* enhancer (through CRISPR or homologous recombination strategies) for adult specific deletion of *dim.K1* through adult induction of Flp recombinase in Tv neurons. Such *cis*-FRT excision has proven very reliable and will be used to determine the requirement for *dim.K1* in maintaining *dim* transcription [103].

4.5.1.2 Long-term goals

We have only explored the role of known developmental Tv1 and Tv4 terminal selectors in the regulation of *dimm* maintenance, and at this point we do not know, for example, what factors are maintaining *dimm.K1* reporter activity in Th3 Tv1 neurons (**Figure 19**). To identify additional *trans*-regulatory factors that regulate *dimm* maintenance we could employ a multi-step approach. First, we could observe the response of fluorescent Nplp1 and FMRFa transcriptional reporters to adult-specific RNAi knockdown of candidate factors. This approach would identify factors involved in maintaining neuropeptide expression and those involved in maintaining any of the terminal selectors. To identify factors that maintain *dimm*, we could observe the response of a fluorescent reporter gene driven from the native *dimm* locus to adult-specific knockdown of factors identified with the first approach. To pair factors that maintain *dimm* with their corresponding CRM(s) we could subsequently observe the response of fluorescent reporter genes driven by individual CRM(s) to adult-specific knockdown of factors shown to maintain *dimm*.

We may also be interested in identifying the direct regulators of *dimm* CRM(s) and characterizing the sites at which they bind to exert initiation and/or maintenance activity. This may, for example, reveal closely associated but separable initiation and maintenance elements within regulatory regions such as *dimm.K1*. Electrophoretic mobility shift assays can be used to show the ability of transcription factors to bind directly *in vitro*. *In vitro* experiments can be complimented with *in vivo* studies; for example, the requirement for direct binding of maintenance-specific factors can be demonstrated by showing that deletions of their putative binding sites has no effect on initiation, but abrogates maintenance of reporter expression driven by *dimm.K1*. It would be difficult to use this approach, however, to assess the maintenance-specific requirement for direct binding sites of transcription factors such as Ap that regulate both initiation and maintenance of gene expression. If Ap binds at the same site to both initiate and maintain gene expression, to unambiguously demonstrate the necessity of that binding site for

maintenance the site would need to be disrupted specifically in adult neurons, which at this time is technically intractable. In such situations, a technique like DNA in situ hybridization-proximity ligation assay (DNA-ISH-PLA), which facilitates the detection of DNA-protein interactions *in vivo*, would be better suited to characterizing maintenance-specific binding configurations [104].

If further evidence suggests that *dimm.K1* does indeed collaborate with other CRMs in a cell-specific manner in order to maintain *dimm* expression it will be important to identify these additional CRMs. In our first approach to identify CRMs involved in initiation and/or maintenance of *dimm* expression, we assessed the *sufficiency* of intergenic regions to drive reporter expression at Stg17 and A5 and no regions other than that encompassing *dimm.K1* were sufficient to drive expression in Tv neurons at either stage. Initiation through *dimm.K1*, however, may be required for other CRMs to exert their maintenance-specific activities. Thus a *necessity* approach should be used to identify any maintenance-dedicated CRMs. CRISPR could be used to facilitate a tiling deletion analysis through an endogenous *dimm* locus in which a fluorescent reporter gene has been knocked in to replace the native *dimm* gene. The deletion of a maintenance-dedicated CRM should have no effect on initiation of *dimm* expression at Stg17, but result in the loss or downregulation of *dimm* expression in adult neurons. I recognize that this approach is limited by the fact that CRMs for a gene are not necessarily located within the closest intergenic regions, as long-range enhancers can be brought close to promoters through the complex organization of chromosomes in the nucleus. Thus, if necessary, we may wish to explore the use of chromosome conformation capture technologies as a means of identifying CRMs that interact with the *dimm* promoter. This alternative approach would require us to isolate our individual cells of interest. We could explore the use of laser capture microdissection for these purposes, as this strategy has been used successfully to isolate individual *Drosophila* neurons [105]. If we can establish a reliable system for isolating individual Tv1 and Tv4

neurons, techniques such as RNA-seq and ChIP-seq would also be open to us, and these approaches may facilitate our identification of novel *dimm* regulators and our characterization of transcription factor-CRM binding configurations (discussed above), respectively. It is important to recognize, however, that optimizing a system to isolate the Tv1 and Tv4 neurons and generating enough material for these approaches are not trivial tasks. Generating enough material should become less of a limitation over time, however, as single-cell applications of these technologies continue to improve [96,106-108].

