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ΙΩΑΝΝΑ ΠΙΤΣΙΔΙΑΝΑΚΗ
ΠΤΥΧΙΟΥΧΟΣ ΤΟΥ ΤΜΗΜΑΤΟΣ ΒΙΟΛΟΓΙΑΣ ΚΡΗΤΗΣ

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INSTITUTE OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

STUDY OF THE CIS-REGULATORY MODULES OF DROSOPHILA MELANOGASTER HEY GENE

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Bsc in BIOLOGY

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Abstract

Hey gene encodes a basic-Helix-Loop-Helix transcription factor transiently expressed in the newborn neurons of *Drosophila melanogaster* central nervous system. It has been shown that *Hey* expression is Notch dependent in some regions, while in others it is Notch independent. In addition, homozygous *Hey* deletion is late embryonic lethal and the rare escapers die as 1st or 2nd instar larvae. Bioinformatics analysis revealed two potential enhancer elements for *Hey*, containing suppressor of Hairless binding sites, one region is located in the second intron of the gene sequence and the other upstream of the promoter region. In the present work we provide a mutation analysis for both enhancer elements, by generating transgenic reporter fly lines, in an attempt to dissect the functional role of each binding site of these two regulatory modules. Furthermore, we attempted deleting the region of the intronic enhancer in the endogenous sequence of *Drosophila melanogaster Hey* gene in order to decipher the importance or a potential redundancy of the two enhancers, without successful results. Concluding, the immunohistochemistry analysis of the transgenic reporter fly lines provide robust proof of the regulatory role of the fragments of *Hey* enhancer elements. The present study contributes to a better understanding of Notch signaling in the context of *Hey*, a necessary for viability of *Drosophila melanogaster*, gene.

Περίληψη

Το γονίδιο *Hey* κωδικοποιεί έναν μεταγραφικό παράγοντα της οικογένειας βασική έλικα-βρόγχος-έλικα (basic Helix-Loop-Helix, bHLH), που εκφράζεται παροδικά στους νεογέννητους νευρώνες του κεντρικού νευρικού συστήματος της *Drosophila melanogaster*. Σε προηγούμενη δουλειά του εργαστηρίου είχε δειχθεί η ελεγχόμενη από Notch σηματοδότηση, έκφραση του *Hey*, αλλά και ανεξάρτητη από Notch έκφραση σε κάποιες περιοχές του εγκεφάλου της φρουτόμυγας. Επιπλέον, η απαλοιφή του *Hey* σε ομόζυγη κατάσταση είναι θνησιγενής σε προχωρημένο εμβρυικό στάδιο, ενώ ελάχιστα άτομα που επιβιώνουν στο στάδιο της προνύμφης πεθαίνουν στο πρώτο ή δεύτερο στάδιο. Βιοπληροφορική ανάλυση της περιοχής του γονιδίου του *Hey* αποκάλυψε δύο πιθανές ρυθμιστικές περιοχές - ενισχυτές, με βάση την παρουσία μοτίβων πρόσδεσης (binding sites) του συν-παράγοντα του Notch, suppressor of Hairless. Η μία εξ αυτών βρίσκεται ανοδικά του υποκινητή του γονιδίου και η άλλη εντός του δεύτερου ιντρονίου της γονιδιακής αλληλουχίας. Στη παρούσα εργασία πραγματοποιούμε μία ανάλυση διαγραφής στοιχείων των δύο ρυθμιστικών περιοχών, κατασκευάζοντας διαγονιδιακές φρουτόμυγες που φέρουν γονίδια αναφοράς για τις τροποποιημένες αλληλουχίες αυτών των ρυθμιστικών περιοχών, με στόχο να αποκαλύψουμε το ρυθμιστικό ρόλο που έχουν στην έκφραση του γονιδίου. Ακόμη, έγινε προσπάθεια, χρησιμοποιώντας τη μέθοδο γενετικής τροποποίησης, CRISPR/Cas9, να διαγραφεί ο ενισχυτής που βρίσκεται στην ιντρονική περιοχή, χωρίς όμως επιτυχές αποτέλεσμα. Τέλος, η ανοσοϊστοχημική ανάλυση των διαγονιδιακών σειρών παρέχει πληροφορίες για τη ρύθμιση του *Hey* στο κεντρικό νευρικό σύστημα της *Drosophila melanogaster,* κατά το εμβρυικό και προνυμφικό στάδιο. Η παρούσα μελέτη συνεισφέρει στη καλύτερη κατανόηση της σηματοδοτησης Notch στη ρύθμιση της έκφρασης του γονιδίου *Hey* της *Δροσόφιλας*.

Introduction

Hes and Hey, proteins belong to a subfamily of bHLH-O transcription factors, which in vertebrates have been characterised as repressors. They consist of a basic domain that provides specific DNA binding, preferably on E-box sequences (CANNTG), as well as, HLH and Orange domains that mediate dimerisation and protein - protein interactions, respectively. On the carboxy-terminal end of Hes proteins there is a highly conserved motif of a tetra-peptide WRPW that recruits groucho-type co-repressors (Courey & Jia, 2001), while on the carboxy- terminal end of Hey proteins a YRPW motif is found, with an unknown function so far (Iso, Kedes, & Hamamori, 2003; Iso et al., 2001). Both *Hes and Hey genes* are direct transcriptional targets of the Notch signaling pathway (Iso et al., 2003), while the function of these proteins are essential in many Notch-dependent processes , including repression of key cell fate determinants and cell cycle regulators (Fischer & Gessler, 2007; Louvi & Artavanis-Tsakonas, 2006).

Notch signaling is a conserved, cell – cell communication pathway shared by many multicellular organisms, with a significant role in multiple developmental processes (Lai, 2004) (Artavanis-Tsakonas, Rand, & Lake, 1999). Notch protein is a single pass transmembrane receptor protein with an extracellular part able to bind ligands and an intracellular part that has a role as a transcription factor upon Notch signaling. There are four different Notch receptor proteins (Notch1-4) in mammals, while there is a single one in Drosophila , containing 36 Epidermal Growth Factor (EGF) domains (Fleming, 1998) and 3LNR domains (K. Sakamoto, Chao, Katsube, & Yamaguchi, 2005). Notch receptor contains a hetero-dimerization domain on the extracellular part, while the intracellular domain is composed by six tandem ankyrin repeats, facilitating protein - protein interactions and a glutamate-rich domain (OPA domain). On the carboxy-terminal end of Notch receptor, a PEST (proline - glutamate - serine - threonine rich) domain is found, considered to act as a protein degradation signal (Spencer, Theodosiou, & Noonan, 2004) (Figure A adapted from Gordon et al., 2008). Notch ligands are the DSL proteins, Serrate (Ser) and Delta (Dl) in *Drosophila*, Lag2 in *Caenorhabditis elegans,* while Jagged is the Serrate orthologue in mammals. All ligands contain numerous EGF repeats, specifically, Ser and Dl contain 14 and 9 EGF repeats respectively. Specific EGF motifs have been suggested to bind Ca2+ and facilitate the formation of a binding surface mediating protein - protein interactions between receptor and ligand (Rand et al., 2000). Moreover, Notch ligands contain a well-conserved, disulphide-rich DSL-domain (Delta-Serrate-Lag2) in the extracellular part (Fig.A) which is a degenerate EGF-like repeat, necessary but not sufficient, for interactions with Notch (Shimizu et al., 1999) (Gordon, Arnett, & Blacklow, 2008; Gray et al., 1999).

Binding of the ligand by the receptor leads to two cleavage events on Notch receptor, one by Kuzbanian, an ADAM metalloprotease extracellularly (Brou et al., 2000) and one by γ-secretase in the intracellular part (De Strooper et al., 1999). When the intracellular part of Notch receptor (NICD: Notch intracellular domain) is released it is transferred in the nucleus. Once it is in the nucleus, it acts as an activation and recruitment element of Notch signaling effectors, the CSL DNA-binding protein (*C*BF-1/RBPJ-κ in *Homo sapiens*/*Mus musculus*, *S*uppressor of Hairless in *Drosophila melanogaster*, *L*ag-1 in *Caenorhabditis*

elegans), which is a DNA-binding protein (Bray & Bernard, 2010; Fortini & Artavanis-Tsakonas, 1994) and mastermind, a nuclear protein, (Smoller et al., 1990), which in turn, direct the assembly of transcriptional complexes that initiate target-gene expression, such as *Hes* and *Hey* transcription factors (Fig. A).

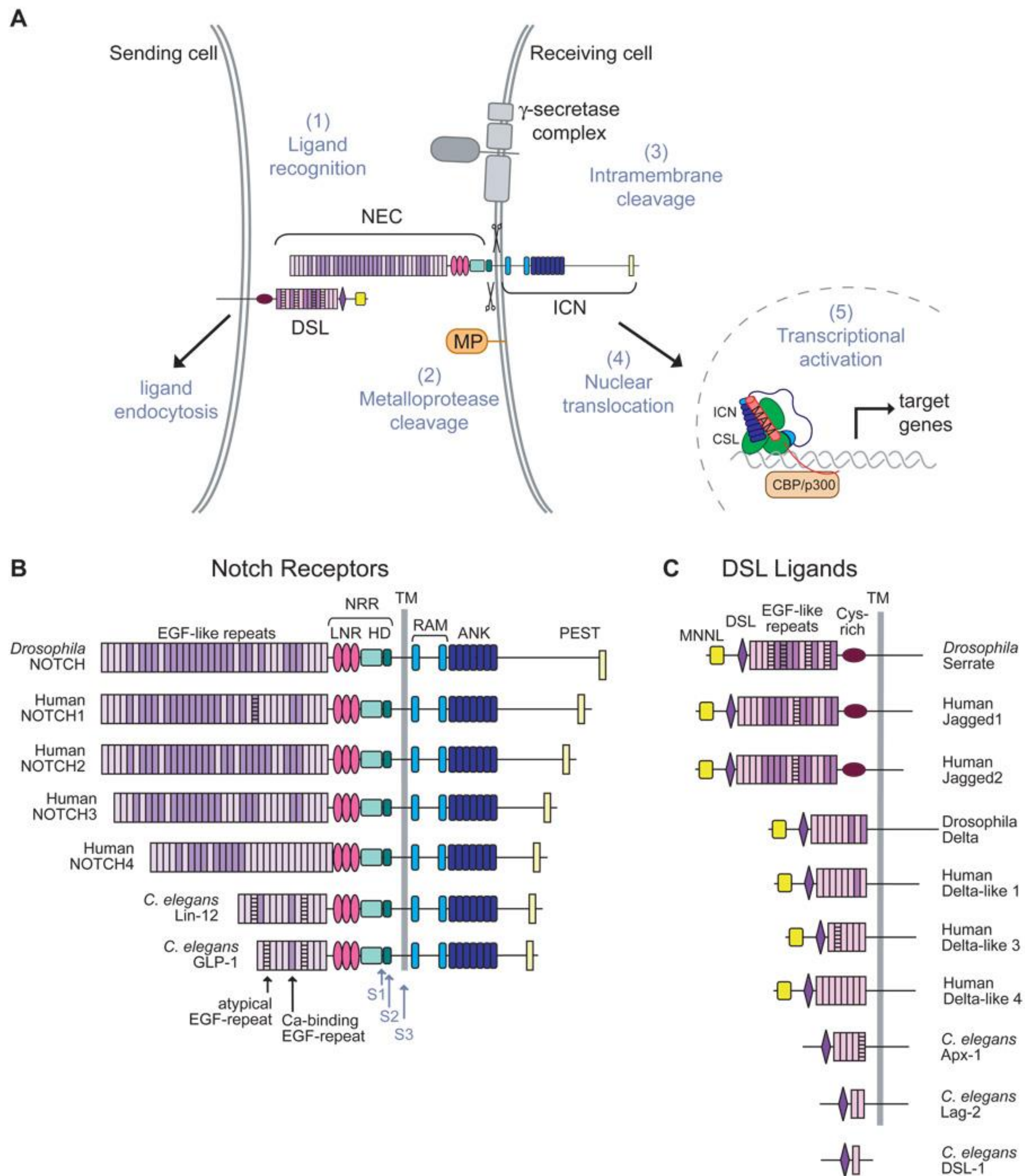


Figure A. Notch signaling, receptor and ligand structures. (A) Model for the major events in the Notch signaling pathway. Signals initiated by the binding of ligand (1) lead to metalloprotease cleavage (MP) at site S2 (2). This proteolytic step allows the cleavage of Notch by the γ-secretase complex at site S3 within the transmembrane domain (3), and release of intracellular notch (ICN) from the membrane (4). ICN translocates to the nucleus where it enters into a transcriptional activation complex with CSL and Mastermind (MAM). (B, C) The domain organization of Notch receptors (B) and DSL-family ligands (C) from fly, human and worm. (Gordon et al., 2008)

Notch signaling in mammals targets *Hes* and *Hey* genes. In human, rat and mouse genomes, three *Hey* paralogues are found, Hey1, Hey2 and HeyL , that are also found in publications as *Hrt1,2,3, Hesr1,2, Herp1, 2* or *Chf1, 2* (Fischer & Gessler, 2007; Steidl et al.,

2000). Hey proteins are implicated in many processes of vertebrates' development, especially in cardiovascular and neural system, while *Hey1* deletion has no defects in mouse development, loss of Hey2 leads to cardiovascular defects with high postnatal lethality (Fischer, Schumacher, Maier, Sendtner, & Gessler, 2004; Sakata et al., 2006). Over-activation of Notch2 signaling, or over-expression of either *Hey1* or *Hey2*, is shown to repress Bone morphogenetic protein-2 (Bmp2) and lead to cardiac defects, too (Rutenberg et al., 2006). In addition, *Hey2* deletion in heterozygous mice leads to extended fibrosis and increased apoptosis upon hemodynamic stress, as well as, increase of *GATA4* expression. GATA4 is a known transcriptional activator of cardiac hypertrophy, in cardiomyocytes (Liu, Yu, Wu, & Chin, 2010), thus linking *Hey2* mutations to cardiac hypertrophy. In the nervous system, overexpression of mouse *Hey1* and *Hey2* in early mouse embryonic stages prevented neural precursors from differentiating, while in later stages, promoted gliogenesis (M. Sakamoto, Hirata, Ohtsuka, Bessho, & Kageyama, 2003). On the other hand, homozygous null mutation of *HeyL* prevents the physiological neuronal development, a defect that is rescued upon *Hey1* homozygous deletion (Mukhopadhyay, Jarrett, Chlon, & Kessler, 2009). Moreover, it should be noted that the three mammalian *Hey* orthologues are redundant and have been shown to be expressed in osteoblasts, fibroblasts, primary sertoli cells and pillar cells of the inner ear (Behr & Kaestner, 2002; Doetzlhofer et al., 2009; Takata & Ishikawa, 2003; Zanotti & Canalis, 2016).

In *Drosophila,* Notch pathway has a vital role in neurogenesis, signaling the lateral inhibition of the pro-neural clusters (Collier, Monk, Maini, & Lewis, 1996) and preventing neural cell differentiation. In absence of Notch signaling numerous ectodermal cells are mis-specified as neuroblasts leading to a hyperplastic central nervous system (CNS) during embryonic development. Specifically, Notch signaling acts via its transcriptional targets of the E(spl) complex. These are Hes like transcriptional repressors, which are expressed in the cells surrounding the neuroblasts, antagonize proneural proteins and prevent neural cell fate (Wurmbach, Wech, & Preiss, 1999). In a similar way, Notch signaling prevents the neural precursor cell fate in the peripheral nervous system of *Drosophila*. In addition, Notch signaling is implicated in the retina, heart precursor and cardioblast, as well as, in the midgut epithelial cell differentiation (reviewed in (Delidakis, Monastirioti, & Magadi, 2014)).

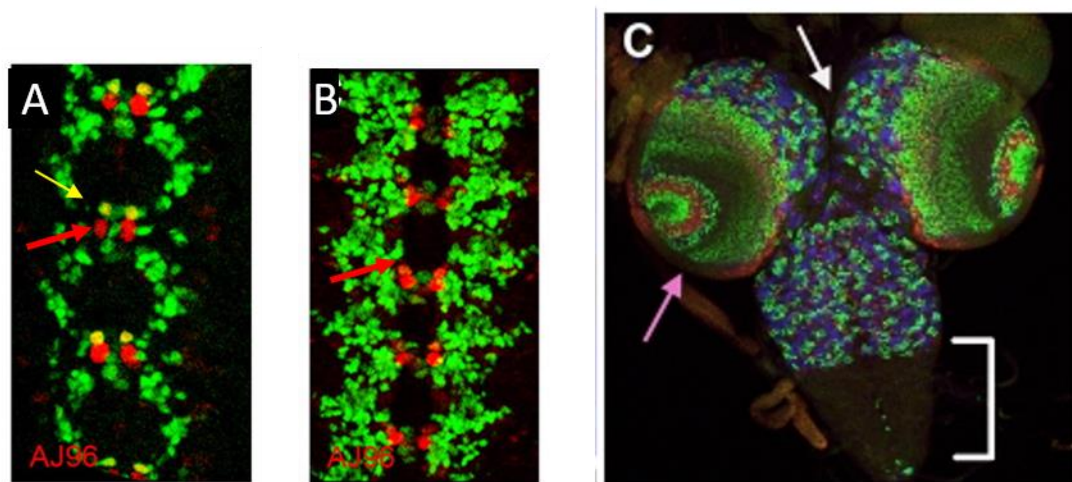


Figure B. In image A is the CNS of a stage 12 embryo and in B, a stage 15. In green is endogenous Hey, red is an enhancer trap reporter for ventral and dorsal MP2 neurons. Hey expression is evident in the vMP2 cell that allows Notch signaling upon its birth (A). This expression is transient and it is lost during embryo ageing (compare to B). Image C presents a confocal projection

of 3rd instar larva CNS. Red: Dpn, neuroblast marker, Blue: Pros, GMCs and neurons marker, Green: Hey (Monastirioti et al., 2010).

Drosophila genome encodes Hairy and Enhancer-of-split (E(spl)) genes similar to mammalian *HES* and a single Hey gene. *Drosophila* Hey gene is expressed in the central nervous system (CNS) and the peripheral nervous system (PNS), during embryonic development (Fig. B), and during 3rd instar larva (Fig. B). *Hey* has a Notch dependent and independent expression in the central nervous system (Monastirioti et al., 2010) primarily in neurons but in some glia cells as well. During nervous system development neuroblasts divide asymmetrically to give rise to a ganglion mother cell (GMC) (an intermediate precursor cell) and a neuroblast (Yu, F, Kuo CT, Jan YN, 2006). Most of the GMCs divide also asymmetrically giving birth to two neurons or in some cases to glia. In the case of the two neurons, different levels of Notch signaling lead the two sibling neutrons to adopt different cell fates. The neutron receiving high levels of Notch signaling develops into type “A” neuron, while the sibling receiving low levels adopts type “B” neuronal fate (Skeath and Thor, 2003).

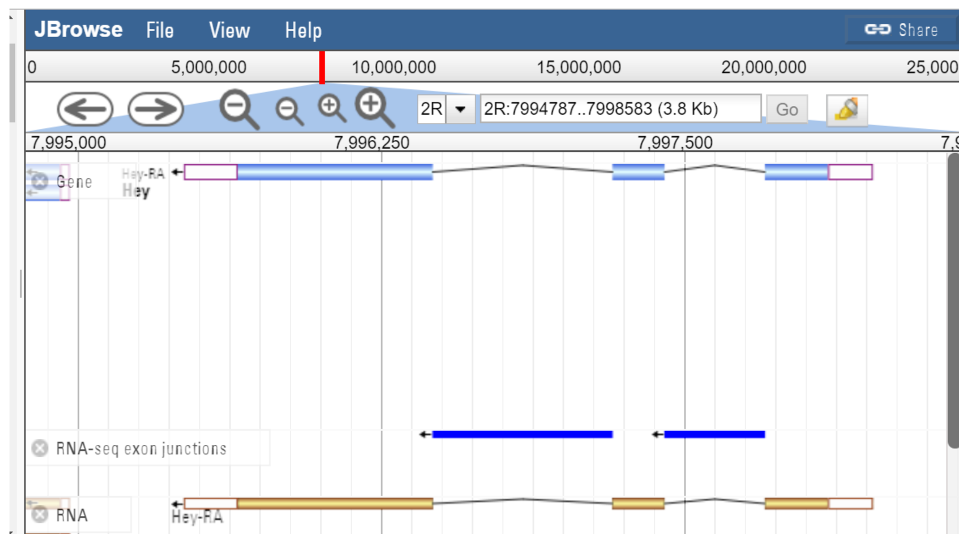


Figure C. Scheme depicting Hey gene from flybase genome browser.

In 2010, *Hey* was shown to be expressed in the new-born type “A” neurons upon Notch signaling and to contribute to the “A” cell fate determination, as by ectopic *Hey* expression the fate of type “B” neurons turns into type “A” (Monastirioti et al., 2010). *Hey* expression is transient and it is evident from embryonic stage 10 until stage 16 (Monastirioti et al., 2010). Besides nervous system, *Hey* is also expressed in cells of the developing midgut and adult midgut as well, the identity of which has not been specified yet (unpublished observations). *Hey* is an essential gene since *Hey* homozygous deletion is late embryonic lethal, with rare escapers producing very inactive larvae that do not reach pupation. Hey molecular function in Drosophila is not yet clear, although there is a recent report suggesting that it acts as a transcriptional activator (Stampfel G., et al, 2015). Stampfel et al., examined the regulatory contribution of transcription factors, either activation or downregulation of target gene expression and the level of this phenomenon. By performing enhancer complementation assays and validating their results, taking into consideration the binding site context of the enhancers of selected transcription factors, including Hey, they concluded that the regulatory

function observed was related to the context of the enhancers. Specifically, upon Hey presence, the experimental results showed activation of expression of the reporter gene located downstream of the enhancer sequence bearing Hey binding sites.

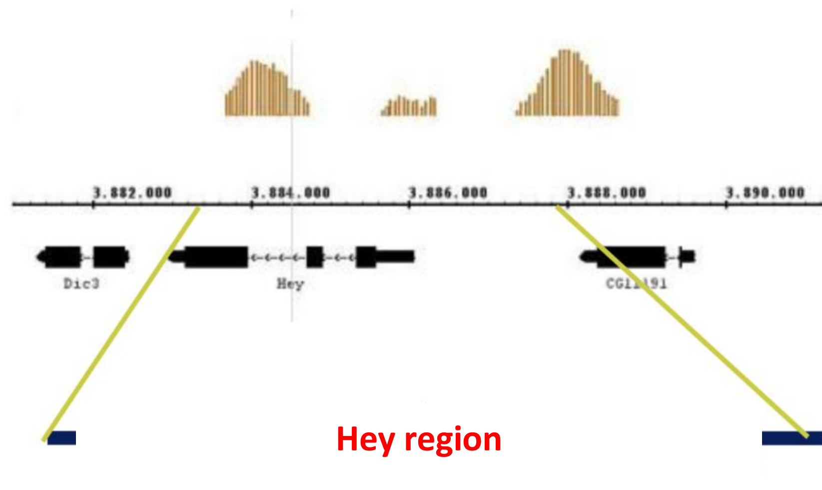


Figure D. Chip-on-chip experiments for Su(H) revealed two regions containing Su(H) binding sites, one upstream of Hey promoter region and one in the second intron of the gene (M.Monastirioti, Neurofly, 2016).