4.5.2 *ap*

4.5.2.1 *Immediate goals*

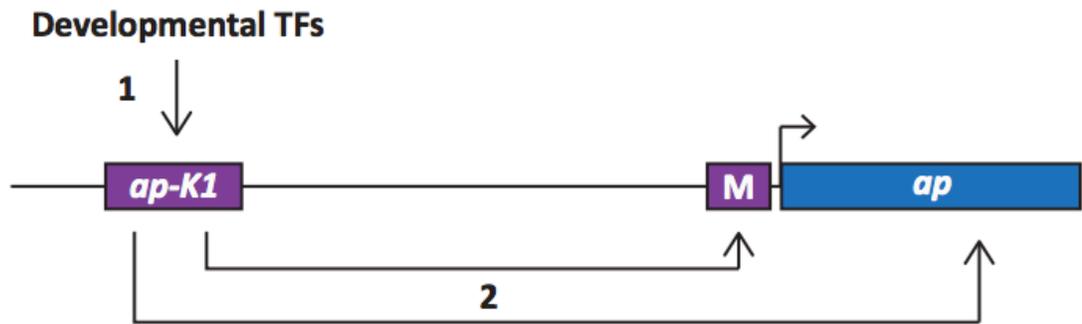
We have data suggesting that *ap.K1* is necessary for *ap* expression in Tv1 and Tv4 neurons, using *Nplp1* and *FMRFa* expression as indirect read-outs. A fly line in which endogenous *ap* has been GFP-tagged is now available and we have confirmed the fidelity of its expression pattern in the VNC at Stg17 and in adults [91, 110]. If we re-create the stable *ap.K1* deletion in this *ap-gfp* background we can improve our necessity data by assessing a direct read-out of *ap* expression. Also, to unambiguously determine whether *ap.K1* is necessary for maintenance of *ap* expression and thus distinguish between the possible models for *ap* maintenance presented in **Figure 24**, we must assess *ap* expression following deletion of *ap.K1* specifically in adult neurons, after development. We are planning to FRT-flank the *ap.K1* enhancer (through CRISPR or homologous recombination strategies) in an *ap-gfp* background for adult-specific deletion of *ap.K1* through adult induction of Flp recombinase in Tv neurons.

4.5.2.2 *Long-term goals*

We have made progress in characterizing the *cis*-regulatory architecture that governs initiation and maintenance of *ap* expression in Tv1 and Tv4 neurons. We have not investigated

further the *trans*-regulatory factors involved and how these *trans*-regulatory factors interact with the corresponding *cis*-regulatory architecture. To accomplish this we can perform a screen using the same approach that was discussed for *dim* in section 4.5.1.2.

A. Initiation of *ap* expression



B. Maintenance of *ap* expression: Model A



C. Maintenance of *ap* expression: Model B



Figure 23. Models for *ap* initiation and maintenance in Tv1 and Tv4 neurons.

(A) Developmental transcription factors act through *ap.K1* to initiate gene expression and ‘activate’ the maintenance element (M). (B) Possible model for *ap* maintenance in which both *ap.K1* and M are required to maintain *ap* expression. (C) Alternative model for *ap* maintenance in which M functions alone to maintain *ap* expression.

5. CONCLUSIONS

We have made progress in understanding how transcription factor networks are maintained in mature neurons. Using *Drosophila* Tv1 and Tv4 neurons as a model, we have shown that various mechanisms, distinct from those used at initiation, can be employed to maintain terminal selectors in a network. Furthermore, we have shown that, for a single transcription factor, initiation mechanisms that are shared by different cell subtypes can diverge into cell-specific maintenance mechanisms that differ between cell subtypes, and also exhibit difference between individual neurons of a single subtype.

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APPENDIX A. SUPPLEMENTARY TABLES AND FIGURES

	Tv1				Tv4			
	Dimm		<i>dimm.K1-nDsRed</i>		Dimm		<i>dimm.K1-nDsRed</i>	
	Th1	Th3	Th1	Th3	Th1	Th3	Th1	Th3
A20								
A1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	7.8%	6.6%	3.7%	8.5%	8.7%	6.6%	16.8%	9.7%
A20	76.9	78.3	135.9	150.2	113.2	120.4	206.7	330.8
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	7.6%	8.3%	5.8%	10.7%	11.5%	8.7%	21.6%	34.7%
P value	0.04	0.047	<0.0001	0.0008	0.4	0.07	0.0004	<0.0001
A45								
A1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	13.1%	10.4%	5.9%	7.9%	7.0%	12.1%	5.2%	12.6%
A45	95.7	140.4	186.4	267.7	167.2	183.5	637.8	485.0
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	11.0%	14.3%	9.1%	18.5%	11.2%	22.6%	84.1%	43.3%
P value	0.8	0.03	<0.0001	<0.0001	0.0001	0.0041	0.0001	<0.0001

Table 2. Quantification of anti-Dimm and *dimm.K1-nDsRed* intensities at A20 and A45, relative to A1.

Relative intensities are presented as the % mean of A1 expression levels +/- SEM. Student's two-tailed t-tests were used to compare each type of neuron between the two ages indicated.

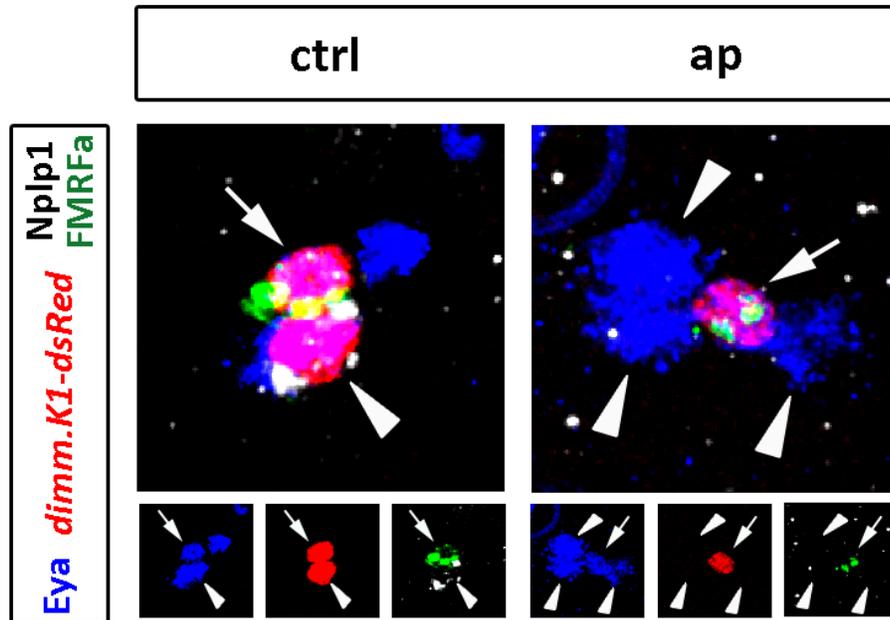


Figure 24. Loss of *dimm.K1-nDsRed* expression in the Stg17 Tv1 neurons of *ap* mutants.

Representative Th3 Tv clusters are shown. Tissue was stained with anti-Eya to visualize the Tv clusters, anti-Nplp1 to visualize the Tv1 neurons and anti-FMRFa to visualize the Tv4 neurons.

In controls, robust *dimm.K1-nDsRed* expression is exhibited in Tv1 (arrow heads) and Tv4 (arrows) neurons. In *ap* mutants, *dimm.K1* reporter intensity is reduced in Tv4 neurons

(quantified in **Figure 12B**) and lost in Tv1 neurons. **Genotypes.** Control: *dimm.K1-*

nDsRed/dimm.K1-nDsRed. Experimental: *ap^{Gal4}*, *dimm.K1-nDsRed/ap^{P44}*, *dimm.K1-nDsRed*