Drosophila Hey gene contains three exons and two introns (Fig. C). Chip-on-chip experiments for Su(H) binding (a Notch co-activator) (Zacharioudaki et al., 2016) revealed two regions containing Su(H) binding sites, indicating them as putative regulatory regions of the gene (Fig. D). One such region is upstream of the gene promoter and the other in the sequence of the second intron of *Hey* gene (Fig. D). Indeed, bioinformatics analysis revealed 3 Su(H) binding sites in the upstream and 4 in the intronic region of *Hey* (Fig. E).

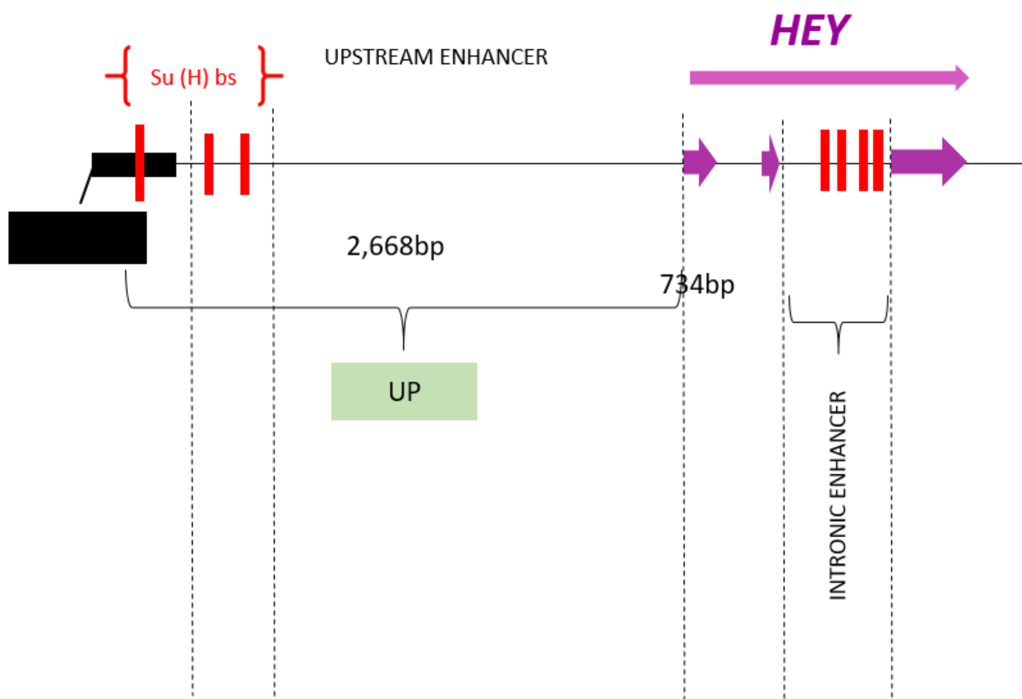


Figure E. Scheme depicting the genetic region of Hey along with the two enhancer elements.

Ioanna Koltsaki a previous member of the lab subcloned the two putative enhancer regions of *Hey* in vectors upstream of a minimal Hsp70 promoter, driving the expression of a reporter gene, either nuclear GFP or nuclear beta galactosidase. The expression pattern of these reporters was previously studied in the 3rd instar larval CNS (I. Koltsaki, N.Batsiotos) and the results of these experiments suggested that both enhancer elements were capable of driving the reporter gene expression. In the present thesis work, I extend the study of these reporters during all embryonic stages of neuronal development. Moreover, I examined the expression in the peripheral nervous system and the developing midgut of the embryos.

In order to better understand the regulation of *Hey* gene under Notch signaling, we designed a series of mutations on both potential enhancer elements identified in Hey gene region and studied their expression by generating transgenic reporter fly lines. The mutations were either deletions including the Su(H) binding sites of the upstream enhancer element or point mutations on the Su(H) binding sites of the intronic enhancer. Finally, I attempted to generate deletions within the second intron of *Hey* by CRISPR/Cas9 methodology in order to elucidate the contribution of each one of the two enhancer regions in the regulation of Hey gene expression.

Materials and Methods

Reporter constructs

In the present study 8 different reporter lines were studied.

a) The first two lines were generated by Ioanna Koltsaki (previous lab member). PCR amplification of HeyUP (2665 bp) and HeyINT2 (733 bp) regions was performed using the following primer pairs that also bear cloning sites EcoRI, KpnI and XbaI (underlined sequences). The respective EcoRI-KpnI and XbaI-KpnI fragments were cloned in GFP or beta-galactosidase reporter expression vectors that carried an attB recombination site for integration in attP transgenic flies. The resulting constructs were HeyUP beta-gal (named M11m2), HeyUPGFP (M4m3) and HeyINT2 beta-gal (M5m1), HeyINT2GFP, respectively and were integrated in pole cell genome after injection, via site specific phiC31 integration ({Groth, 2004 #3}). Transgenes were obtained and used to establish HeyUP and HeyINT2 reporter lines.

FOR_Heyup: 5' CA GAATTC CGG CGG CTT TGT AAC CTC CAC TTT GCG 3'
REV_Heyup: 5' CT GGTACC GAG GCA GAT GCA GTT GGC GGC TAG 3'
Hey intron-forward XbaI: 5' CGC TCTAGA ATT ACC AAG CCC ACT TGA GC 3'
Hey intron-reverse KpnI: 5' GC GGTACC CTG CAA CAA GAT ACG AGG AGG 3'

b) Concerning Hey upstream deletion analysis, fragments of the Hey upstream enhancer were PCR amplified and cloned in GFP reporter expression vectors. pattb nls eGFP, which carried a minimal hsp70 promoter upstream of the reporter gene sequence, was used to clone the EcoRI-KpnI fragment of the upstream enhancer (primer pair: Hey-forward EcoRI-REV_Heyup) including the 2 Su(H) binding sites, that resulted in the 2+3SH construct and transgenic reporter fly line. The EcoRI-KpnI fragment of the upstream region, product of UPFip and REV_Heyup, was cloned in the GFP reporter expression vector HSB-GsFpH-Stinger_attB1, which does not contain any promoter region, resulting in the NSH construct and transgenic reporter fly line.

For PCR amplification of the 2+3SH element the following primer set was used:
Hey-forward EcoRI: 5' GGA ATT CGT GTG TAT GTG TGT GCG GC 3'
REV_Heyup: 5' CT GGTACC GAG GCA GAT GCA GTT GGC GGC TAG 3'

For PCR amplification of the NSH element the following primer set was used:
UPFip: 5' CAA GAATTC TGG GTT CGT CCC GTT GTC GGT GG 3'
REV_Heyup: 5' CT GGTACC GAG GCA GAT GCA GTT GGC GGC TAG 3'

c) To construct the HeyINT2 lines carrying mutations in different combinations of the 4 Su(H) binding sites of Enron 2, the mutated intronic regions generated by Ioanna Koltsaki were digested using EcoRI- KpnI restriction sites and were sub cloned in a GFP reporter expression

vector (pattb nls eGFP) with an attB recombination site. The exact location of the suppressor of Hairless binding sites within Hey Intron 2 is depicted in figure 43 of the results section.

The Sm14 carried the mutations:
1st Su(H) binding site: tGTGGGAA >>> tG***TCGCGA*** (Nru I)
4rth Su(H) binding site: cGTGGGAA >>> c***GTGCAC***C (ApaL I)
The Sm124 carried the mutations:
1st Su(H) binding site: tGTGGGAA >>> tG***TCGCGA*** (Nru I)
2nd Su(H) binding site: tGTGGGAA >>> tG***AGGCCT*** (Stu I)
4rth Su(H) binding site: cGTGGGAA >>> c***GTGCAC***C (ApaL I)
The Sm134 carried the mutations:
1st Su(H) binding site: tGTGGGAA >>> tG***TCGCGA*** (Nru I)
3rd Su(H) binding site: TTCCCACg >>> T***CCCGGG***g (Sma I)
4rth Su(H) binding site: cGTGGGAA >>> c***GTGCAC***C (ApaL I)
The Sm123 carried the mutations:
1st Su(H) binding site: tGTGGGAA >>> tG***TCGCGA*** (Nru I)
2nd Su(H) binding site: tGTGGGAA >>> tG***AGGCCT*** (Stu I)
3rd Su(H) binding site: TTCCCACg >>> T***CCCGGG***g (Sma I)

Drosophila genetics

For the experiments of HeyUP-GFP, crossed to HeyINT2-GFP the lines M4m1 and M1m3, respectively, were used.

For the mutant *Delta Serrate* background, a fly line homozygous for HeyINT2-GFP reporter was crossed to mutant background flies (HeyINT2-GFP/HeyINT2-GFP ; III/III X II/II ; $Dl^{-}Ser^{-}$/TM3) and the non-stubble progeny flies (HeyINT2-GFP/II ; $Dl^{-}Ser^{-}$/III) were collected and crossed to generate embryos with HeyINT2 reporter and homozygous Delta Serrate mutant background (HeyINT2-GFP/II ; $Dl^{-}Ser^{-}$/ $Dl^{-}Ser^{-}$). In the same manner were performed the experiments for HeyUP reporter, using a homozygous HeyUP-GFP line.

Drosophila melanogaster Fly lines

The reporter constructs were injected in embryos of the transgenic fly line 25709, genotype: y1 v1 P{nos-phiC31\int.NLS}X; P{CaryP}attP40, offered by Dr Pawel Piwko, that carried the attP40 integration site on second chromosome and expressed the phiC31 integrase under nanos control region.

For the CRISPR/Cas9 genetic modification the y1 M{nos-Cas9.P}ZH-2A w* (stock number 54591, Bloomington Drosophila Stock Center) fly line was used.

Genetic modification by CRISPR/Cas9 genome editing

In order to delete HeyINTR2 region by applying CRISPR/Cas9 genome editing, we designed two guide RNAs targeting the region of the second intron of Hey gene. The gRNAs were designed using the online tool Optimal Target Finder (Gratz et al., 2014) (tools.flycrispr.molbio.wisc.edu/targetFinder/) of Wisconsin University, for a 20nt gRNA, with high stringency and setting PAM: NGG. The sequence of the gRNA targeting the 5'end of the intron was: 5' – CTTC_GAGCAAATAGAGGGTTAACT- 3', while the sequence of the gRNA targeting the 3'end of the second intron was: 5' - AAAC _GAACTGGGATTAGACAGCTC- 3', that are appropriate for

efficient expression from the U6 promoter. Each guide RNA sequence was ordered in both sense and anti-sense strands and were cloned into pU6-BbsI-chiRNA by Addgene (https://www.addgene.org/45946/) according to Gratz et al (Gratz, Rubinstein, Harrison, Wildonger, & O'Connor-Giles, 2015). The resulting gRNA expression vectors were injected in Cas9 transgenic flies and the resulting flies were crossed to Hey deletion flies or yw flies. The offspring of these crosses were screened for deletion events using various sets of primers described below.

PCR screening primers

The sets of PCR primers used for screening of deletion events in the offspring of the injected flies were:

CDSF: 5' CT GAGCTCA TGG ATC ACA ACA TGC ACG TCA ATG 3'

CDSR: 5' CA CAGCTGC CAC AAT CAA TAG GCC ATC TCG G 3'

INT2F: 5' CGC TCTAGA ATT ACC AAG CCC ACT TGA GC 3'
INT2R: 5' GC GGTACC CTG CAA CAA GAT ACG AGG AGG 3'

Immunohistochemistry

Embryo stainings: Embryos were collected overnight or for certain time window in order to obtain specific embryonic stages and dechorionated using 50% bleach for 3 minutes. Fixation followed using 4% formaldehyde solution in 1x PEM (0,1M PIPES Disodioum salt, 2mM EGTA, 1mM MgSO4, pH 6,95 with HCl) (Clemons et al., 2010) and vigorous shaking for 20 minutes. Fixative was removed and the embryos were extracted from the vitelline membrane by vigorous shaking in a 50% heptane, 50% methanol solution. The vitelline membranes were removed, followed by three embryo washes in methanol. Next embryos were stored in methanol at -20C or immediately prepared for immunostaining. Preparation for immunostaining included three washes in PT solution (1xPBS-Phosphate-buffered saline buffer (PBS), 0,2% Triton) and blocking in PBT (1xPBS, 0,2% Triton, 0,5% BSA) solution, incubating the embryos for 2 hours at room temperature (RT). Next the blocking buffer was removed and embryos were incubated over night at 4°C with the primary antibodies mix (the antibodies were diluted in PBT solution). After primary antibody incubation, three washes in PBT solution were performed and incubation with secondary antibodies followed at RT for 2-4 hours. The immunostained embryos were washed three times in PBT and a final wash in PBS before mounting on imaging slides using 0.5% n-Propyl gallate in PBS solution.
Larval CNS staining: 3rd instar larvae were collected and dissected under a Leica stereoscope, in cold PBS solution. The dissected larvae were fixed in a 4% formaldehyde solution, 1x PEM (PIPES Disodioum salt, EGTA, MgSO4) (Clemons et al., 2010) on mild agitation for 20 minutes. Fixation solution is removed and three washes in PT solution are performed. The blocking of the tissue for 2 hours at RT is performed in PBT solution and the brains were incubated overnight at 4C with the primary antibodies mix. Next day the mix is removed and three washes in PBT solution follow. Incubation with the secondary antibodies is performed at RT for 2 to 3 hours after which three washes in PBT and one in PBS solutions follow. Finally, the CNS was detached off the dissected larvae under the stereoscope and mounted on imaging slides, using 0.5% n-Propyl gallate in PBS solution.

Primary antibody dilutions:

guinea pig anti-hey: 1:100
rat anti-Elav: 1:100
rabbit anti-GFP: 1:100
mouse anti-Even skipped: 1:100
rabbit anti b-gal: 1:100
Secondary antibody dilutions:
anti-guinea pig 647: 1:200
anti-guinea pig 488: 1:200
anti-guinea pig Cy5: 1:400
anti-guinea pig 555: 1:200
anti-rat 555: 1:1000
anti-rat 633: 1:1000
anti-mouse 555: 1:1000
anti-mouse 647: 1:1000
anti-rabbit 488: 1:200
anti-rabbit Cy3: 1:200

Confocal imaging:
The images provided in the present thesis were obtained using an SP8 confocal microscope by Leica. The microscope files were converted in figures using the FIJI software.

Results

Two Hey enhancer elements are functional during embryonic development

As it is mentioned in the introduction Hey expression starts late at stage 10 of embryonic development and is present until stage 16. To study whether the two enhancer elements found by searching for suppressor of Hairless (su(H)) binding sites followed the same expression pattern as Hey, reporter lines were constructed by I. Koltsaki, a previous lab member. In the present thesis, the expression of either β galactosidase or GFP, driven by Hey upstream enhancer or Hey intronic enhancer was studied in various embryonic stages of the respective transgenic lines in comparison to Hey protein expression.

Hey upstream enhancer reporter (HeyUP reporter) contains a 2665 bp fragment with all sequence upstream of transcription start site plus 5'UTR of Hey gene (figure 5 of introduction), driving the expression of beta galactosidase. Embryos of the HeyUP reporter fly line were collected and the expression of the transgene was studied by immunohistochemistry and confocal microscopy. The reporter expression pattern covers most of Hey CNS pattern in a stage 13 embryo (Fig 1,2 showing representative stacks of sections), it presents particularly strong expression in specific cell groups in the dorsal side of the CNS (yellow arrows in Fig.1) while there are cells presenting weak (Fig 2) or no reporter expression on the ventral side of the CNS. During later stages the expression of HeyUP continues to follow the Hey pattern in the vast majority of expressing cells, presenting strong and weak expression, while it is absent from some cells as shown from a stage 15 embryo (Fig. 3).

The Hey expression analysis performed in the past, revealed Hey positive cells that belong to various neuronal lineages (Monastirioti et al., 2010). In order to seek the identity of the strongly reporter expressing HeyUP cells, we used the lineage specific marker even-skipped (eve) that was previously used to characterize Hey expression pattern (Monastirioti et al., 2010). As shown in fig4, the strongly expressing HeyUP-bgal cells indeed belong to cells of the eve lineages and in particular the pCC and U cells.

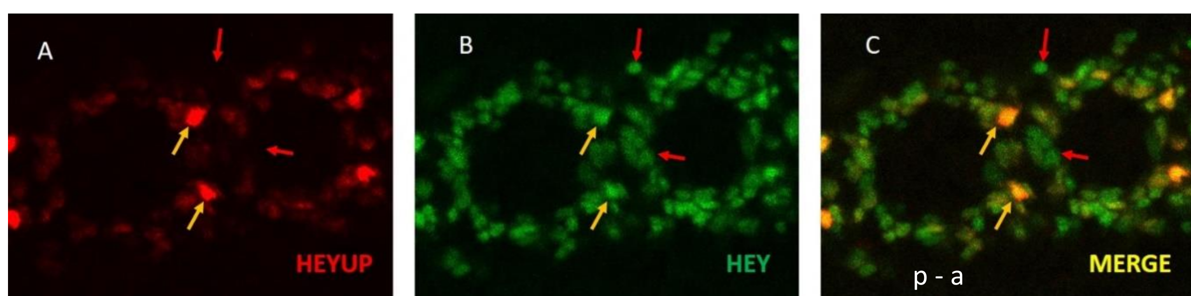


Figure 1. The expression pattern of HeyUP - beta –galactosidase reporter (red) presents strong expression in specific cells (yellow arrows) during early embryonic CNS development. The reporter has weak or no expression at other cell lineages, compared to Hey expression intensity (green) as shown by red arrows. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Image of representative Z sections stack ontwo segment of a stage 13 embryo.

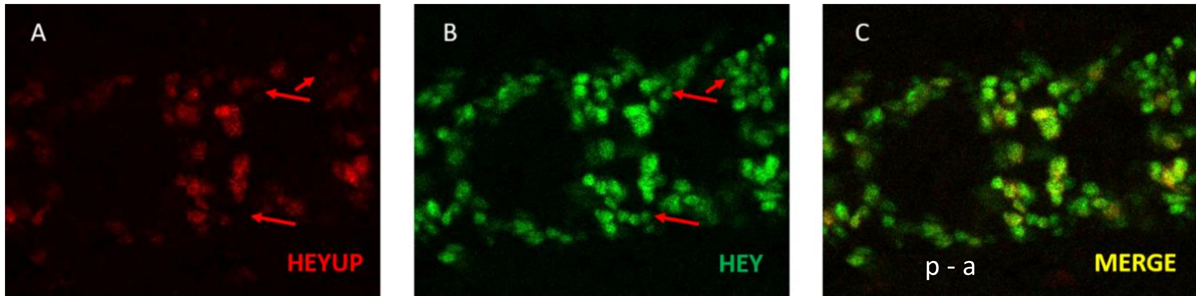


Figure 2. The expression pattern of HeyUP beta –galactosidase reporter (red) is weak in many cell lineages and absent from others as indicated by arrows. Hey expression is shown in green, "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are Z stack projections of 2 Z stacks from the CNS of a stage 13 embryo (same embryo in figure 1).

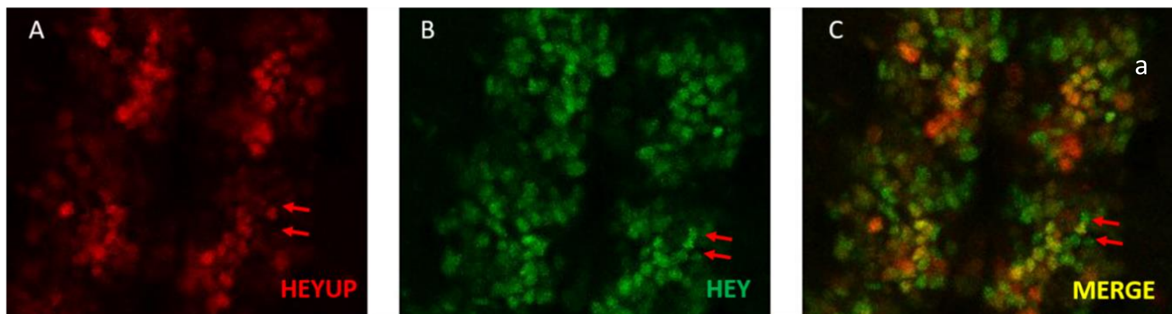


Figure 3. The expression pattern of HeyUP beta –galactosidase reporter (red) includes strong and weak expressing cells, while it is absent from some cells that are Hey positive (green), as indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are projections of representative Z stacks of a stage 15 embryo.

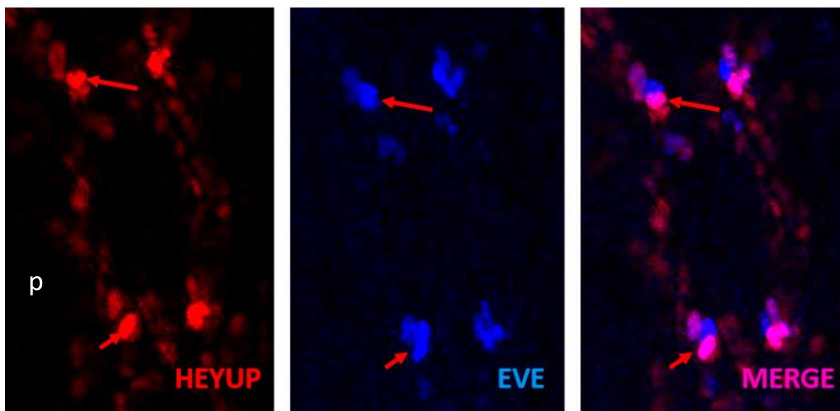


Figure 4. The strongly HeyUP beta –galactosidase reporter (red) expressing cells belong in the eve (blue) neuron al lineages. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are projections of representative Z stacks of a stage 11 embryo.

HeyUP enhancer expression is also present in the peripheral nervous system (PNS) of *Drosophila* embryos at stages, 13 to 16, in most of the Hey positive cells (yellow arrows in Fig. 5). However, the reporter is also ectopically expressed in Hey negative cells (red arrow Fig.5). Furthermore, in embryos of stage 14 and 15, Hey expression is observed in the developing midgut followed by the reporter expression of HeyUP enhancer (Figure 6). In figure 7 low magnification images present the HeyUP reporter expression in a young embryo of stage 12 on the left and an older one of stage 16 on the right panel. During earlier embryonic stages (before germ band retraction) HeyUP reporter exhibits ectopic expression in a segmented pattern of cells which putatively correspond to PNS primordia (Fig.7a), while in later stages the ectopic expression in the PNS is evident (Fig.7b).