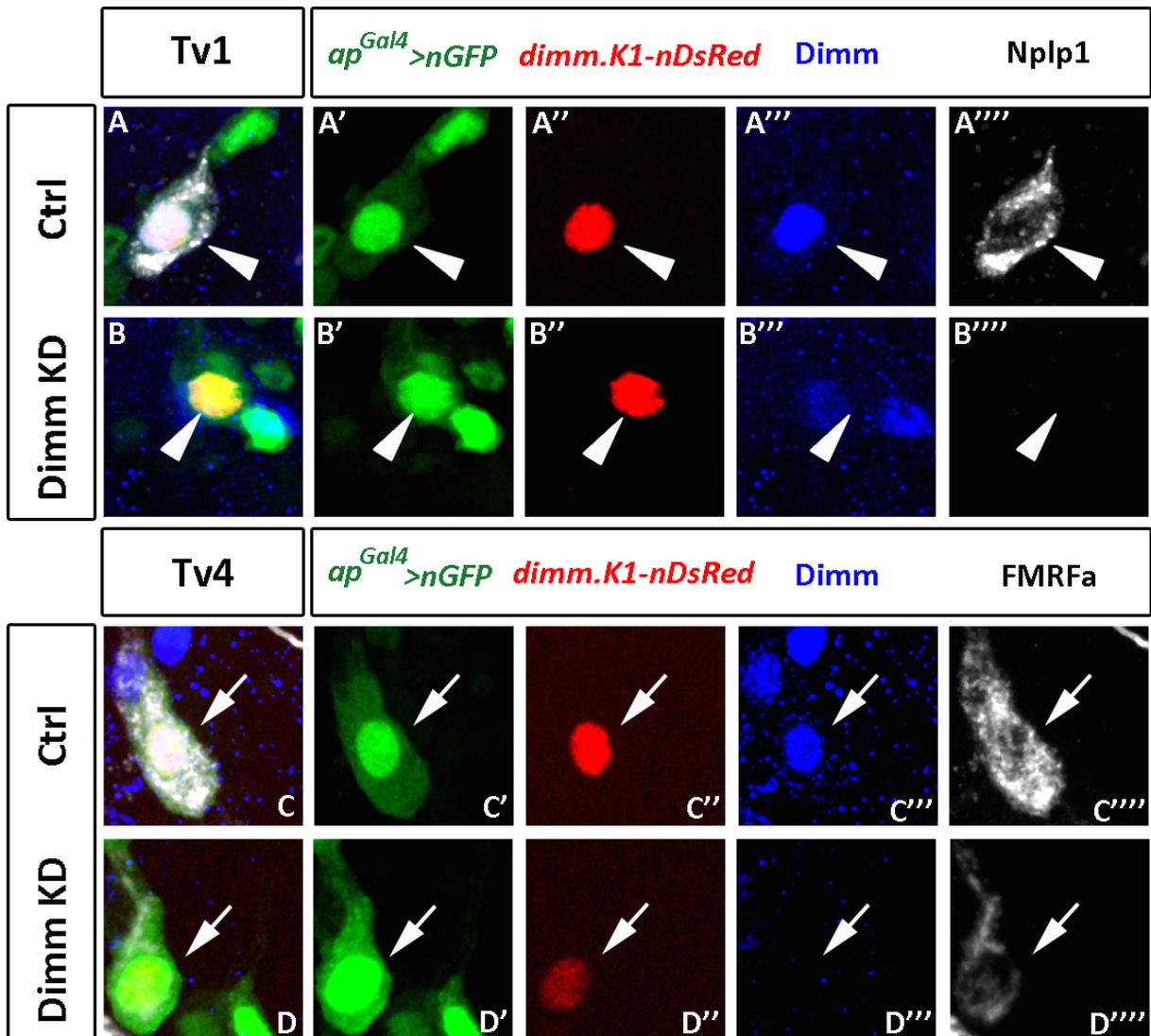


Figure 25. Confirmation of successful Dimm knockdown in Tv1 and Tv4 neurons.

The TARGET system was used to knockdown (KD) (*dimm^{dsRNAi}*) Dimm from A1 to A10 in Tv1 and Tv4 neurons, after normal development. Dimm immunoreactivity was lost from all Tv1 and Tv4 neurons in Dimm KD animals. Representative Th3 neurons are shown. **Genotypes:** Ctrl (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*), Dimm KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/rev4*, *UAS-dimm^{dsRNAi}*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*).

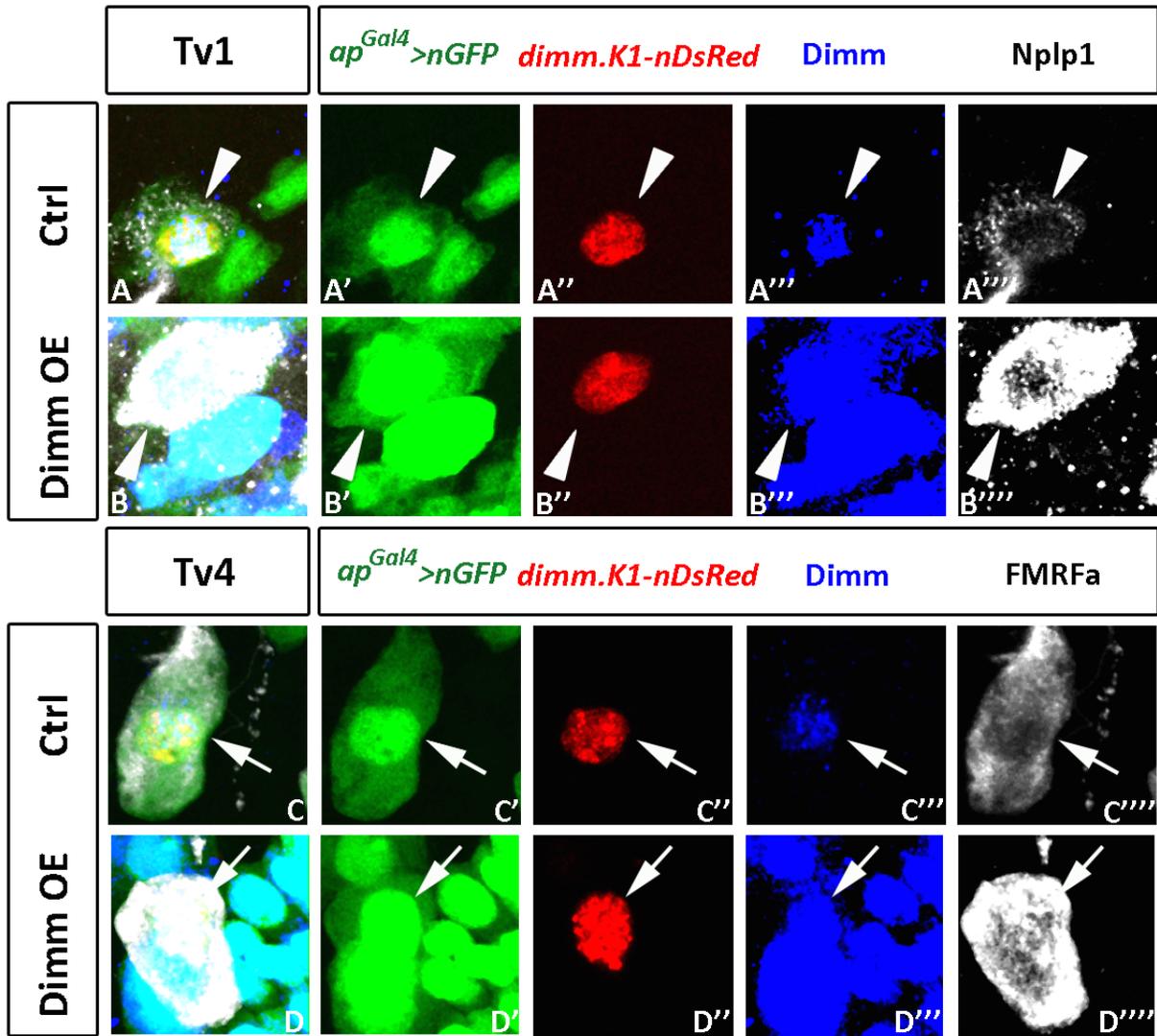


Figure 26. Confirmation of successful Dimm over-expression in Tv1 and Tv4 neurons.

The TARGET system was used to over-express (OE) (*UAS-dimm*) Dimm from A1 to A5 in Tv1 and Tv4 neurons, after normal development. Dimm immunoreactivity was successfully increased in all Tv1 and Tv4 neurons in Dimm OE animals. Representative Th3 neurons are shown.

Genotypes: Ctrl (*ap^{Gal4}, dimm.K1-nDsRed/+ ; tubP>Gal80^{TS}, UAS-nEGFP*), Dimm OE (*ap^{Gal4}, dimm.K1-nDsRed/UAS-dimm ; tubP>Gal80^{TS}, UAS-nEGFP*).