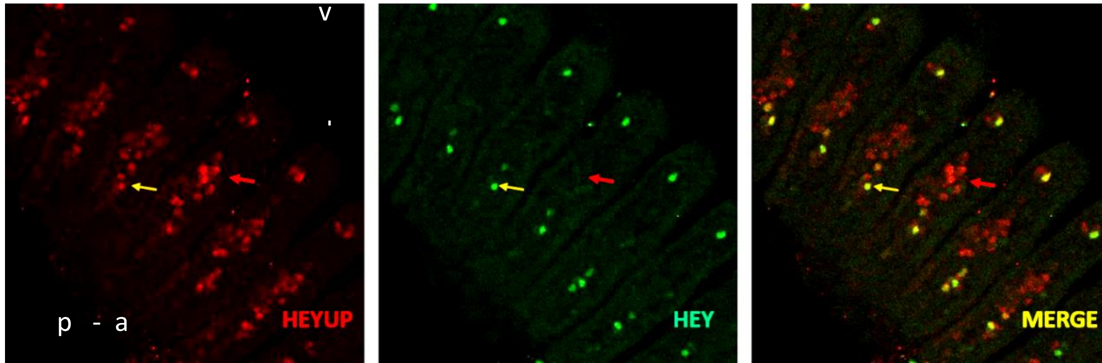


Figure 5. The expression pattern of the HeyUP enhancer reporter co-localizes with Hey expression pattern (yellow arrows) in PNS. Ectopic reporter expression in non-neuronal cells is indicated by red arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo and "v" and "d", stand for ventral and dorsal side. Images are projection of representative stacks of a stage 14 embryo.

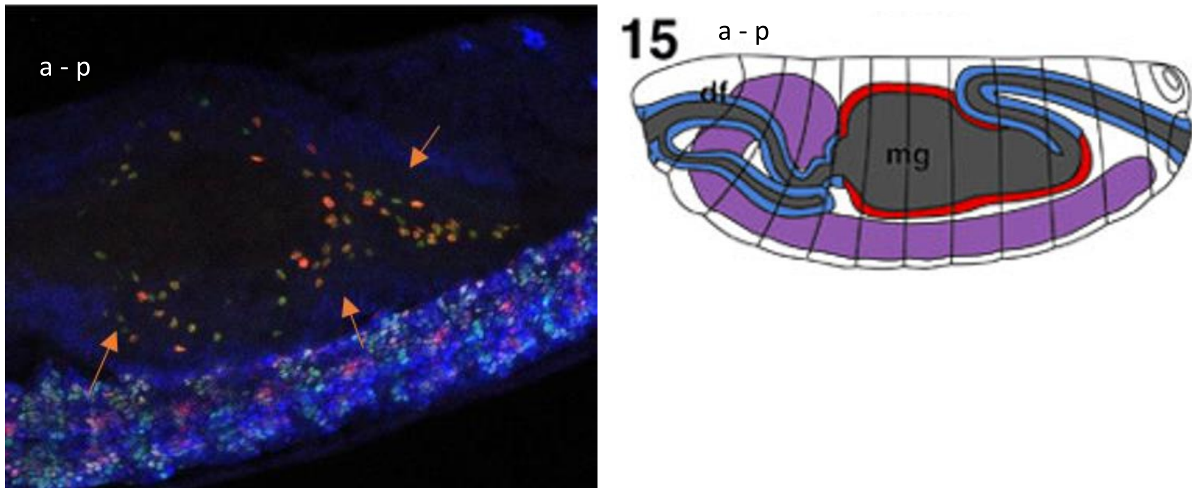


Figure 6. Left: The expression pattern of the HeyUPbgal reporter (red) co-localizes with Hey expression pattern (green). Elav positive cells (neural cells) are marked in blue. Expression of the reporter in the developing midgut is indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo. Image of representative stacks of a stage 15 embryo. Right: scheme presenting a stage 15 embryo. In red is the endodermal origin midgut, in blue the ectodermal origin gut, in purple the CNS (modified image from Atlas of Drosophila development, Volker Hartenstein).

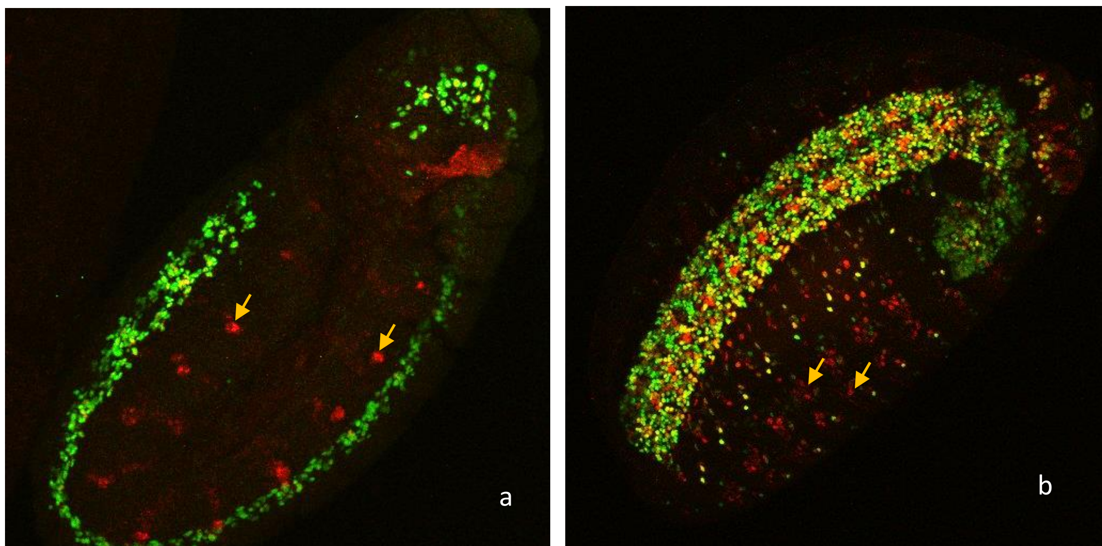


Figure 7. The expression pattern of the HeyUP bgal reporter (red) co-localizes with Hey expression pattern (green), however, ectopic expression is detectable from early to late stages in the PNS. The image presents the maximal projection of the sagittal view of a late stage 11 (a) and a stage 16 (b) embryo. Arrows indicate the ectopic expression in the PNS.

Hey intronic enhancer reporter (HeyINT2 reporter) includes the whole intron 2 region of Hey gene (733 base pairs), driving the expression of beta galactosidase. The study of the reporter expression in transgenic lines was performed by immunohistochemistry and confocal microscopy. Analysis of the images obtained revealed that HeyINT2 expression pattern coincides with the endogenous Hey pattern, during the early stages of development and the cells present uniform and strong reporter expression (Fig.8). In later stages HeyINT2 expression covers the whole Hey pattern, (Fig.9) and, in addition, it exhibits ectopic expression in some Hey negative cells which are glia according to the glia marker repo staining (Fig. 10). In developing PNS the expression of Hey and HeyINT2 reporter co-localize while HeyINT2 depicts ectopic expression as well in more cells (Fig.11). In older embryos HeyINT2 reporter expression was observed in the developing midgut, where it also presents strong ectopic expression in a stripe of big nuclei which based on their position in the middle of the midgut primordia in stage 15 embryos correspond to copper cells (Fig.12). In figure 13, low magnification images of HeyINT2 reporter expression in representative early and late stages of embryogenesis illustrate the expression pattern in CNS and early midgut, before even the fusion of anterior and posterior midgut primordia (st 12/13-Fig. 13a) and in an older embryo HeyINT2 expression in CNS and PNS (Fig.13b).

At this point we wanted to know if the expression of both reporters combined would lead to recapitulation of the complete *Hey* expression pattern. Indeed, after crossing a HeyUP-GFP reporter line to HeyINT2-GFP line we observed the expected Hey pattern, as well as the ectopic expression of the reporters in the PNS (Fig. 14).

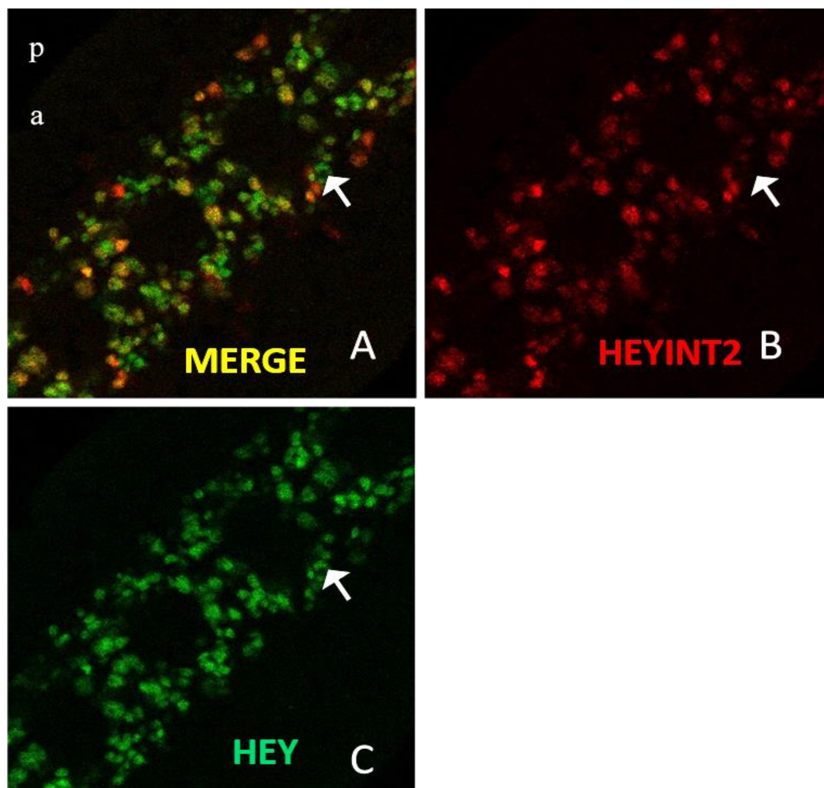


Figure 8. The expression pattern of the HeyINT2 bgal reporter (red) B, co-localizes with the Hey endogenous expression pattern (green) C. In image A there is the merge of the two patterns, arrows indicate the Hey positive cells that are negative for the reporter expression. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are projections of a single Z stack of a stage 12 embryo.

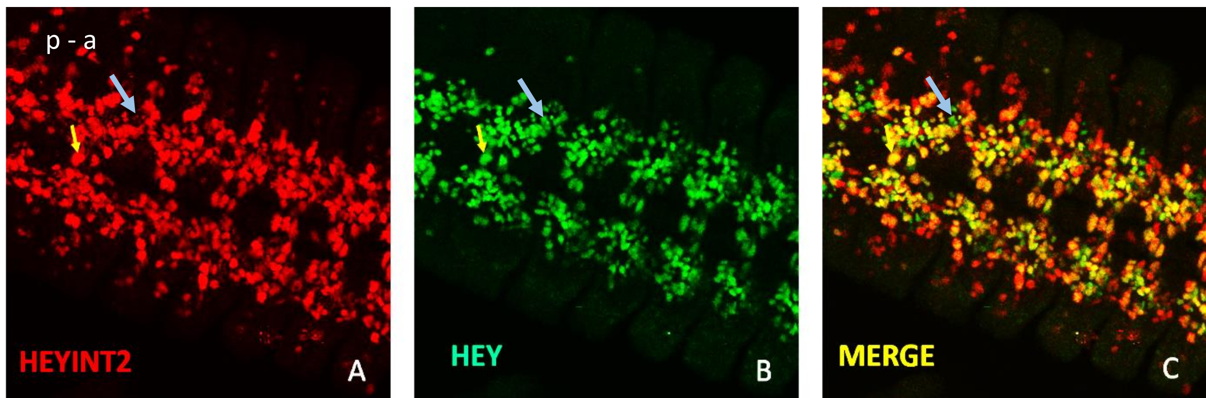


Figure 9. The expression pattern of the HeyINT2 bgal reporter (red) B, co-localizes with the Hey endogenous expression pattern (green) C. In image C there is the merge of the two patterns, yellow arrows indicate an example of strong reporter expression and blue arrows indicate an example of weak HeyINT2 reporter expression. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are projections of Z stacks of a stage 14 embryo.

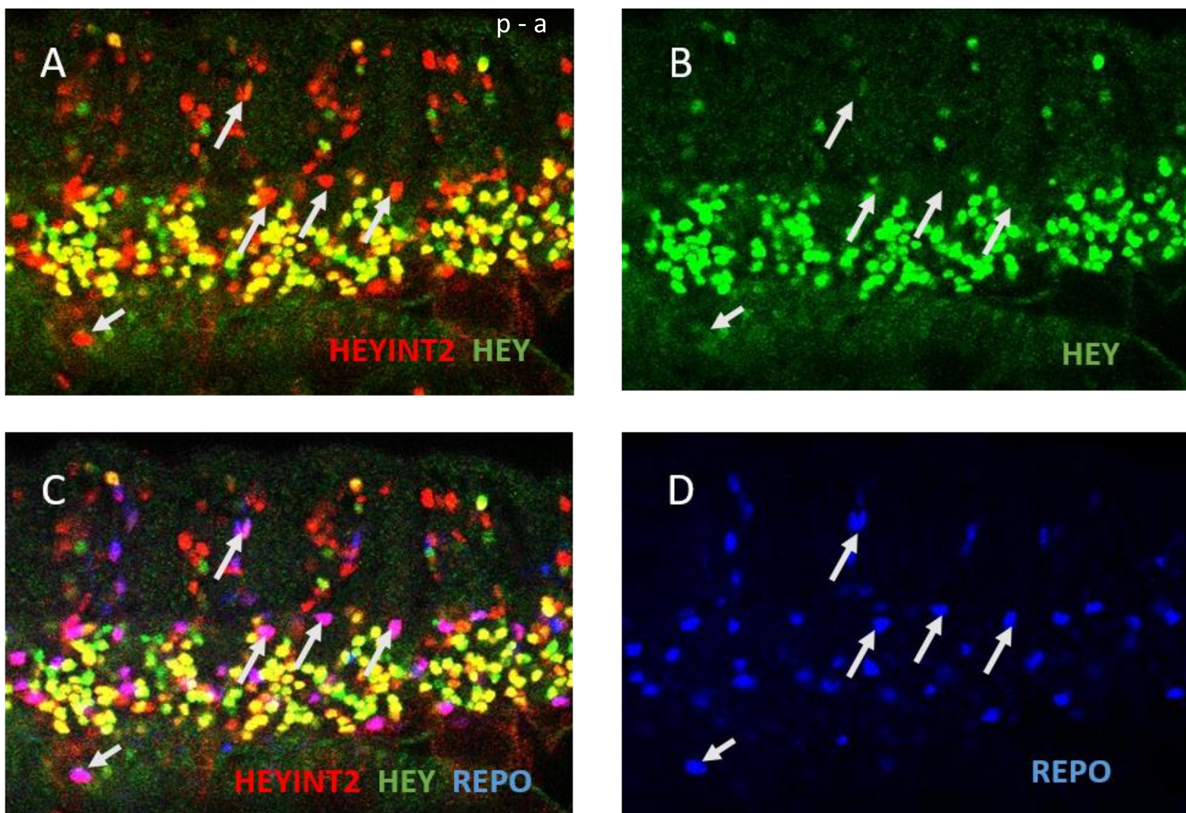


Figure 10. HeyINT2 presents an ectopic expression in cells that express the glia marker repo. HeyINT2 is in red, endogenous Hey (B) is in green and repo (D) is in blue. "a" and "p" indicate the anterior and posterior orientation of the embryonic CNS. Images are a single Z stack of a stage 15 embryo.

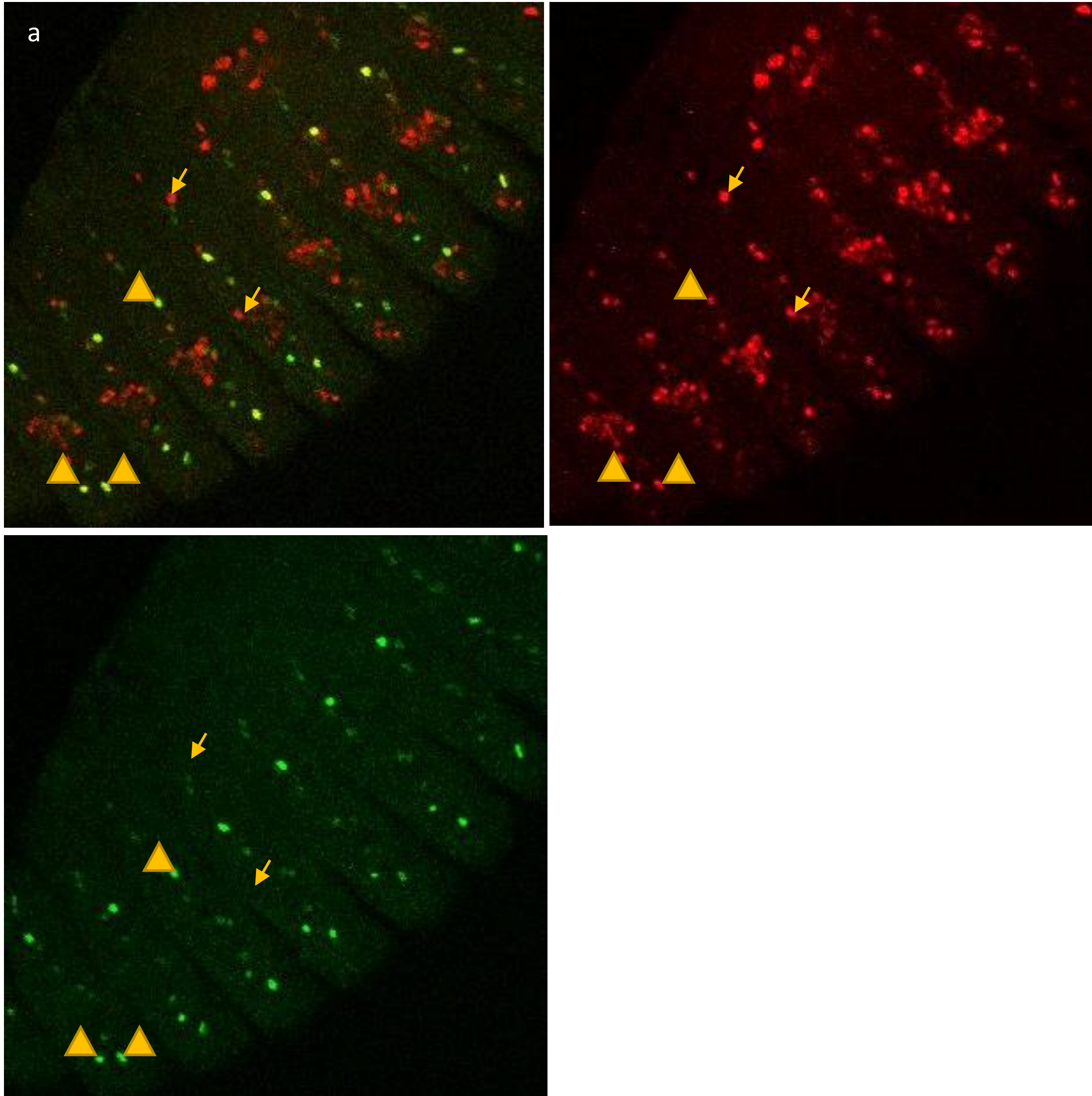


Figure 11. The expression pattern of the HeyINT2 –bgal reporter (red) co-localizes with Hey expression pattern (green), in PNS as indicated by arrowheads. Ectopic reporter expression is indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo. Images of representative stacks of a stage 15 embryo.

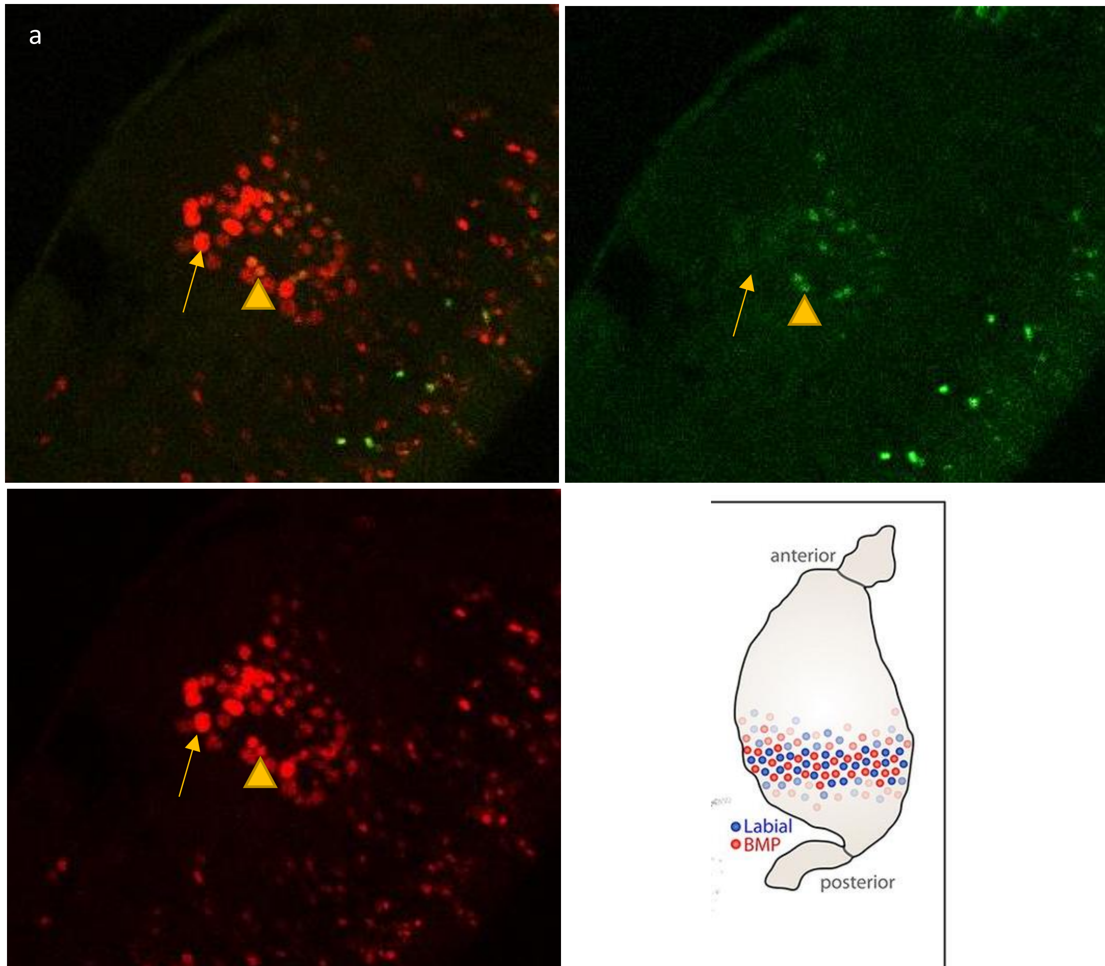


Figure 12. The expression pattern of the HeyINT2 enhancer reporter (red) co-localizes with Hey expression pattern (green), in embryonic midgut, as indicated by arrowheads. Strong ectopic reporter expression in Hey negative cells is indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo. Images of representative Z stacks of a stage 15 embryo. The cartoon on the bottom right presents copper cells of the midgut 24 hours after pupal formation, since labial and BMP signaling are localized to the area of these cells (Driver & Ohlstein, 2014).