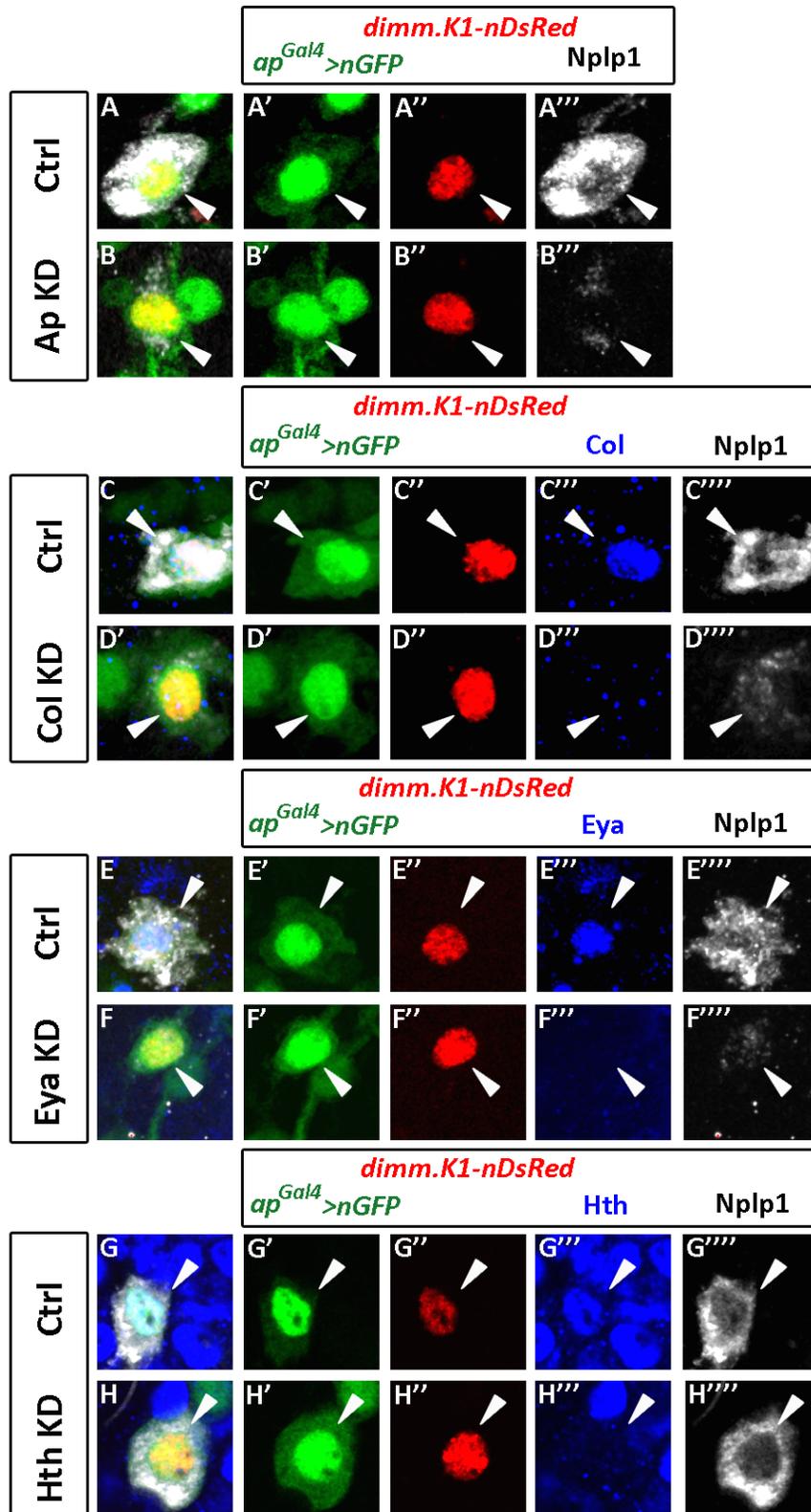


Figure 27.

Figure 27. Confirmation of successful transcription factor knockdown in adult Tv1 neurons.

The TARGET system was used to knockdown (KD) each Tv1 transcription factor after A1.

Successful Ap KD was assessed indirectly through reduction of Nplp1 immunoreactivity.

Genotypes: Ctrl (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*),

Ap KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-*

ap^{dsRNAi}), Col KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*, *UAS-*

nEGFP/UAS-col^{dsRNAi}), Eya KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*; *tubP>Gal80^{TS}*,

UAS-nEGFP/UAS-eya^{dsRNAi}), Hth KD (*UAS-dicer2/Y*; *ap^{Gal4}*, *dimm.K1-nDsRed/+*;

tubP>Gal80^{TS}, *UAS-nEGFP/UAS-hth^{dsRNAi}*).