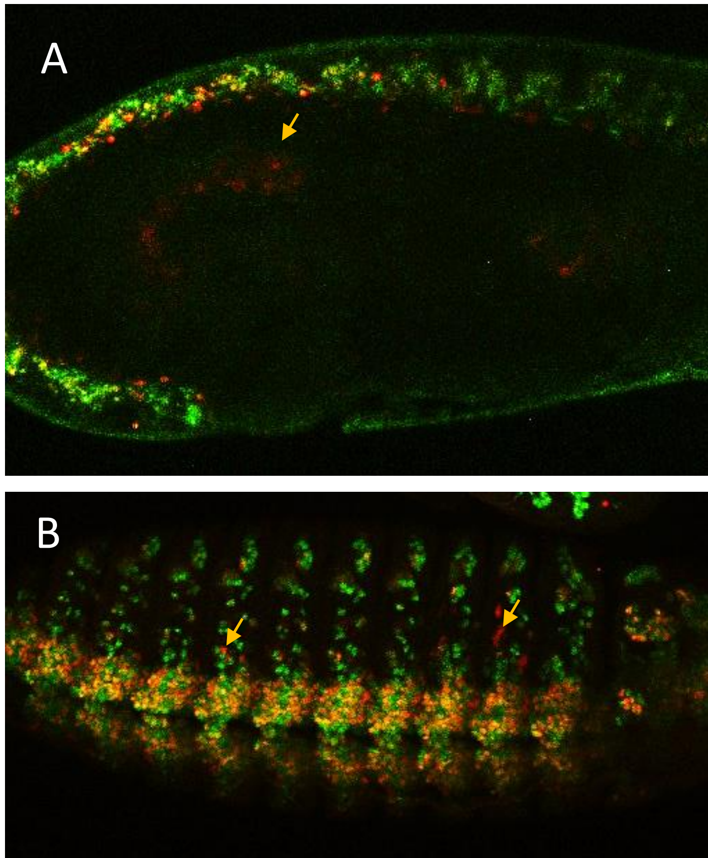


Figure 13. The expression pattern of the HeyINT2 bgal reporter (red) co-localizes with Hey expression pattern (green). The images present the maximal projection of the sagittal view of a stage 12 (A) and a stage 15 (B) embryo. Arrows indicate the ectopic expression in the developing midgut (A) and the ectopic expression in the PNS (B).

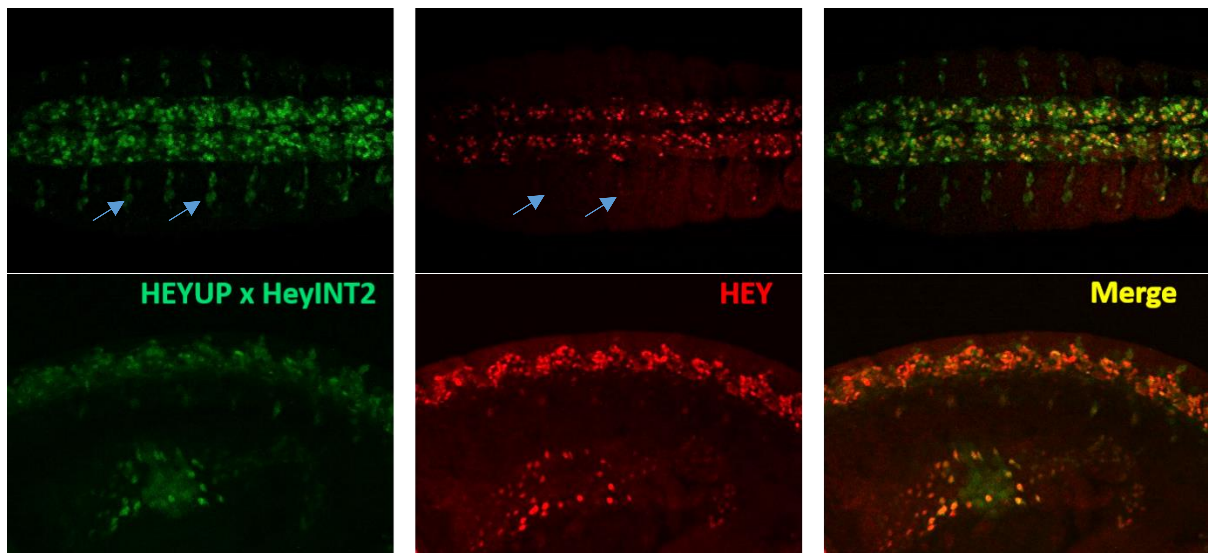


Figure 14. HeyUP-GFP crossed to HeyINT2-GFP leads to a complete Hey pattern. GFP in green, Hey in red. Ectopic expression of the reporters in the PNS is indicated by arrows. The image is projection of representative stacks of the CNS of a stage 14 and 13, respectively, Drosophila embryos.

Hey enhancer reporter expression is distorted IN Delta/Serate mutant background

In order to comprehend the responsiveness of each enhancer element to Notch signaling we introduced the two Hey enhancer reporters, into a Delta/Serate mutant genetic background in which Notch signaling is absent. The expression of the reporters in this condition was studied by applying immunohistochemistry and confocal imagining. The genetic crossing experiments in order to introduce the reporters in the Delta/Serate mutant background were performed by Aliki Grammatikaki, an undergraduate student in the laboratory. Image analysis revealed that in absence of Notch signaling the HeyUP reporter expression is distorted, following the remaining pattern of the endogenous Notch-independent Hey expression in embryonic CNS (Fig. 15). However, there is a cell population in the midline of the CNS in which weak Hey expression is maintained while, the HeyUP reporter expression is not detected (Fig.15).

Expression of the HeyINT2 enhancer reporter is strongly affected, in the Delta/Serate mutant genetic background. In most of the mutant embryos the reporter expression is completely abolished, while there is one example where weak, ectopic expression of the reporter was observed (Fig.16 E-G). In Figure 14, four embryos of unspecified developmental stage are shown, supporting the observations. Hey expression pattern is presented for comparative purposes, while the neuronal marker Elav is used to indicate the neuronal defect in each case.

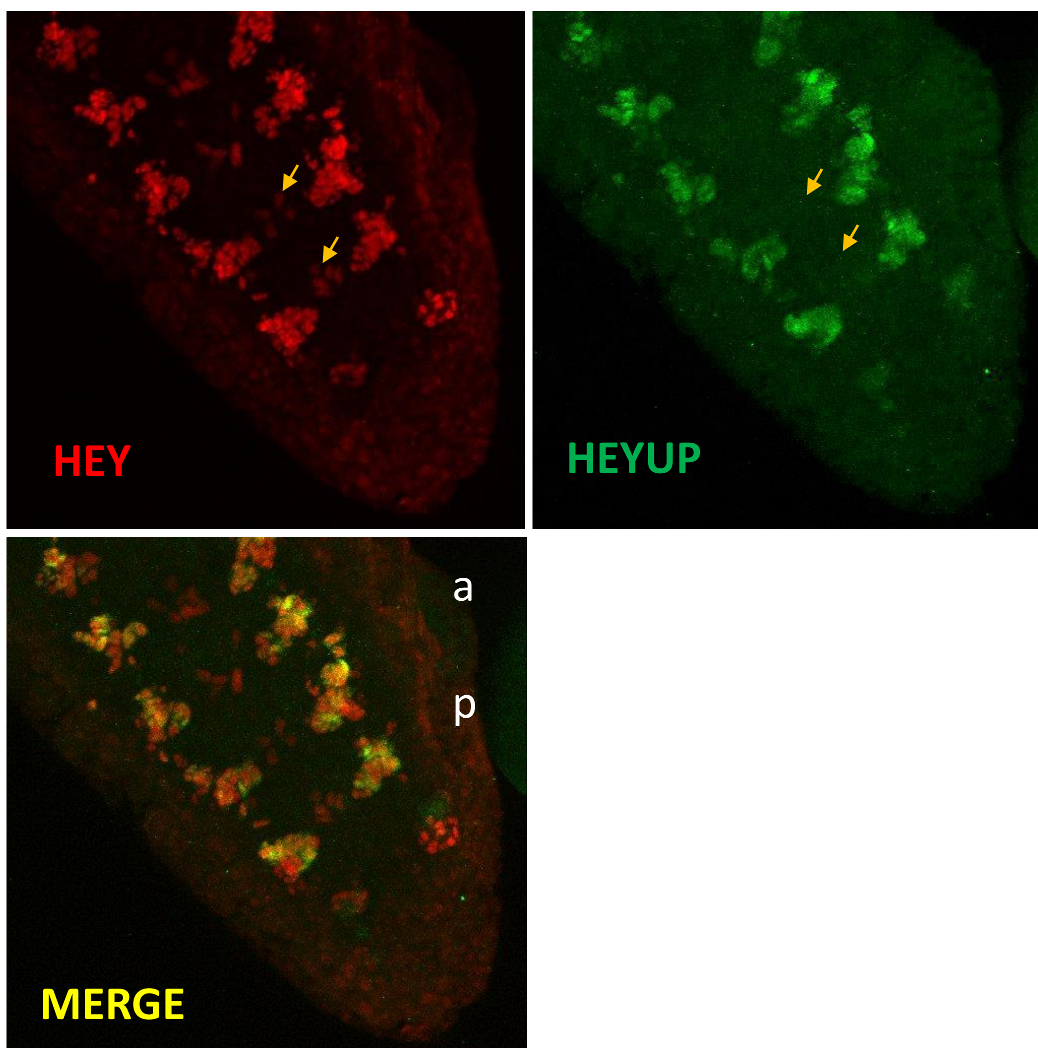


Figure 15. In a Delta/Serate mutant background the Hey expression pattern (red) is distorted. Accordingly, the HeyUP reporter expression pattern is distorted, following Hey expression in most of the Hey positive cells. Hey positive cells that lack reporter

expression are indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo. Images are projection of representative stacks of a Delta/Serate mutant embryo.

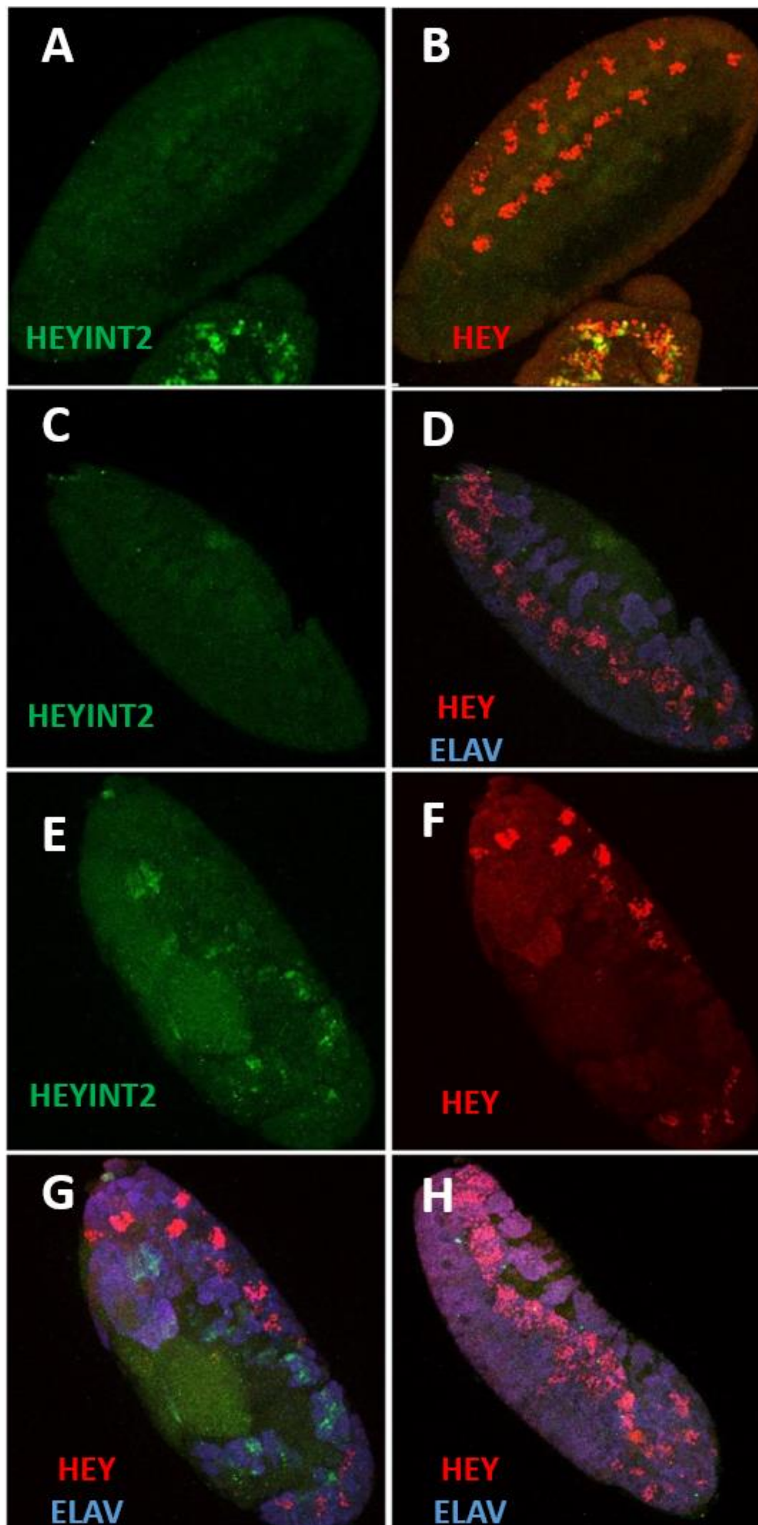


Figure 16. In a Delta/Serate mutant background the Hey expression pattern (red) is distorted. In absence of Notch signaling HeyINT2 reporter expression pattern (green) is abolished. In blue is shown the expression of the neuronal marker Elav as an indicator of the abnormal neuronal development. Images are projection of representative Z stacks of mutant embryos. Images A and B depict the same embryo, C and D a second embryo, in both cases the reporter is not expressed. In images E, F and G

that belong to the same embryo weak ectopic expression of the reporter is observed. In image H a late embryo with no reporter expression is depicted. Due to the severe phenotype of the mutant background the stages of the embryos could not be specified.

Upstream Hey enhancer deletion analysis reveals significant Su(H) binding sites

To further comprehend the response of the two Hey enhancer elements to Notch signaling we studied the expression of these reporters upon deletion or mutation of the Su(H) binding sites identified in the respective sequences. The upstream enhancer element (HeyUP) was studied by deletion analysis, while the intronic enhancer (HeyINT2) was studied by Su(H) binding site mutagenesis.

To study the significance of each of the Su(H) binding sites in the sequence of HeyUP, two different sets of primers were used -as described in materials and methods- to clone shorter fragments of HeyUP reporter into a GFP expression vector. Two new reporter constructs were generated and inserted into Drosophila genome using the Phi C31 integration system (Groth, Fish, Nusse, & Calos, 2004). The two reporter fly lines that were generated contain either the two proximal or none of the three su(H) binding sites that have been identified within the region of HeyUP (Fig.17). The expression of the two reporter lines, namely **2+3SH** and **NSH**, respectively, was studied during Drosophila embryonic development in central nervous system, embryonic midgut and peripheral nervous system. Furthermore, it was studied in the third instar larvae central nervous system.

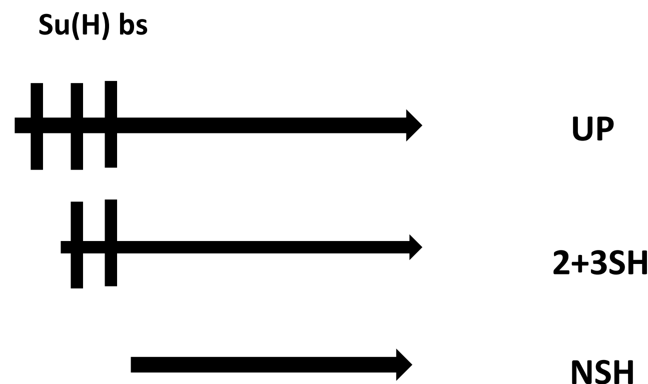


Figure 17. Scheme depicting the three enhancer sequences that were studied in the analysis of the upstream enhancer element.

The expression 2+3SH reporter initiates at the same developmental stage (st 10) as the endogenous Hey expression (Fig.18), presenting strong expression at a subset of cells, while it does not recapitulate the whole pattern of Hey which though could be attributed to a delay in GFP accumulation. On the other hand, the 2+3SH enhancer reporter maintains the same expression pattern as the unmodified HeyUP enhancer reporter during all developmental stages (fig 19) suggesting that the most distal Su(H) bs is dispensable. In contrast, NSH reporter expression is not evident in early stages (stage 10 embryo (Fig.18) and it exhibits a weak and more restricted expression pattern after stage 11 (Fig.19). This weak and restricted expression of NSH is maintained during later embryonic development (Fig.19). Moreover, the enhancer reporter containing the two Su(H) binding sites presents expression in the PNS, including ectopic expression in some cells similarly to the HeyUP reporter, while the NSH reporter is not expressed in PNS (Fig.20). Finally, from the two modified upstream

enhancers, only the 2+3SH seems to maintain midgut expression in Hey positive cells as shown in figure 21.

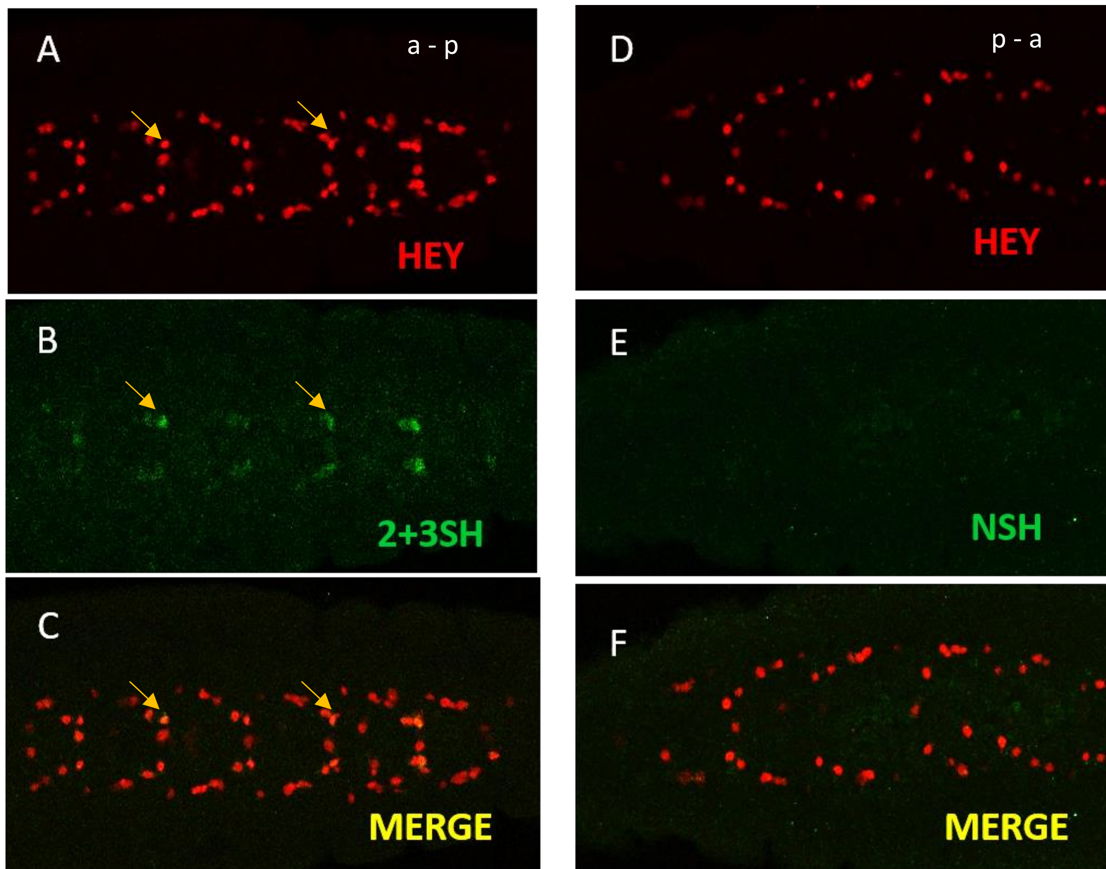


Figure 18. Deletion analysis of the HeyUP enhancer element. The 2+3SH reporter (green) shows strong expression in specific lineages (B, arrows) within the Hey pattern (A, red) on the left panel (A-C). The NSH reporter (E, green) delays to initiate expression as shown on the right panel (D-F). "a" and "p" indicate the anterior and posterior orientation of the embryo, all images are maximal projection of Z stacks of stage 11 and 10 embryos.

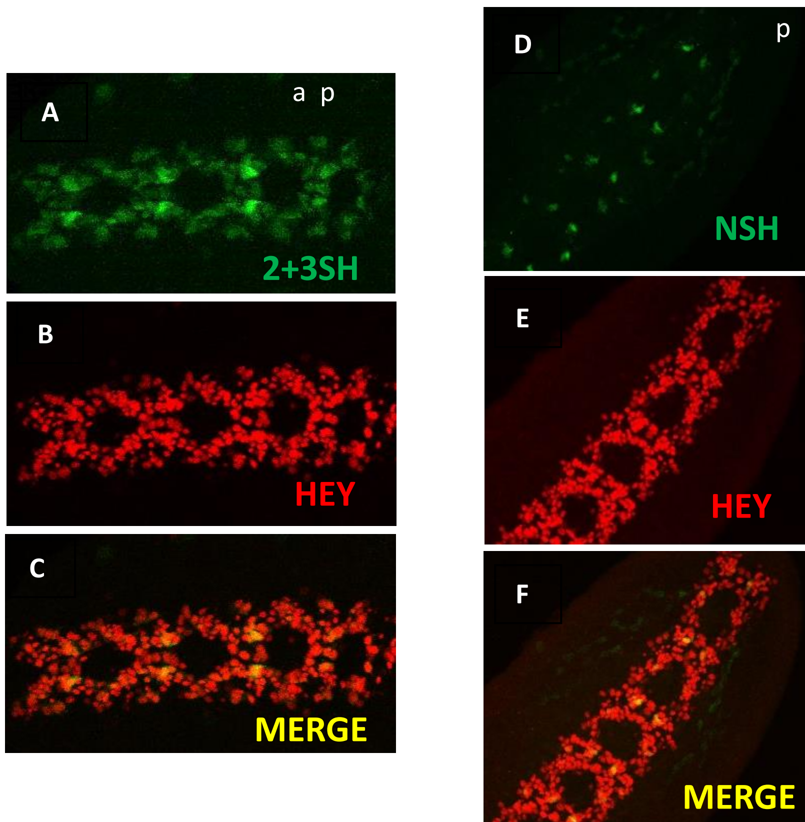


Figure 19. Hey deletion analysis of the upstream enhancer element. The 2+3SH reporter (green) shows strong expression in specific lineages of the Hey pattern (red) on the left panel. The NSH reporter (green) is weak as shown on the right panel. "a" and "p" indicate the anterior and posterior orientation of the embryo. Images of maximal projection Z stacks of late stage 12 embryos.

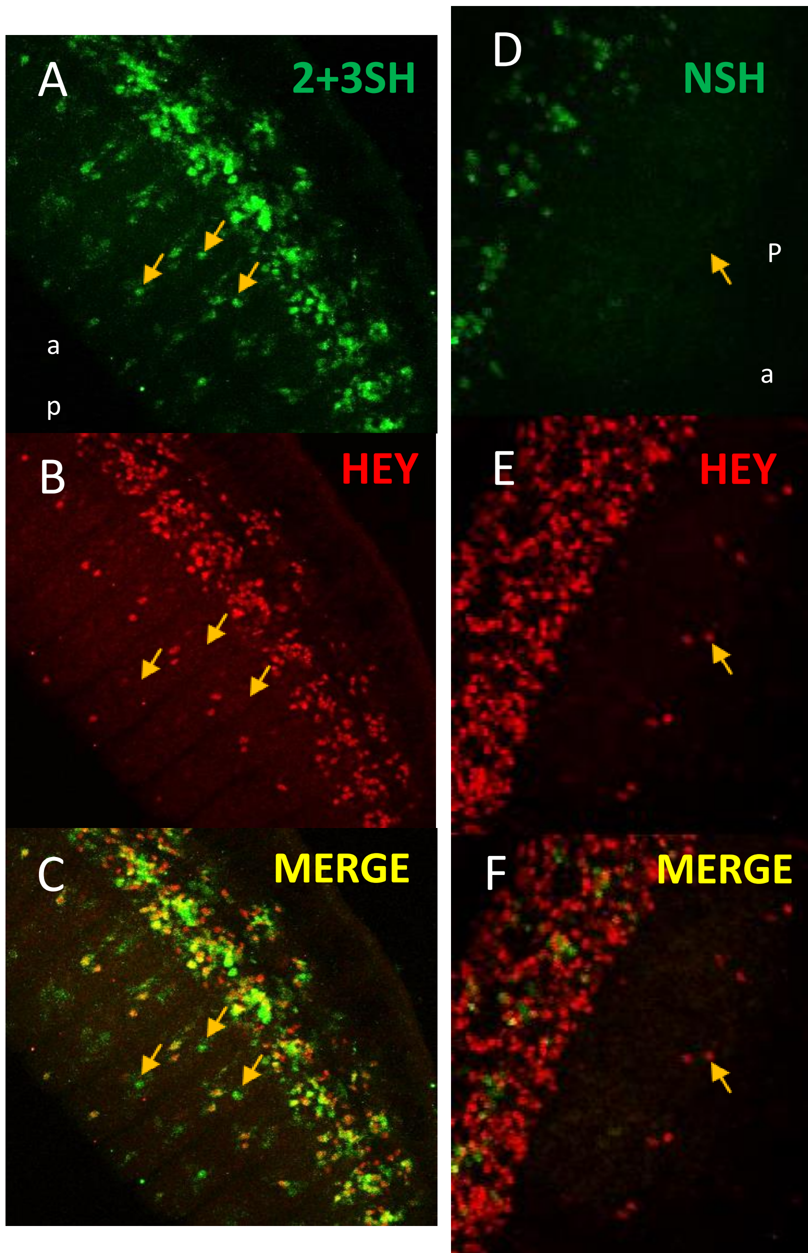


Figure 20. The expression of the 2+3SH upstream reporter (A) presents ectopic expression (arrows) when compared to Hey expression pattern (B) in the drosophila embryo PNS. C is a merge of A and B. On the right panel, the expression of the NSH reporter (D) has a limited expression pattern, with no expression (arrows) in the PNS of the embryo. E presents the endogenous hey expression of the embryo and F is a merge of D and E. Images are projection of representative Z sections of the lateral side of a stage 15 embryos.

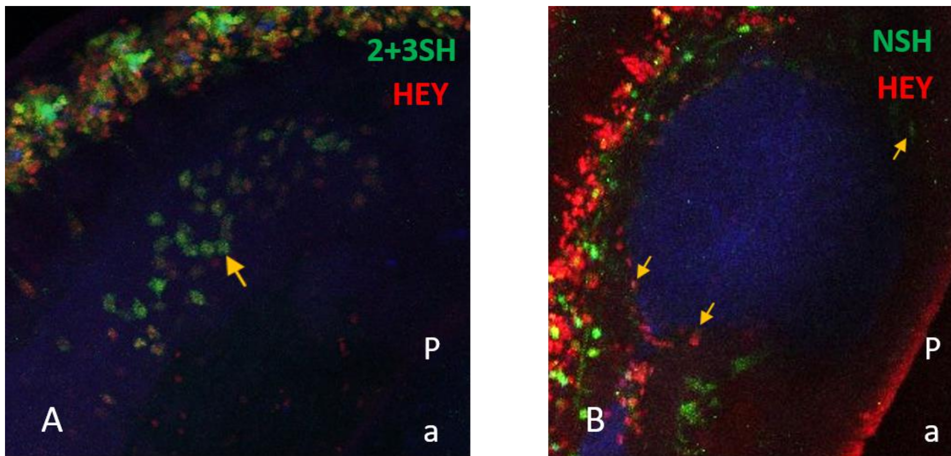


Figure 21. Hey (red) is expressed in midgut cells during embryonic development. 2+3SH upstream enhancer reporter (green) is presented in a stage 12 embryo (A), while in B, the image of a later embryo (stage 13) expressing the NSH upstream enhancer (green) is shown. Reporter expression is indicated by arrows. "a" and "p" indicate the anterior and posterior orientation of the embryo. Images are projection of representative Z stacks.

The expression of the HeyUP enhancer element in third instar larvae CNS (Fig.22) was studied by Nick Batsiotos, a previous lab member. Some of the Hey positive cells are in the lineages of the mushroom bodies neuroblasts, which produce the Kenyon cells and in other brain regions such as the optic lobes and in cells located in the Medulla (for brain regions see (Fig.23), (Kurusu et al., 2002)). We decided to study the two modified upstream enhancer reporters and analyze their expression in the CNS of 3rd instar larvae.

The expression of 2+3SH reporter recapitulates the pattern of HeyUP enhancer reporter in the larval CNS (Fig. 24) and it is present in all Hey positive cells. In the dorsal and ventral sides there are lineages with strong or weak (blue arrow) reporter expression (Fig. 24 A, A', A'', C, C', C''), while particularly strong expression is detected in Kenyon cells (yellow arrow in Fig. 24 A, A', A''). Furthermore, co-localization of Hey and 2+3SH reporter, expression is observed at the optic lobe (Fig. 24 B, B', B''). In a similar manner, the expression pattern of 2+3SH reporter in the VNC, includes all Hey expressing lineages, both in thoracic (Fig.25 up) and abdominal (Fig.25 down) neuromeres presenting strong or weak reporter expression). In addition, the reporter presents ectopic expression in the abdominal interneurons, where Hey is not present (Fig. 25, B).

We then proceeded in studying the expression pattern of NSH reporter in the CNS of third instar larvae. In this case the expression pattern did not cover all the Hey positive cells but mainly cells of the mushroom body lineage (Fig. 26) and part of the optic lobe (Fig. 27). The expression in the lineages located at the Medulla is restricted, while, the reporter presents ectopic expression in older neurons of the mushroom body lineage located in deeper layers relative to the young ones located superficially close to the NBs (Fig. 26 upper and lower panels respectively). In figures 27 and 28 different views of the ventral side of the CNS are presented showing partial reporter expression in Hey positive cells in the optic ganglia and its absence from the majority of the Hey expressing cells, while some Hey negative lineages present an ectopic NSH reporter expression (Fig. 28). In the VNC the expression of NSH reporter is restricted compared to Hey expression, presenting ectopic expression in the

ventral side of the region of the thoracic interneurons and in the dorsal side of the region of the abdominal interneurons (Fig. 29).

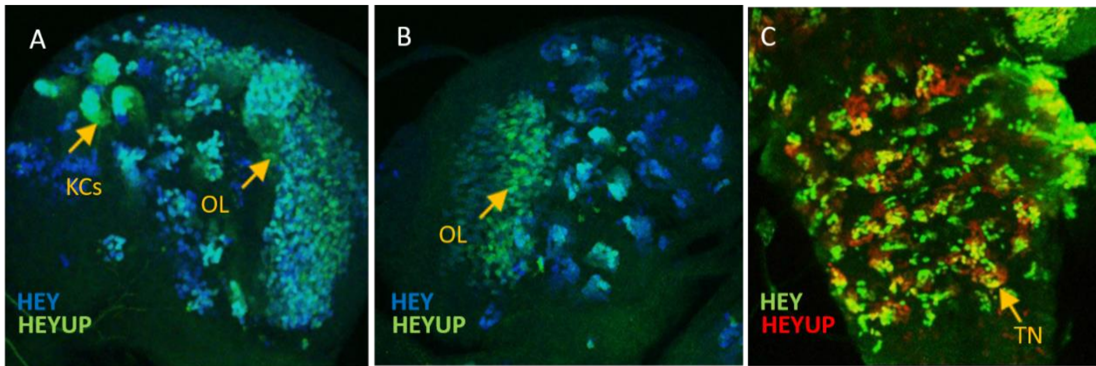


Figure 22. The expression of the HeyUP enhancer reporter in the CNS and VNC of a 3rd instar larva. The reporter expression (green) covers the Hey expression pattern (blue) in CNS (A, B), with weak and strong expression lineages. The images are representative stacks of the dorsal side (A), where the four mushroom body lineages (KCs) are indicated by arrow and the ventral side (B) where the optic lobe is indicated (arrow) and Medulla is marked between lines. The ventral site of the VNC is presented on the right panel (N.Batsiotos).

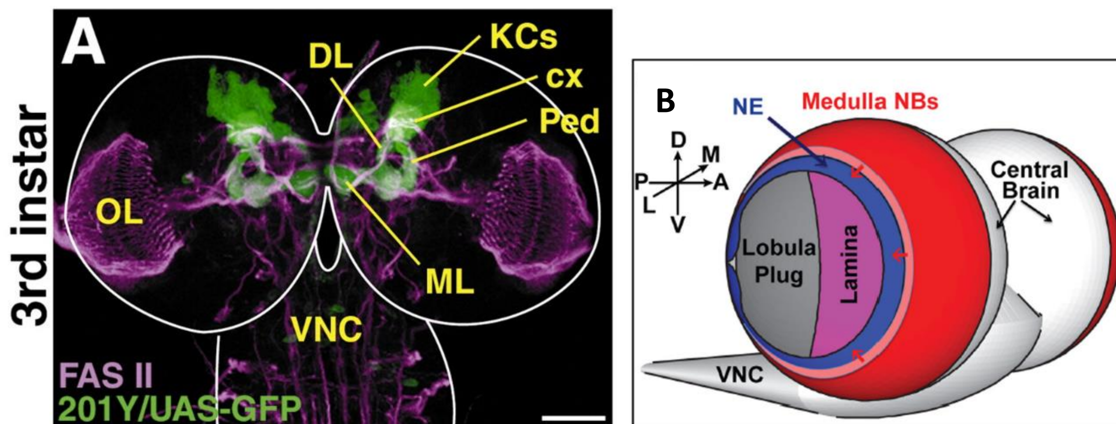


Figure 23. Image modified by (Kurusu et al., 2002) (A) and (Li et al., 2013) (B). (A) Third instar larval brain stained with anti-FAS II (magenta) and GFP (green) driven by a MB GAL4 line, 201Y. Indications are cx, calyx; DL, dorsal lobe; KCs, Kenyon cells; ML, medial lobe; OL, optic lobe; Ped, peduncle; VNC, ventral nerve cord. (B) Model of larval brain.

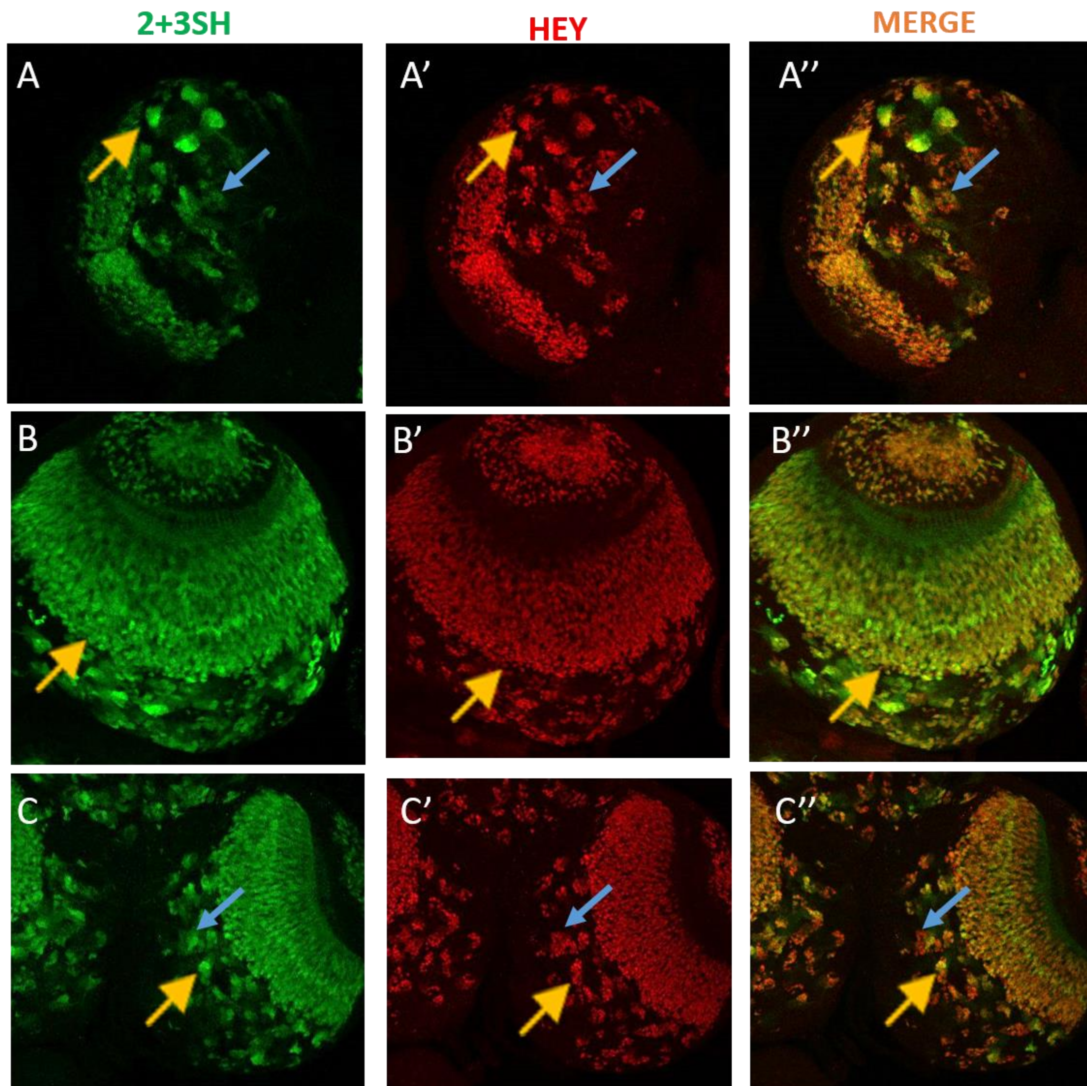


Figure 24. The expression pattern of the 2+3SH reporter (green) co-localizes with the expression pattern of Hey (red) in the CNS of 3rd instar larvae, presenting strong expression (yellow arrows) or weak expression (blue arrows). Images are representative Z stacks of the dorsal side (A, A', A''), the optic lobe (B, B', B'') and the ventral side (C, C', C'').

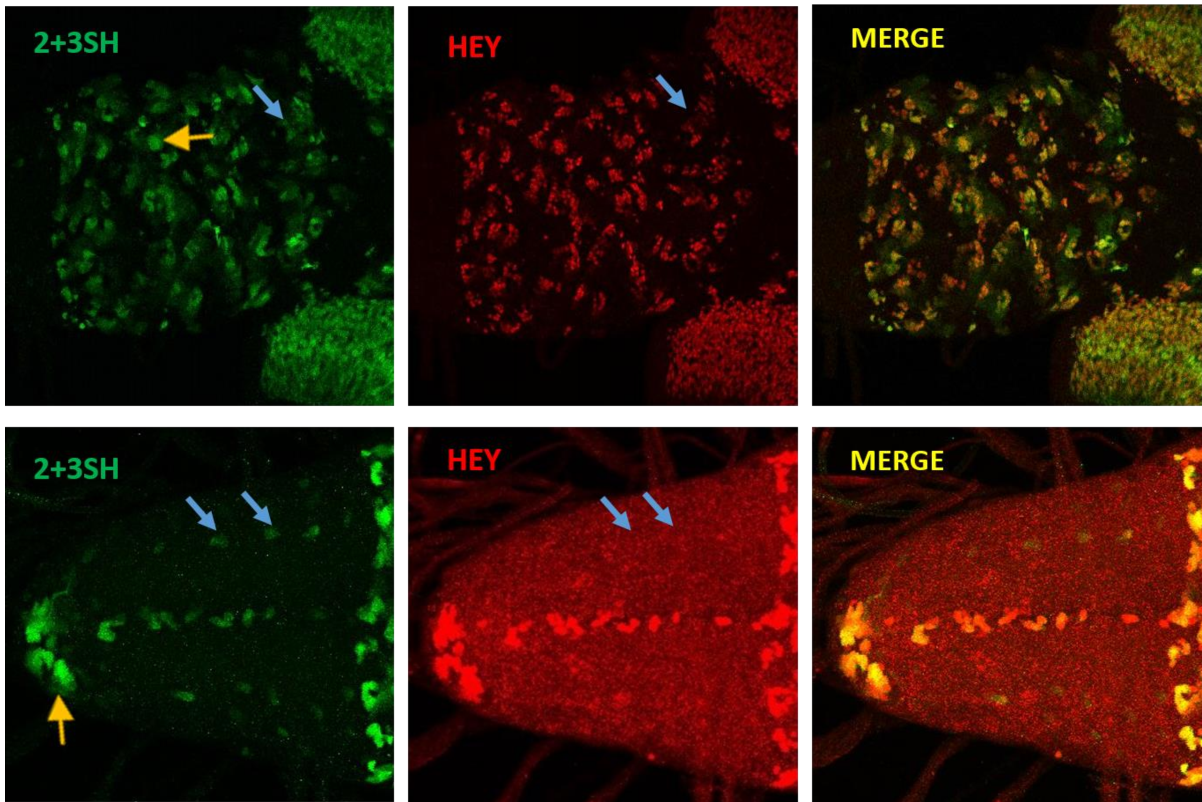


Figure 25. The 2+3SH reporter expression pattern in the region of the thoracic interneurons (up) and of the abdominal interneurons (down) of the VNC has no difference to the expression pattern of the full upstream reporter (Figure 20). Yellow arrows indicate strong reporter expression and blue arrows indicate weak 2+3SH reporter expression. Images are projections of representative Z-sections in the VNC.

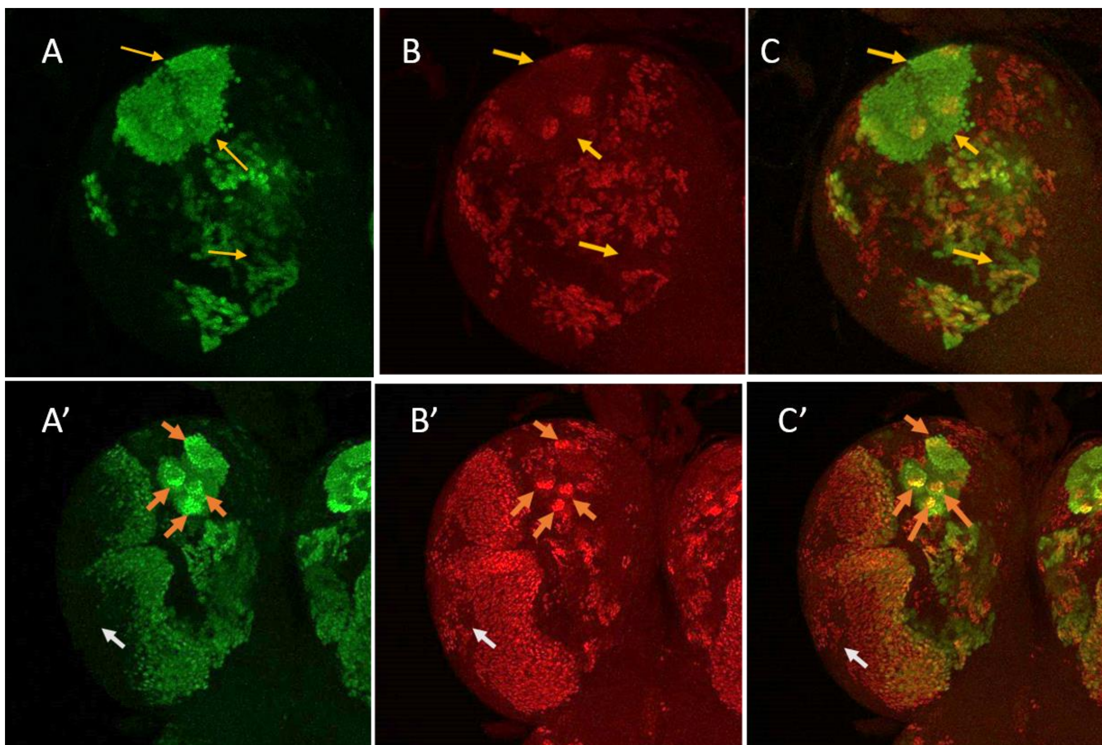


Figure 26. The expression pattern of the NSH upstream enhancer (green) is restricted compared to Hey expression pattern (red), mainly around mushroom-body lineages (orange arrows) and partially on the optic lobe. White arrows indicate the lack of reporter expression in the area of optic lobe, while yellow arrows indicate ectopic expression of the reporter in the region

of mushroom bodies in older neurons of the lineage. Merge of NSH reporter and Hey patterns is shown (C, C'). Images are projection of representative Z stacks of the dorsal side of a 3rd instar larva brain. Images A, B, C are more superficially in comparison to images A', B', C'.