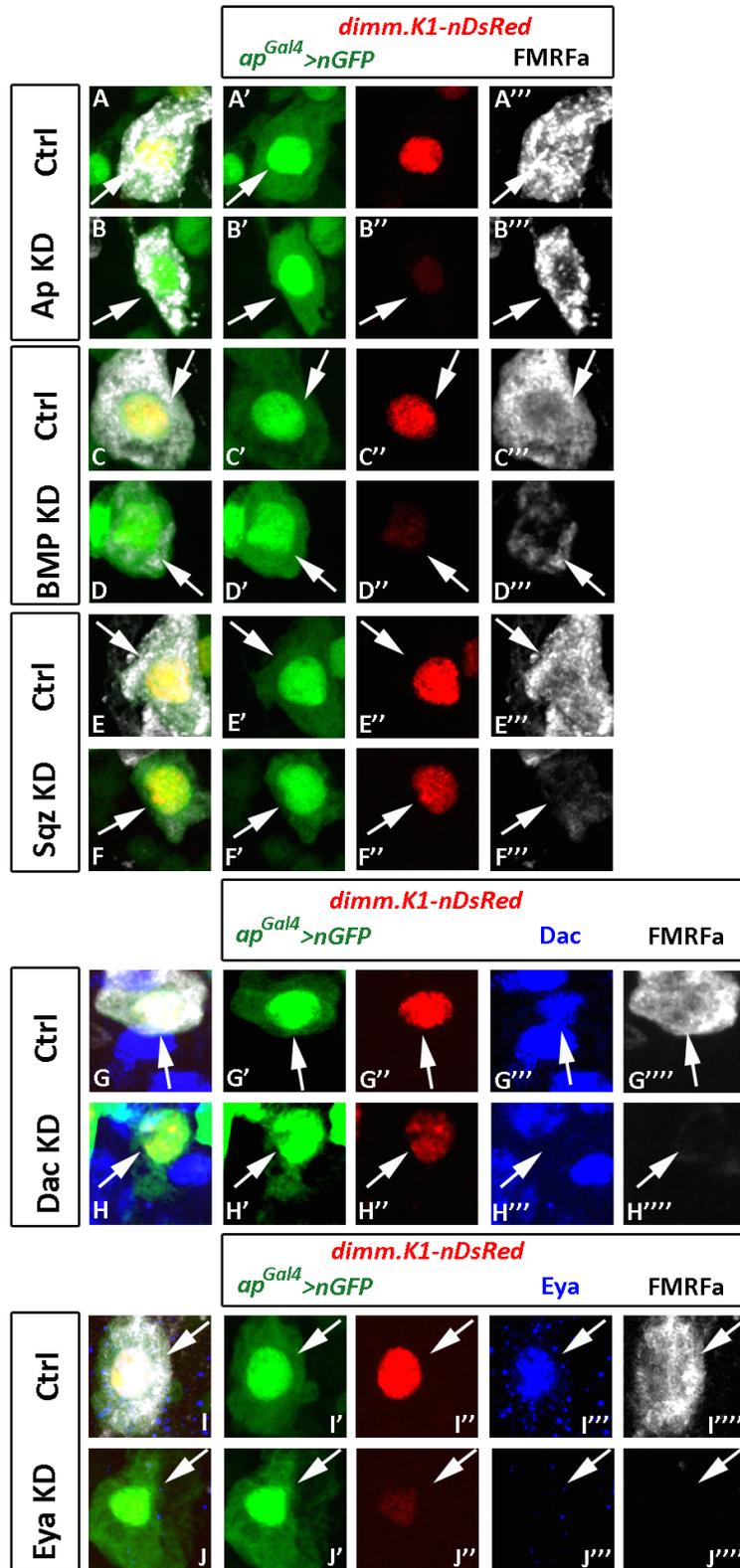


Figure 28.

Figure 28. Confirmation of successful transcription factor knock-down in adult Tv4 neurons.

The TARGET system was used to knock-down (KD) each Tv4 transcription factor after A1.

Representative Th3 neurons are shown. Successful Ap, BMP and Sqz KDs were assessed

indirectly through reduction of FMRFa immunoreactivity. **Genotypes:** Ctrl (*UAS-dicer2/Y* ;

ap^{Gal4}, *dimm.K1-nDsRed/+* ; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*), Ap KD (*UAS-dicer2/Y* ; *ap^{Gal4}*,

dimm.K1-nDsRed/+ ; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-ap^{dsRNAi}*), BMP KD (*ap^{Gal4}*, *dimm.K1-*

nDsRed/UAS-tkv^{DN} ; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-wit^{DN}*), Dac KD (*UAS-dicer2/Y* ;

ap^{Gal4}, *dimm.K1-nDsRed/dac³*, *UAS-dac^{dsRNAi}* ; *tubP>Gal80^{TS}*, *UAS-nEGFP/+*), Eya KD (*UAS-*

dicer2/Y ; *ap^{Gal4}*, *dimm.K1-nDsRed/+* ; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-eya^{dsRNAi}*), Sqz KD

(*UAS-dicer2/Y* ; *ap^{Gal4}*, *dimm.K1-nDsRed/+* ; *tubP>Gal80^{TS}*, *UAS-nEGFP/UAS-sqz^{dsRNAi}*).