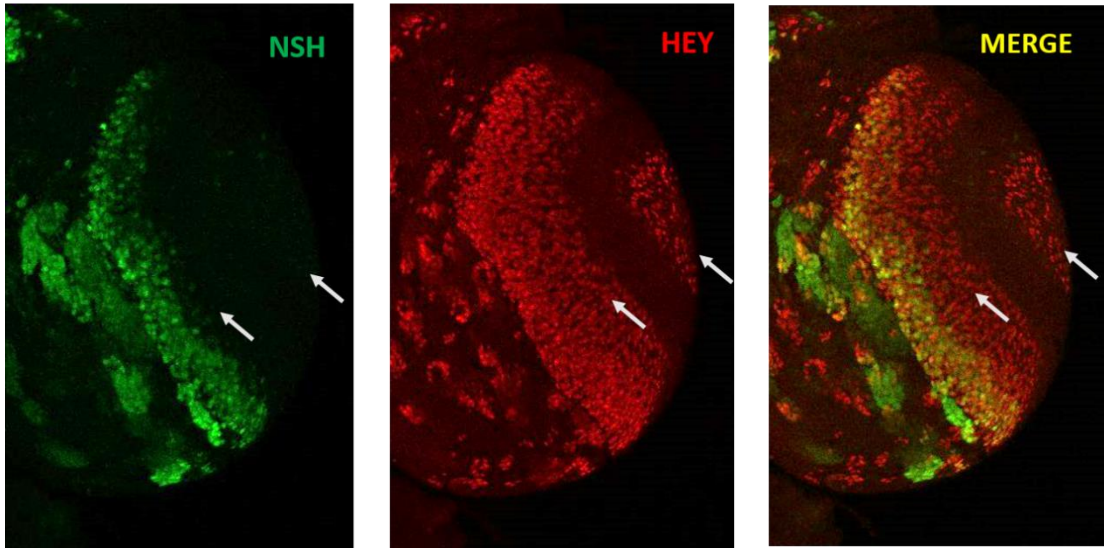


Figure 27. The expression of the NSH reporter (green) is restricted compared to the Hey expression pattern (red) in the lineages of the optic lobe as indicated by arrows. Images are projection of representative Z- stacks of a 3rd instar larva brain lobe.

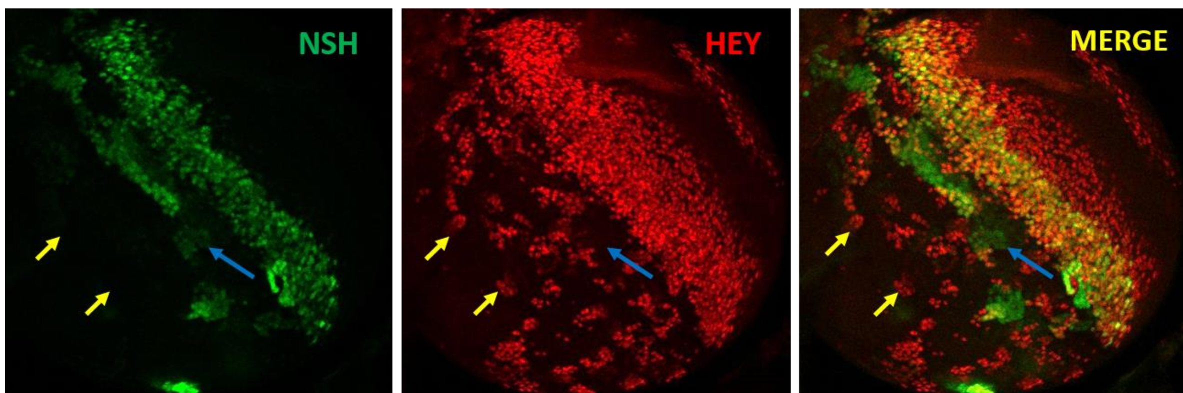


Figure 28. On the ventral side of the Drosophila larvae brain lobe the expression of the NSH reporter (green) is not present in all Hey expressing cells as indicated by yellow arrows. On other lineages an ectopic reporter expression is observed (blue arrows). Images are projection of representative Z stacks.

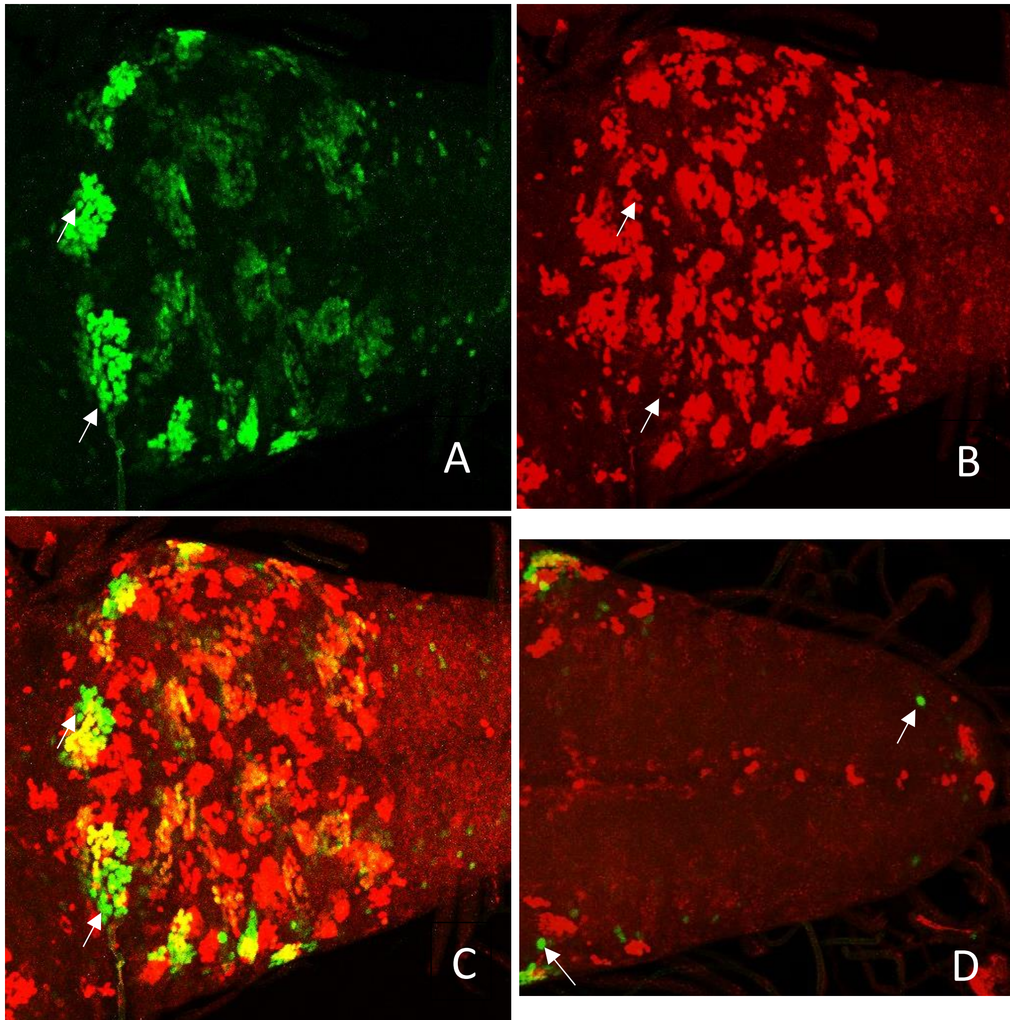


Figure 29. The expression of NSH Hey bgal reporter (A) in the VNC of 3rd instar larvae is restricted compared to the Hey expression pattern (B). The reporter presents ectopic expression (arrows) in the ventral side, in the region of the thoracic interneurons (A-C) and in the region of the abdominal interneurons (D) of the VNC. Images are projection of representative Z stacks of the ventral side (A-C) and the dorsal side (D) of a 3rd instar larval VNC. C is merge of A and B.

Hey intronic enhancer point mutation analysis indicates Su(H) binding site 2 as the most important

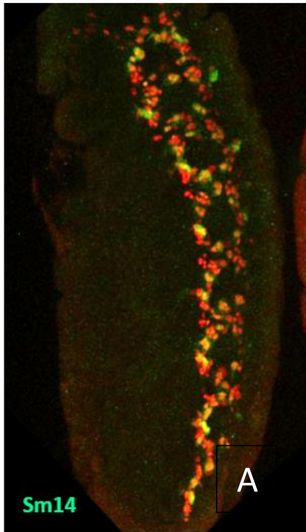
To study the response to Notch signaling of the intronic Hey enhancer a previous member of the lab generated a Hey intronic enhancer reporter that carried mutations in all four Su(H) binging sites (SALL reporter), identified in that enhancer element. Study of this reporter showed that there was no expression upon mutagenesis (I. Koltsaki), (data not shown). This result indicates that one or more of the Su(H) binding sites that was mutated is responsible for the expression pattern of the intronic reporter. In order to specify which of the Su(H) binding sites were functional, four reporter lines were generated. Each of the lines had a different combination of Su(H) binding site point mutations. Line **Sm14** has Su(H) 2 and 3 wild type binding sites. Line **Sm123** contains non mutated Su(H) binding site 4 and **Sm124** the wild type binding site 3. Last but not least, in line **Sm134**, Su(H) binding site 2 remains non mutated. The lines were generated by cloning the modified sequences in a GFP expression vector and integrating them in fly genome using the Phi C31 integration system (Groth et al., 2004).

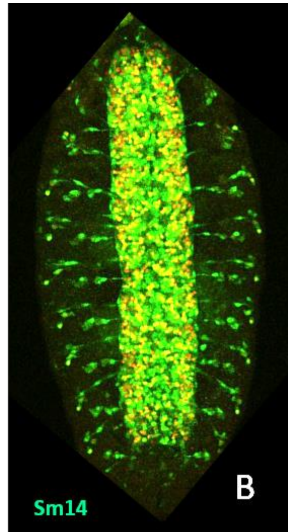
The expression of the reporters was studied in the CNS and midgut during embryonic development and later in the CNS of 3rd instar larvae. All the images for this experiment were obtained applying the same settings on a confocal laser scanning microscope for comparative reasons and samples were prepared under the same conditions to achieve the least possible variability. Representative images of early and late embryos are provided (Fig. 30, 31). Moreover, expression in the PNS and the developing midgut are provided. In addition, in figure 33, images of embryos expressing the non-mutated intronic enhancer are provided for comparative purposes.

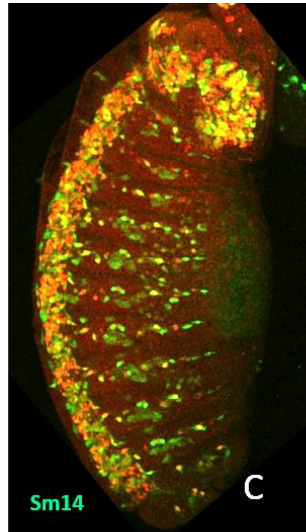
The imaging results of the reporters indicate the significance of Su(H) binding site 2, that upon mutation of all three binding sites (Sm134) it could generate the full expression pattern of a non-mutated intronic reporter. In a similar manner Su(H) binding 3 recapitulates the non-mutated intronic reporter expression, even though, weaker expression is observed (Fig. 30, 31). In more detail, The Sm14 mutant line presents a late initiation of expression in the early stages of hey expression which becomes more prominent in later stages (Fig. 30 A-C, 31 A-C). The expression of this reporter is also observed in the PNS of embryos, co-localizing with Hey expression but also presenting the ectopic expression (Fig. 30C, 31C) that is observed in HeyINT2 reporter (Fig. 8). The expression of Sm124 is very weak throughout all embryonic developmental stages, maintaining the same expression pattern with HeyINT2 reporter (Fig. 30 D-F, 31 D-F). Ectopic expression is observed in the PNS of Sm124 embryos, which is weak as is the rest of the expression pattern (Fig. 30F 31F). Images of the Sm134 reporter indicate that the expression pattern of the reporter even from the early stages of development co-localizes with Hey expression pattern in a similar manner as HeyINT2 reporter. The intensity of expression of the reporter is similar to the non mutated intronic enhancer, but weaker than the endogenous Hey expression. Sm134 does not recapitulate the complete Hey expression pattern and during late embryonic stages enhanced ectopic expression is observed (Fig. 30 G-I, 31 G-I). Finally, images of the Sm123 mutated enhancer reporter present a weak and delayed expression (Fig. 30 J-L, 31J-L). Specifically, expression initiates from stage 13, is weak and later, during stage 15 an ectopic expression in the PNS is observed (Fig. 30L, 31L).

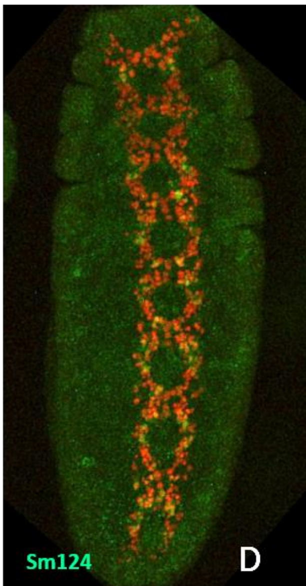
The expression of the mutated intronic enhancer reporters in the midgut of embryos is presented in sagittal plane images of stage 14 to 16 embryos (Fig. 32). Sm14 and Sm134 present a weak expression in the midgut, while the expression of Sm123 is almost undetectable. The expression of Sm124 reporter in the midgut was not studied, as the corresponding data were not sufficient.

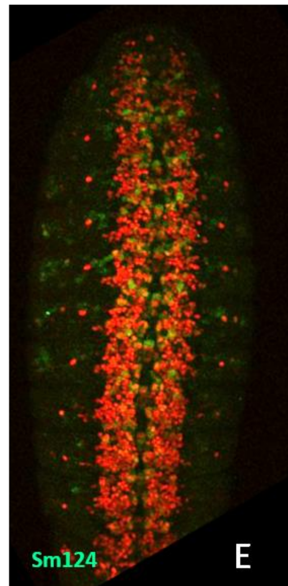
Early stages (10-12) **Late stages (13-15)** **PNS**

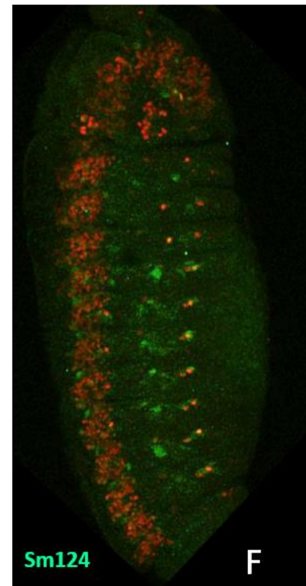












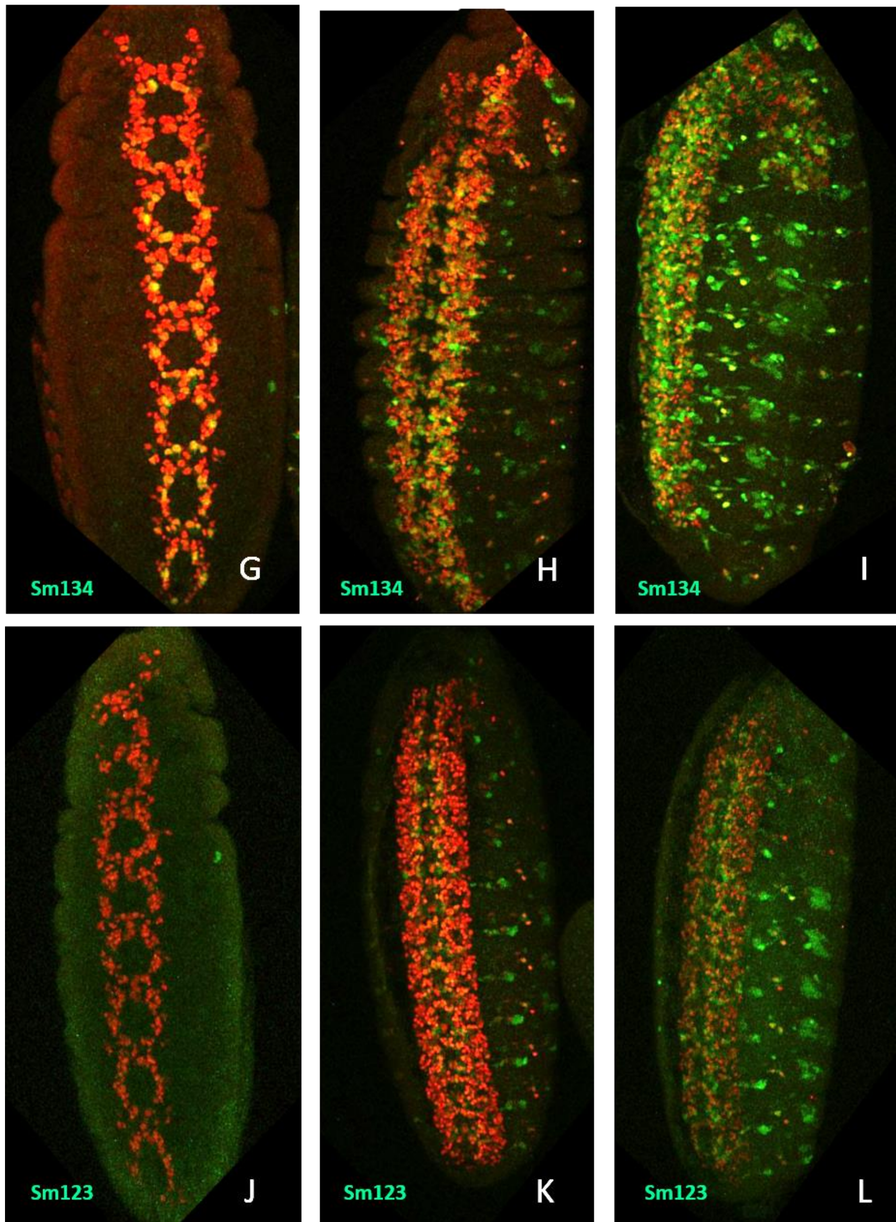
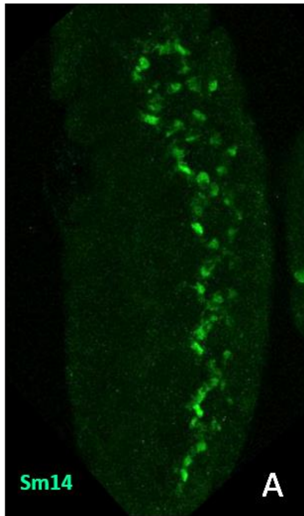
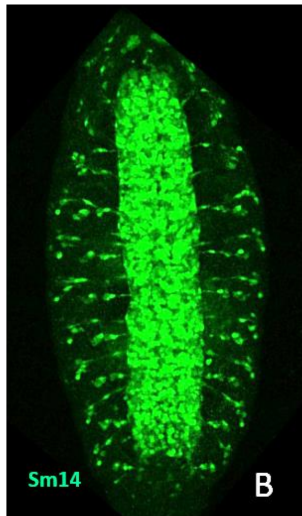
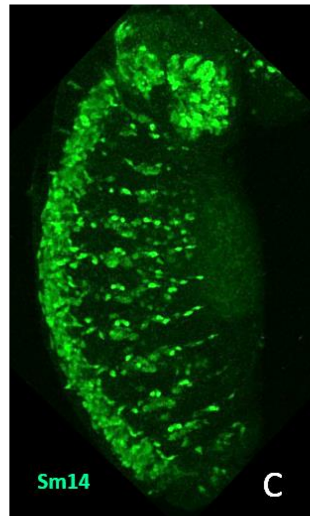


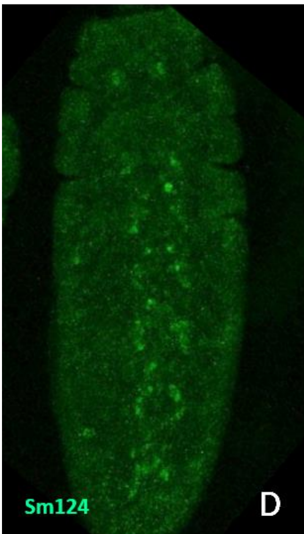
Figure 30. The expression pattern of the mutated intronic enhancers (green), Sm14, Sm124, Sm134, Sm123, merged with expression of the endogenous Hey (red). Images are maximal projections of embryo Z-scans. Images have been acquired using the same settings and the same conditions were applied for all sample preparation.

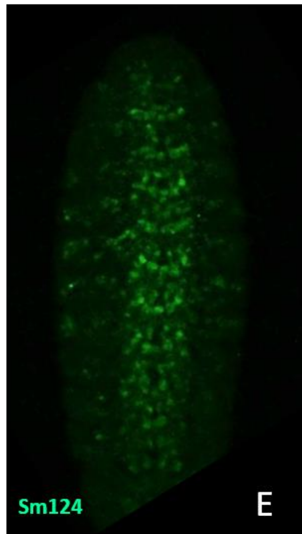
Early stages (10-12) | **Late stages (13-15)** | **PNS**

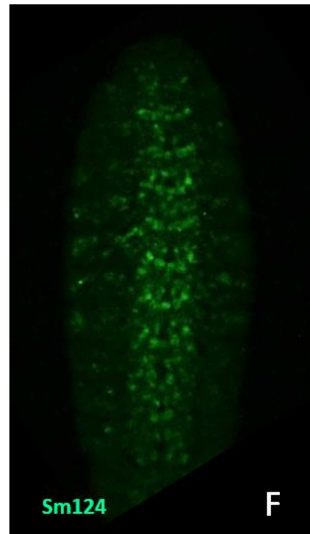












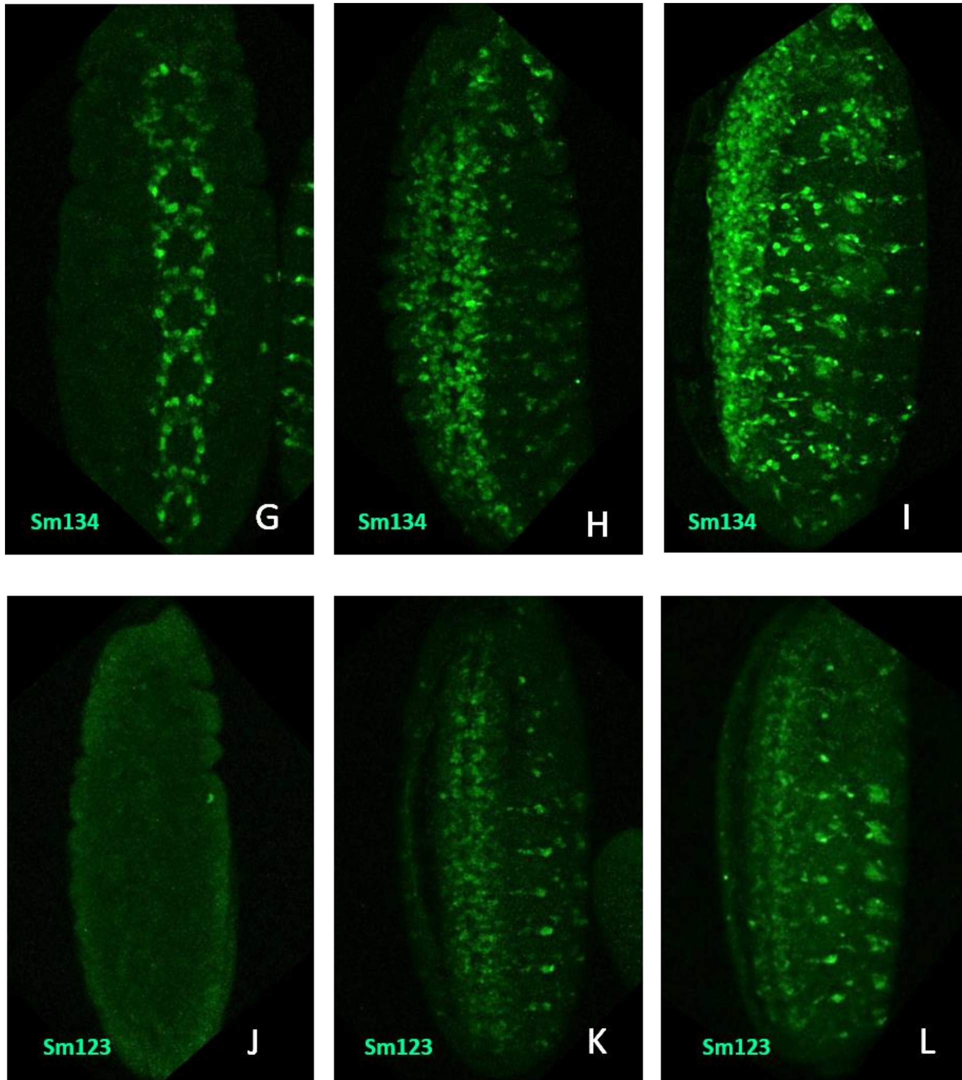


Figure 31. The expression pattern of the mutated intronic enhancers (green), Sm14, Sm124, Sm134, Sm123. Images are maximal projections of embryo Z scans and all embryos are positioned with the antero - postirior axon vertical to the figures. Images have been acquired using the same settings and the same conditions were applied for all.

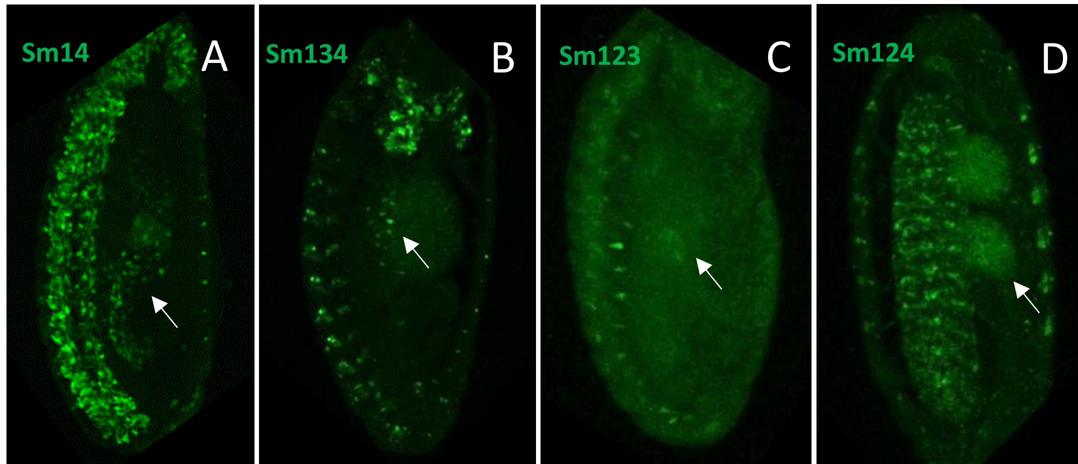


Figure 32, The expression of the mutated intronic enhancer reporters in the developing gut (arrow).Images A-C are of stage 14 embryos, while image D is of a stage 16 embryo. A) Sm14, B) Sm134, C) Sm123, D) Sm124. The images are maximal projections of embryo Z- scans. The same settings have been applied for acquisition of all the images and the same conditions were applied for all sample preparation.

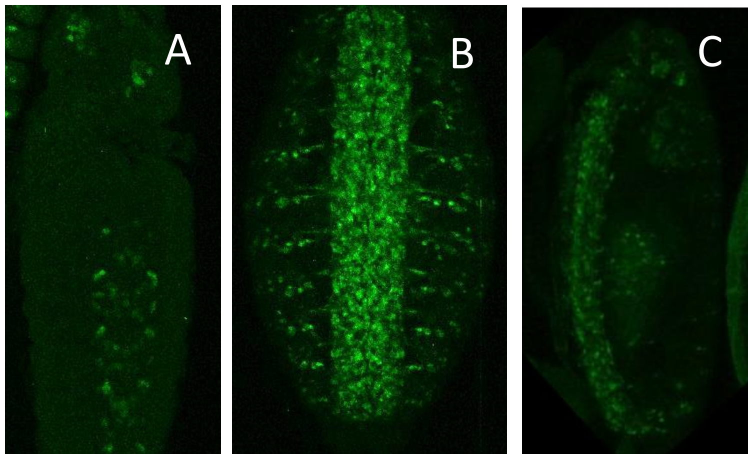


Figure 33. The expression of HeyINT2 enhancer reporter in early and late embryonic development is provided. A) a stage 12 embryo, B) expression of the reporter in the PNS of a late embryo, C) the expression of HeyINT2 in the developing midgut. Images are maximal projections of embryo Z- scans. The same settings have been applied for acquisition of all the images and the same conditions were applied for all sample preparation.

Following the analysis of the mutant intronic reporters during the embryonic development, I proceeded to the analysis of the expression in the CNS of the 3rd instar larva. Images of the dorsal side of the brain lobes, with emphasis on the mushroom body region, as well as, images of the ventral side of the brain lobes and the VNC, for each mutant, reporter line. Images of the non mutated reporter are provided, in figure 34.

The Sm14 enhancer reporter line presents a moderate expression in the lineage of the four mushroom body neurons but not in the new born ones similarly to the non-mutated enhancer (Fig. 35). On the ventral side of the brain lobes there are regions with strong or weak expression (Fig. 36) and persistent ectopic expression in cells of unspecified identity. In the VNC, ectopic expression is observed mainly in the abdominal neuromeres (Fig. 37 D, E), while regions with reporter expression of variable intensity are observed in the region of the thoracic neuromeres (Fig. 37A-C).

Immunostaining of larval CNS tissue of the Sm124 mutant intronic reporter line, revealed a more restricted pattern on both dorsal (Fig. 38 A-C) and ventral (Fig. 38 D-F) side of the brain lobes. The expression is completely abolished from the optic lobe (Fig. 38 D-E) and ectopic expression is observed in cells of non neuronal fate as they are not stained with Elav, neuronal marker (Fig. 39). In the VNC of Sm124 mutated intronic enhancer, reporter line, there is significantly weak expression in the thoracic neuromeres and strong ectopic expression in the abdominal ones (Fig. 38).

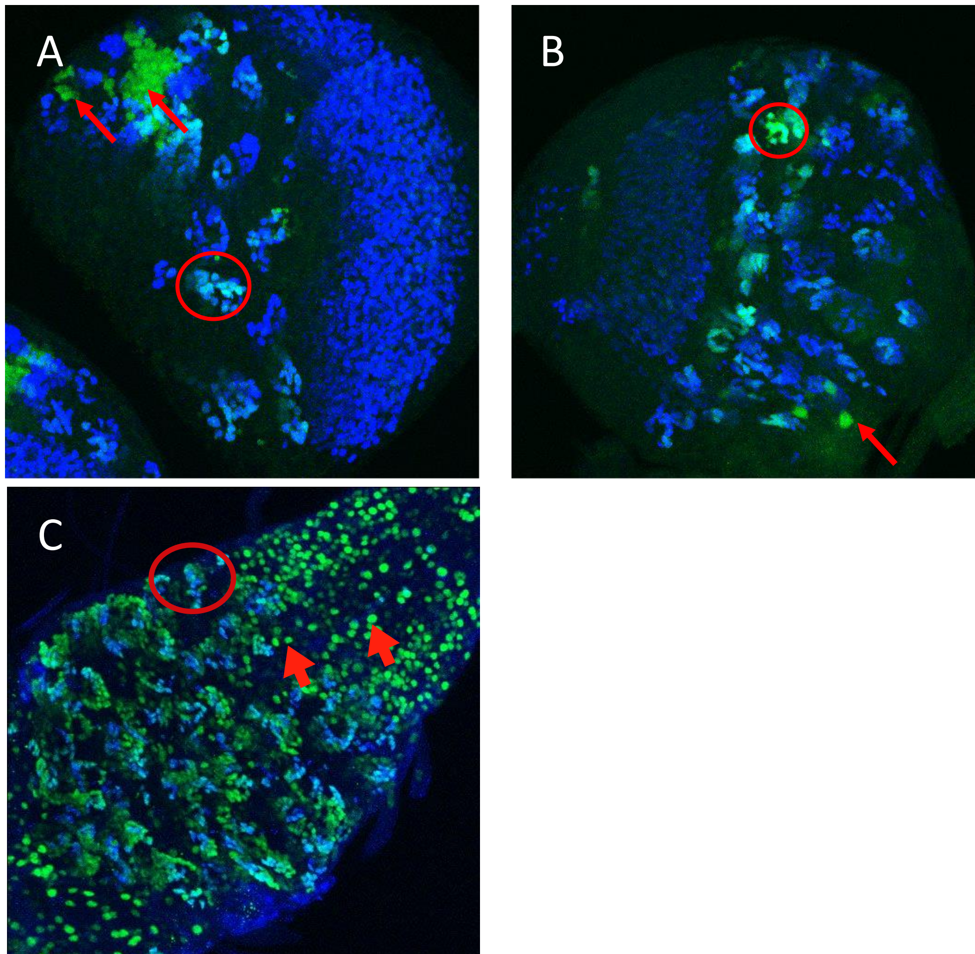


Figure 34. Images of the dorsal, ventral and the VNC of a 3rd instar larva, presenting the expression of HeyINT2 enhancer reporter (green) in comparison to the endogenous Hey expression (blue). Arrows indicate ectopic expression and circles co-localization of Hey and INT2 reporter expression. (N. Batsiotos diploma thesis).

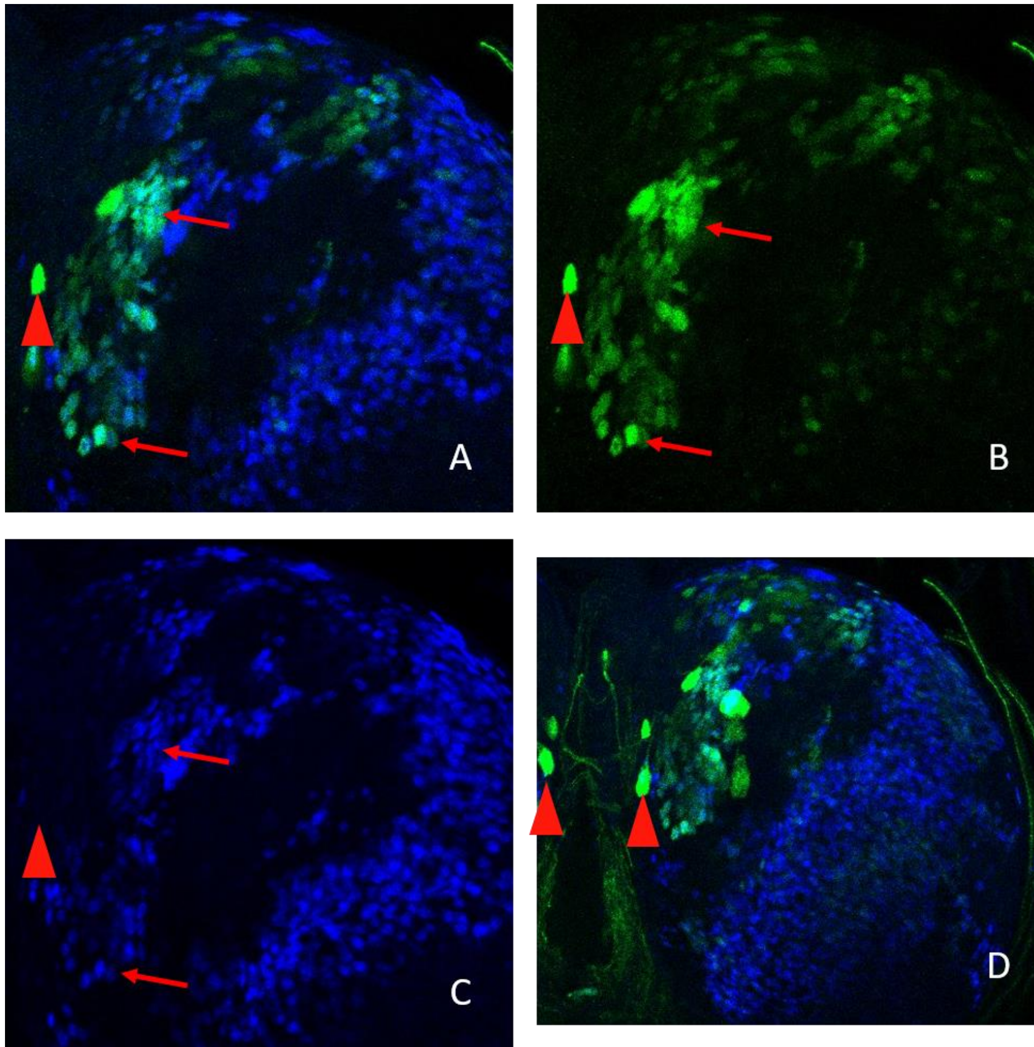


Figure 35. . The Sm14 enhancer reporter line has a moderate expression (A) (green) in the lineage of the four mushroom body neurons as indicated by arrows. B) merge of Sm14 reporter expression and endogenous Hey expression, C) endogenous Hey expression (blue), D) projection of more superficial Z-stacks of the dorsal side. Ectopic expression of Sm14 reporter in cells of unknown identity is indicated by arrowheads. Images are projection of representative Z-stacks of the dorsal side of a 3rd instar larval brain lobe.

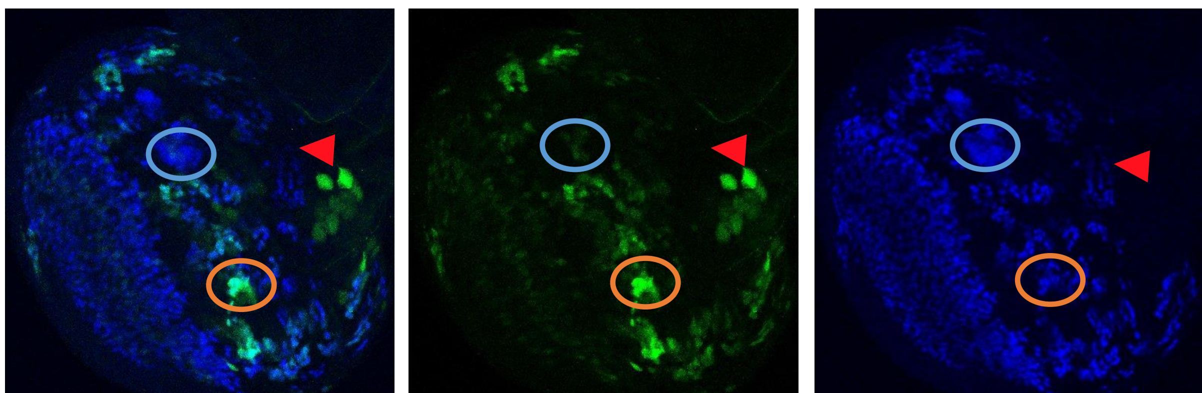


Figure 36. The Sm14 enhancer reporter line presents strong (orange circles) and weak expression (blue circles), in the ventral side of the 3rd instar larval brain lobes. A) Merge of Sm14 and endogenous Hey expression, B) Sm14 expression pattern, C) Hey expression pattern. Ectopic expression is indicated by arrowheads. Images are projection of representative stacks of the ventral region of a 3rd instar larva.

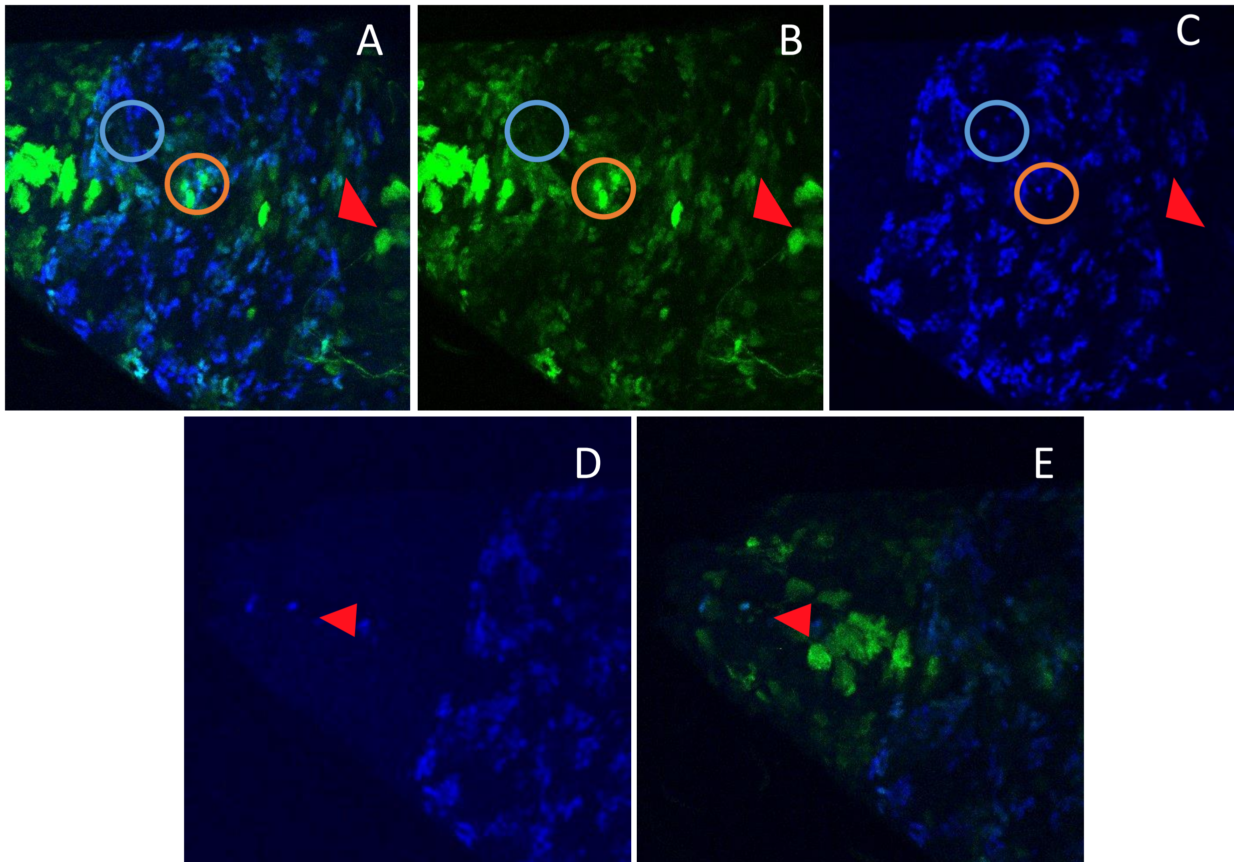


*Figure 37. The **Sm14** enhancer reporter (green) presents strong expression (orange circles) and weak expression (blue circles), in the region of the thoracic interneurons segment of the VNC (A-C). Ectopic expression is indicated by arrowhead and Hey endogenous expression is in blue. In the segment of the abdominal interneurons ectopic expression is indicated (D, E). Images are projection of representative Z-stacks of the dorsal VNC region of a 3rd instar larva.*

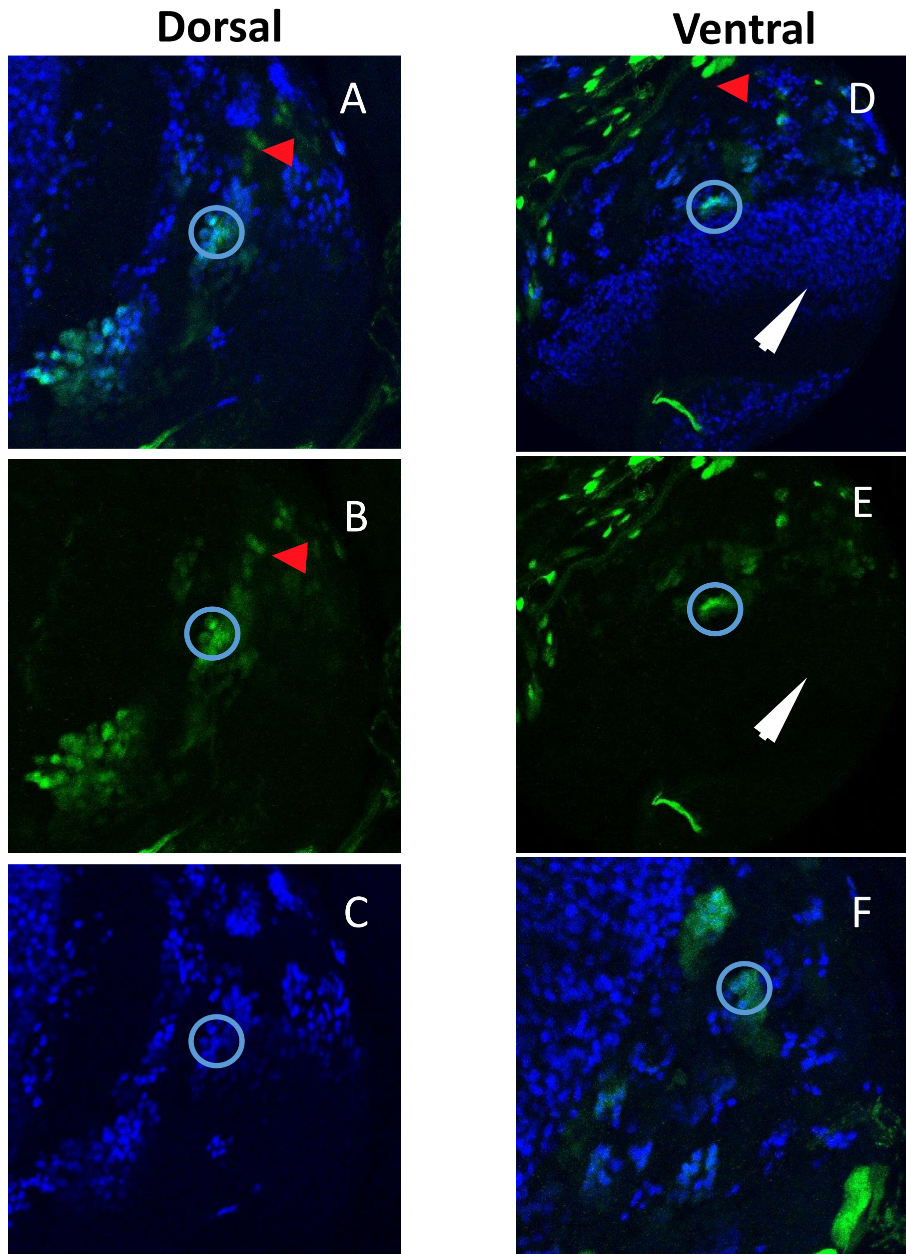


Figure 38. Sm124 mutated intronic enhancer reporter (green) presents a restricted and weak (blue circle) expression pattern. There is no expression of the reporter in the optic lobe region (white arrow), while there is weak ectopic expression in some regions (red arrows). These observations apply on both the dorsal (A-C) and the ventral side (D-F) of the brain lobes. Hey endogenous expression is in blue. Images are projection of representative Z-stacks of high magnification scans on the two sides of brain lobes. Figure F is a higher magnification image on the ventral side.