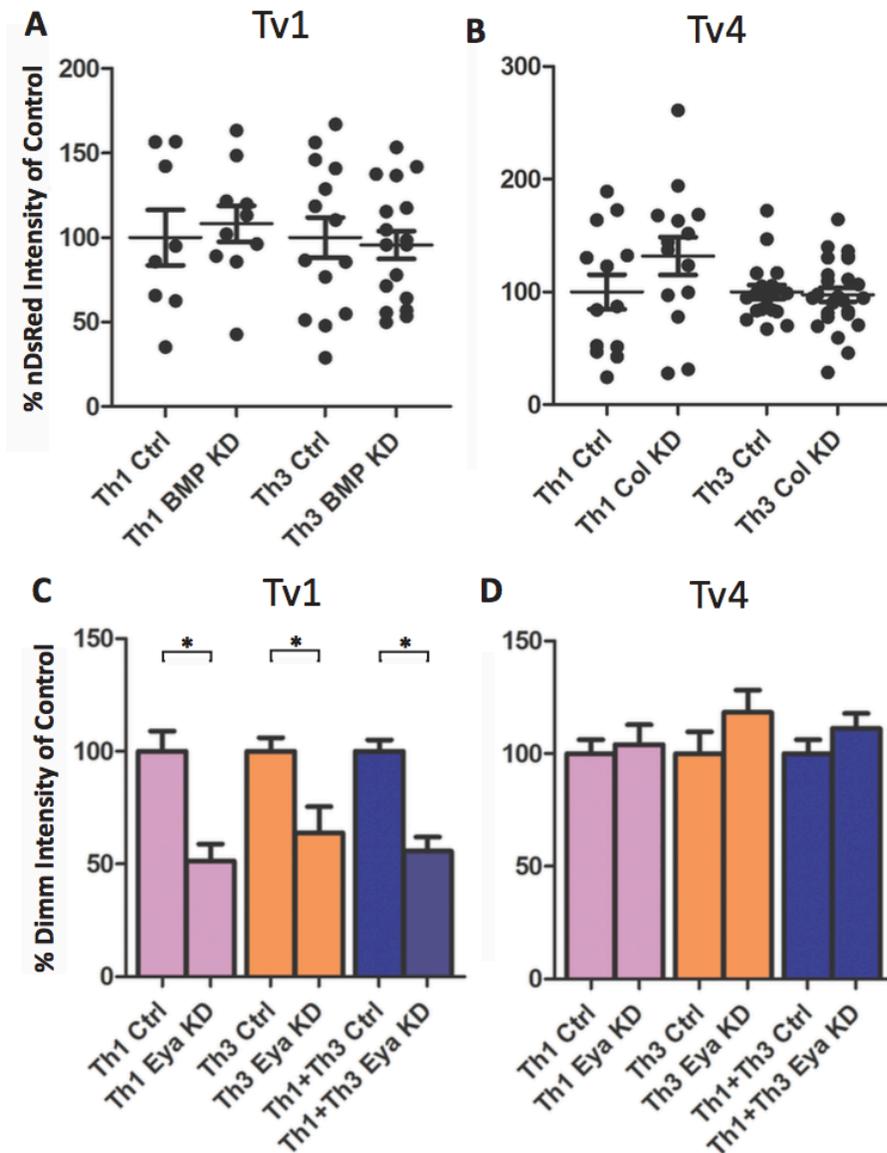


Figure 29. Additional controls for adult-specific transcription factor knockdown experiments.

(A) Knock-down of BMP signaling at A1 had no effect on *dimm.K1* reporter activity in Tv1 neurons by A10. (B) Knockdown of Col at A1 had no effect on *dimm.K1* reporter intensity in Tv4 neurons by A10. (C,D) Following Eya knockdown at A1 we simultaneously measured the response of anti-Dimm and *dimm.K1* reporter intensities at A20; the effects on *dimm.K1* reporter intensities are presented in Figures 14D and 15E, and the effects on anti-Dimm intensities were consistent with previously published work [36]. Results are also presented with Th1 and Th3 neurons pooled, as previously published.

Figure 30. Proposed *cis*-regulatory architecture regulating initiation and maintenance of *eya* expression in Tv1 and Tv4 neurons.

(A) A UCSC genome browser view of the *eya* (blue arrow) locus. (B) A close-up view of the region outlined in red in A. The genomic regions encompassed by the *Janelia* GAL4 lines *eya^{Gal4-GMR21A11}* and *eya^{Gal4-GMR21C02}* are indicated with orange and purple arrows respectively. The genomic region encompassed by *eya^{DsRed-Tv1}* is represented with a pink rectangle. (C) Proposed contributions of each of the regions 1, 2, 3 and 4 (limits denoted with blue dashed lines in B) to initiation and maintenance of reporter expression in Tv1 and Tv4 neurons. (D) A summary of data supporting proposals in C. The ability of the genomic regions *eya^{Gal4-GMR21A11}*, *eya^{Gal4-GMR21C02}* and *eya^{DsRed-Tv1}* to drive reporter expression in Tv1 and Tv4 neurons was assessed at Stg17, A5 and third instar larvae (L3 – an intermediate developmental stage). Region 4 in B encompasses a TRE/PRE identified by Ringrose *et al.* (2003) [109]. A role for this TRE/PRE in maintaining *eya* expression in Tv1 and Tv4 neurons has not yet been investigated.

APPENDIX C. CONSTRUCTION OF *dimm.K1-nDsRed*

A nuclear localized DsRed reporter vector (*pHStinger-nlsDsRed*) was previously generated by SOE PCR to produce an amplicon containing the attB site from UASTattB, a MCS, a HSP70 minimal promoter, *DsRed*, a *tra* nuclear localization signal and the SV40-poly A sequences derived from the *pHStinger* vector. *DsRed* replaced the *EGFP* in the *pHStinger* sequence [73, 111]. The region encompassed by *dimm.K1* was amplified from genomic DNA using the primers listed in **Table 3**. The PCR product was digested with AscI and XhoI and ligated into AscI and XhoI digested *pHStinger-nlsDsRed* vector. Transgenesis was performed by Genetic Services Inc. (Cambridge, MA). A single copy of the transgene was individually and unidirectionally integrated into the attP40 site on the second chromosome of the *D. melanogaster* genome using phiC31 integrase-based transgenesis [73].

	Forward Primer	Reverse Primer
<i>dimm.K1</i>	GGCT GGCGCGCC GTTTTGGCGCGAATTTCTTT	TCCG CTCGAG TTACATTTCCGGTCGCATCTG

Table 3. Primers for PCR Amplification of *dimm.K1*.

AscI restriction digestion sites are depicted in red and XhoI restriction sites are depicted in blue. Preferred flanking nucleotides are depicted in bold.