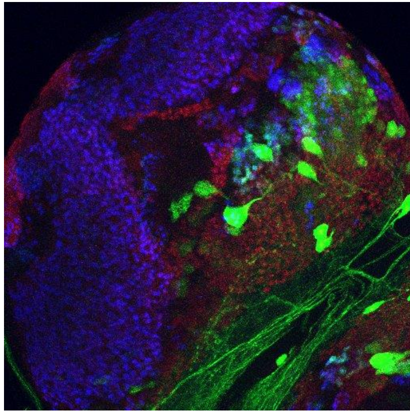


*Figure 39. The ectopic expression observed in the dorsal side of the **Sm124** mutant Hey intronic reporter (green), is not of neural fate. Hey is marked in blue and the neuronal marker Elav, is in red. Images are projection of representative Z-stacks, superficially on the dorsal side.*

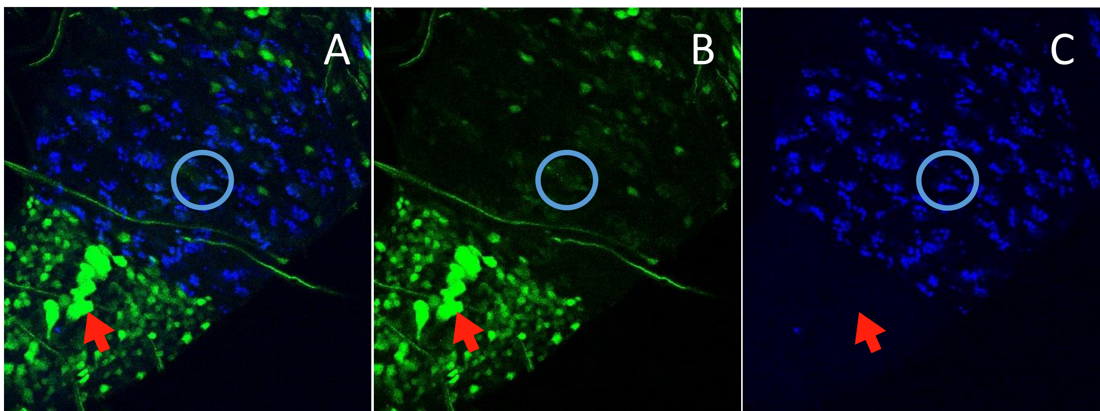


Figure 40. The expression of the mutated intronic enhancer reporter, Sm124, (green) is significantly weak in the region of thethoracic neuromeres t of the VNC (blue circles) and strong ectopic expression in the abdominal ones (arrows). A) Merge of Sm124 and HeyINT2 enhancer reporter patterns, B) Sm124 reporter pattern, C) HeyINT2 enhancer reporter. Images are projection of representative stacks of the ventral side of a larval VNC.

The mutated intronic enhancer Sm134 reporter presents no expression on the dorsal side, while there is weak expression on the ventral side of the brain lobes (Fig. 41-43). Ectopic expression is prevalent on both brain lobe sides and in the VNC (Fig. 43). In a similar mode the mutated intronic enhancer Sm123 reporter line shows restricted and weak expression on the ventral side of the brain lobes (Fig. 44), while on the dorsal side there is strong ectopic expression in the older cells of the mushroom body lineage that do not express Hey. In other regions on the dorsal side there is weak reporter expression (Fig. 44). In the VNC the reporter expression does not co-localize with the endogenous Hey expression but instead persists on ectopic expression in the region of the abdominal interneurons of the VNC (Fig. 46).

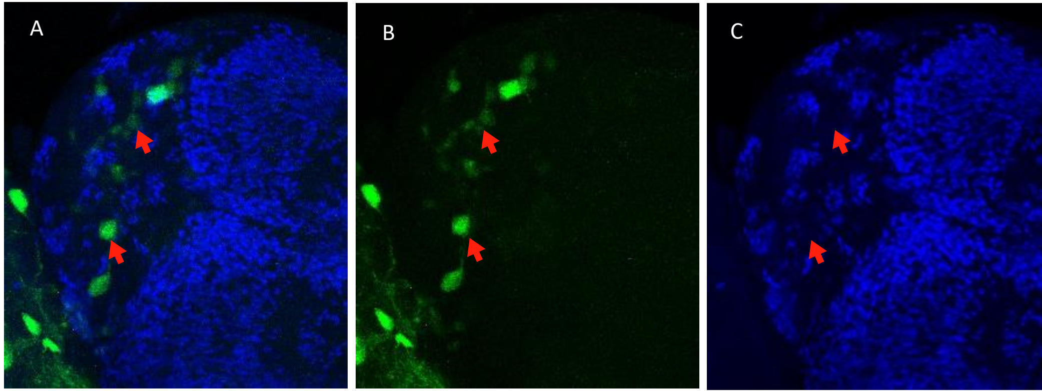


Figure 41. The mutated intronic enhancer Sm134 reporter line presents no expression (green) in Hey positive cells (blue), on the dorsal side of the brain lobes. Ectopic expression is indicated by arrows. A) Merge of Sm134 and Hey expression pattern, B) Sm134 expression, C) Hey expression. Images are projection of representative Z-stacks of the dorsal side of a 3rd instar larva.

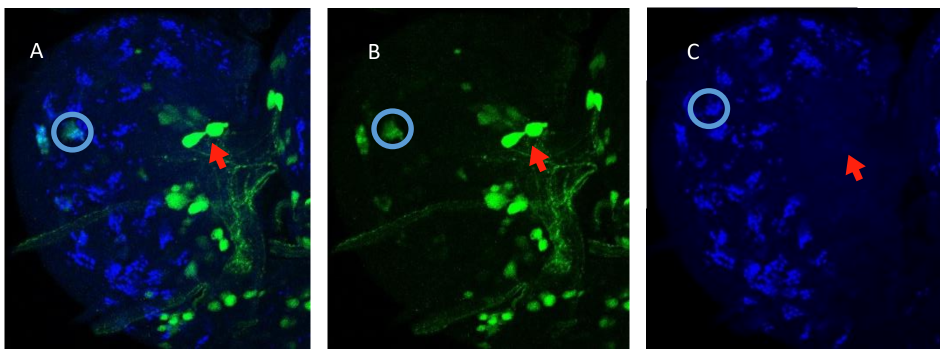


Figure 42. Sm134 reporter line shows restricted and weak expression (green) on the ventral side of the brain lobes (blue circles). Ectopic expression is indicated by arrows. A) Merge of Sm134 and Hey expression patterns, B) Sm134 expression, C) Hey expression. Images are projections of representative Z- stacks on the ventral side.

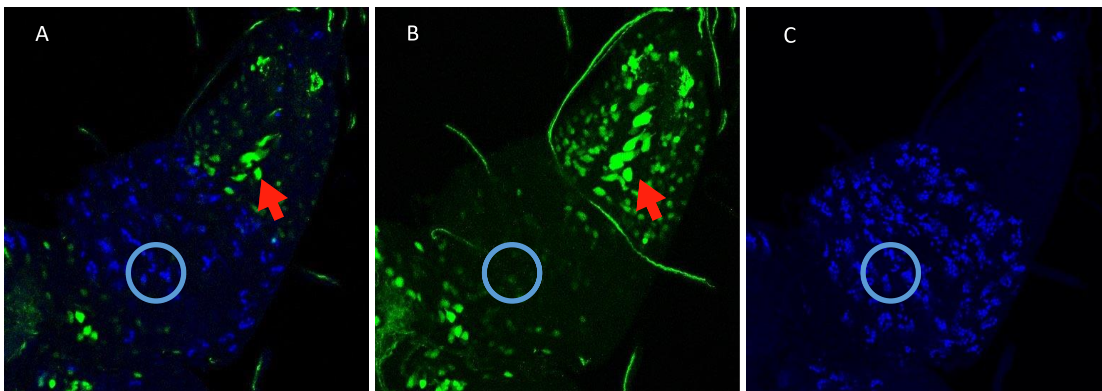


Figure 43. The mutated intronic enhancer Sm134 reporter line shows significantly restricted and weak expression within the Hey expression pattern, on the ventral side of the VNC (circle). Ectopic expression is indicated by arrows. A) Merge of Sm134 and Hey expression patterns, B) Sm134 expression, C) Hey expression. Images are projections of representative Z- stacks on the VNC.

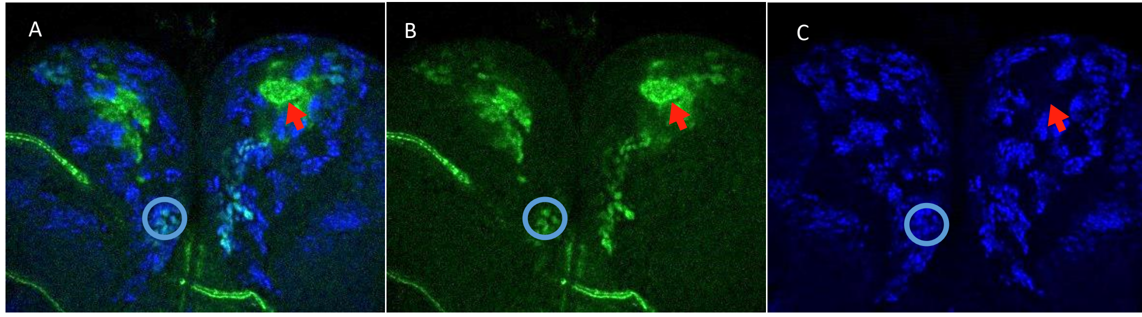


Figure 44. The mutated intronic enhancer Sm123 reporter line (green) shows restricted and weak expression (blue circles) (B), while there is strong ectopic expression in cells of the mushroom body lineage (arrows) that do not express Hey (blue) at this developmental stage. A) Merge of Sm123 reporter expression and endogenous Hey expression, C) Hey expression pattern. Images are projection of representative stacks of the dorsal side.

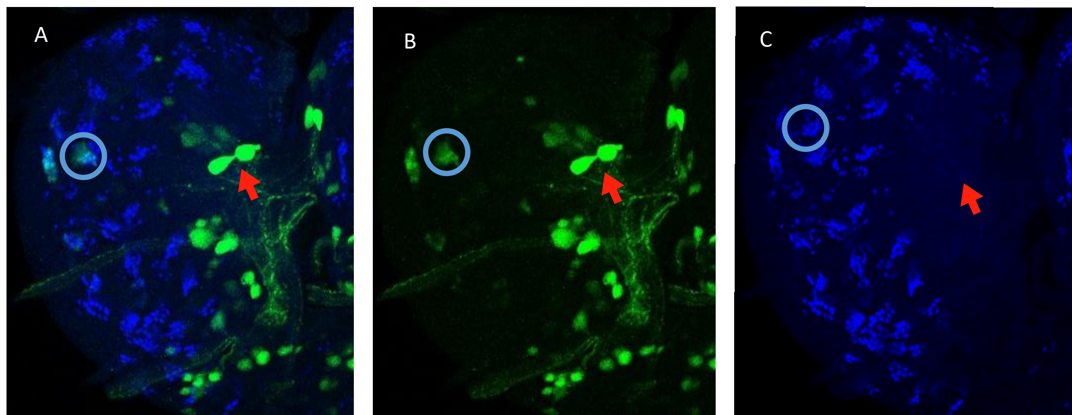


Figure 45. The mutated intronic enhancer Sm123 reporter line shows restricted and weak expression (green) on the ventral side of the brain lobes (blue circles) (A and B). Ectopic expression is indicated by arrows. A) Merge of Sm123 reporter expression and endogenous Hey expression, C) Hey expression pattern. Images are projection of representative stacks of the ventral side.

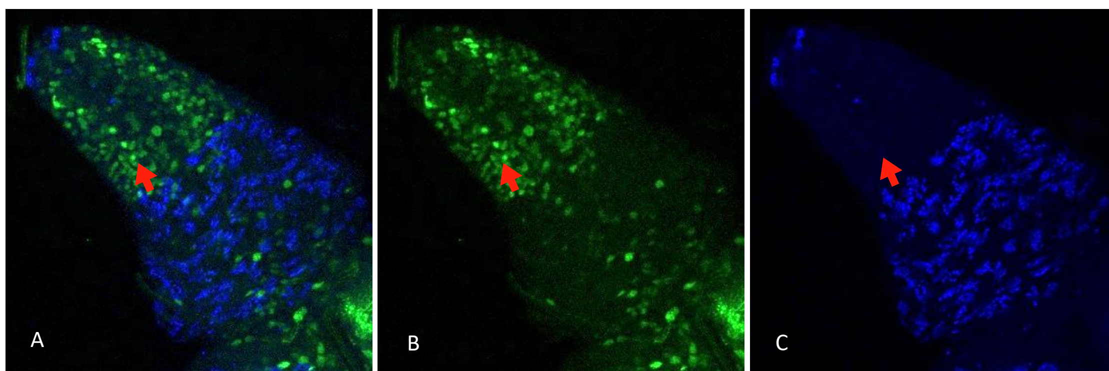


Figure 46. The mutated intronic enhancer Sm123 reporter line expression (green) (B) does not co-localize with the endogenous Hey expression (blue) but instead persists on ectopic expression (arrows) in the region of the abdominal interneurons of the VNC. A) Merge of Sm123 reporter expression and endogenous Hey expression, C) Hey expression pattern. Images are projection of representative stacks of the dorsal side.

Sequence conservation analysis (evo printer)

Considering the variable expression patterns of the modified intronic enhancer reporters, Sm14, Sm124, Sm134, Sm123, I examined the consensus sequence conservation of the four Su(H) binding sites within the sequence of the second intron of Hey gene. The analysis of the sequence was performed applying the EvoprinterHD online tool (https://evoprinter.ninds.nih.gov/) and high conservation was identified among seven *Drosophila* species, for all four Suppressor of Hairless binding sites included in the region (Fig. 47).

D.melanogaster *Hey_INTRON2* Genomic Relaxed *EvoPrint* (Back to Top)

Black capital letters represent bases conserved in all species and colored bases represent sequences present in all species except *D.sechellia*, *D.simulans*, *D.erecta*, *D.yakuba*, *D.ananassae* or *D.pseudoobscura*

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D.melanogaster *Hey_INTRON2* Genomic Relaxed *EvoPrint* (Back to Top)

Black capital letters represent bases conserved in all species and colored bases represent sequences present in all species except *D.sechellia*, *D.simulans*, *D.erecta*, *D.yakuba*, *D.ananassae* or *D.pseudoobscura*

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D.melanogaster *Hey_INTRON2* Genomic Relaxed *EvoPrint* (Back to Top)

Black capital letters represent bases conserved in all species and colored bases represent sequences present in all species except *D.sechellia*, *D.simulans*, *D.erecta*, *D.yakuba*, *D.ananassae* or *D.pseudoobscura*

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gtaagtttaccaagcccacttgagctagcttaaagaggttttaaaaagcaaataggctagctgcaccacagacct  75
cctcactgaagttaaacgcttctatgggtcattaatcacaataggagcaaatagagggttaacttgggcagccaa  150
ctaagttggttaactaaaagaagtaaagtgtaaagaaagtaatttcttatttcgacagttaatattacattaggg  225
cccatttagcgcactaattaatcaaggcatatctgacatacatatgaacttcaatacattataaaaaattggaat  300   Su(H) bs 4
gggaaaaactgcaagcagaatccctaaacgcAAGCcgCTGTGGGAAaCAaTTgAGtaACgAtATTAAAtACAATA  375
TGCGGCAAATTATCACAAATCTGgGTAAACAaTCCTgACCTGTTGTCCCGAaCAGGGTcgaaTtGTGGGAAAATT  450
ATGATtGcCgTAggCAAAgCAGGACcACCACCACCagcgccagcctAgcGCtCaAGTGAATCTCGAATCTCGGGG  525
CTTCCCACGCTcagtagcggcaccaccacgatggcaaccttgtagccaggcgcgggctTTCacCCCCcGAtAgCC  600
ACCTCaCAACaagcCATCccgCCGAAGGACATGGGGGTtGTTcTTTAtgctcGTCGCGTGGGAACAgCTATTTTA  675
ATTGTTTGCCTGAaCtGGGATTAGACAGCTTATTGACTTTCTCCaCCTCCTcgtaTCTTGTTGCAG          750
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Figure 47. Evolutionary conservation analysis of the second intron of Hey gene of Drosophila. Coloured boxes indicate the Su(H) binding sites. The first two are in yellow, while third and forth are in orange. Analysis was performed applying EvoprinterHD.

Deletion of the sequence of HeyINT2, using CRISPR/Cas9 genome editing

Considering the results of the previous experiments and the strong expression of HeyINT2 enhancer reporter during embryonic and third instar larval developmental stages, as well as the responsiveness of this element to Notch signaling, we decided to proceed with the deletion of all four Su(H) binding sites located within the sequence of HeyINT2. Our experimental approach included the application of CRISPR/ Cas9 genome editing, specifically by targeting the nuclease to two sequences either side of the region, containing the four Su(H) binding sites. The two guide RNAs (Fig. 48) were designed to target the 5' and 3' sites of the Hey second intron sequence such as to maintain the branching point of the intron, without disturbing the intronic splicing process. A mix of expression vectors containing the two gRNAs was injected in transgenic fly embryos of the strain y M{nos-Cas9.P}ZH-2A w*, which expressed Cas9 during oogenesis, under control of nanos regulatory sequences (Port et al., 2014). The first round of injections contained each expression vector in 75ng/ul concentration, according to Douris, et al., 2016. For the second round of injections, as the experiment was repeated, we applied a mix of 200ng/ul of each gRNA construct according to Ren, et al., 2014. The seventy-five (75), 1st instar larvae that hatched successfully, were transferred to fly medium and 34 adult files were obtained, which were crossed individually. The single fly crosses were set with flies either carrying a deletion mutation for Hey gene or with yellow white flies. The progeny of each cross was collected and screened in pools of 30 individuals, using different PCR primer sets.

The initial primer set used, CDSF and INT2R, was upstream and downstream of the gRNA-targeting sites, specifically, the forward primer was located at the start of *Hey* coding region (CDSF primer) and the reverse was located on the3' prime of intron 2 (INT2R), generating a fragment of 1815bp on the wild type Hey allele and approximately 1300bp on the allele carrying a deletion event (Fig. 48). In order to ensure that any rare deletion event would not be underestimated by the PCR reaction because of the majority of the wild type alleles in the genomic template, the PCR screening was performed on genomic DNA that was previously digested by SspI. SspI performs a single cut in the Hey intronic region in between the gRNAs, thus PCR product can be produced only in the case of deletion events, since they lack the restriction site, while wild type alleles from the DNA pool sample would not be represented by a PCR product. As it is shown in the screening gel of figure 49, no events were detected.

At this point, we reasoned that if the deletion extended more towards 3' than the expected one, it could not be detected with CDSF-INT2R primer combination as INT2R primer is very close to the gRNA sequence. So we tested the primer sets of CDSF-CDSR, which amplify the full coding region of *Hey* gene (CDSR is located at the 3' end of the coding region of *Hey*), (Fig.48). This primer set produces band fragments of 2438bp on a wild type allele and fragments of less than 1888bp after a CRISPR deletion event. As shown in figure 50 we applied this set of primers on both SspI digested and undigested genomic DNA of 30 flies' pools. Again no product was detected on the reactions with the digested template and only a single band of the wild type allele is evident in the PCR of the undigested DNA indicating that there were not any deletion events. In figure 48 PCR screening reactions of DNA from pooled progeny of more samples, after SspI digest are presented (Fig.51A, 51B). Some of the reactions resulted in unspecific fragments of size less than 1489bp, compared to the lambda sty marker. Moreover, screening of individuals from the progeny of selected injected animals was

performed (Fig. 51C), however again no deletion event was detected. One of the pool samples, pool number 6, presented on SspI digested DNA template a CDSF-CDSR product fragment, of more than 925bp but less than the expected 1888 (Fig.52) indicating that it could be a bigger deletion than the expected one. This DNA template was tested with the primer set INT2F-CDSR, where INT2F is a primer designed at the 5'end of the second intron of *Hey* (Fig. 48) and ensures that the 5' end, necessary for successful splicing of the intron, is intact after any deletion event. This primer combination produces a fragment of 1767bp size when the wild type *Hey* allele is present in the DNA template, while after SspI digest the wild type allele does not produce any fragments and any deletion event product should be of size approximately 1200bp or less. The result of this screening showed that the pool number 6 did not contain any deletion events according to our criteria (Fig. 51). Moreover, on the same PCR screening sample 9, presented a band that was of the expected size for a deletion event, however it was never confirmed by the other primer combinations (data not shown).

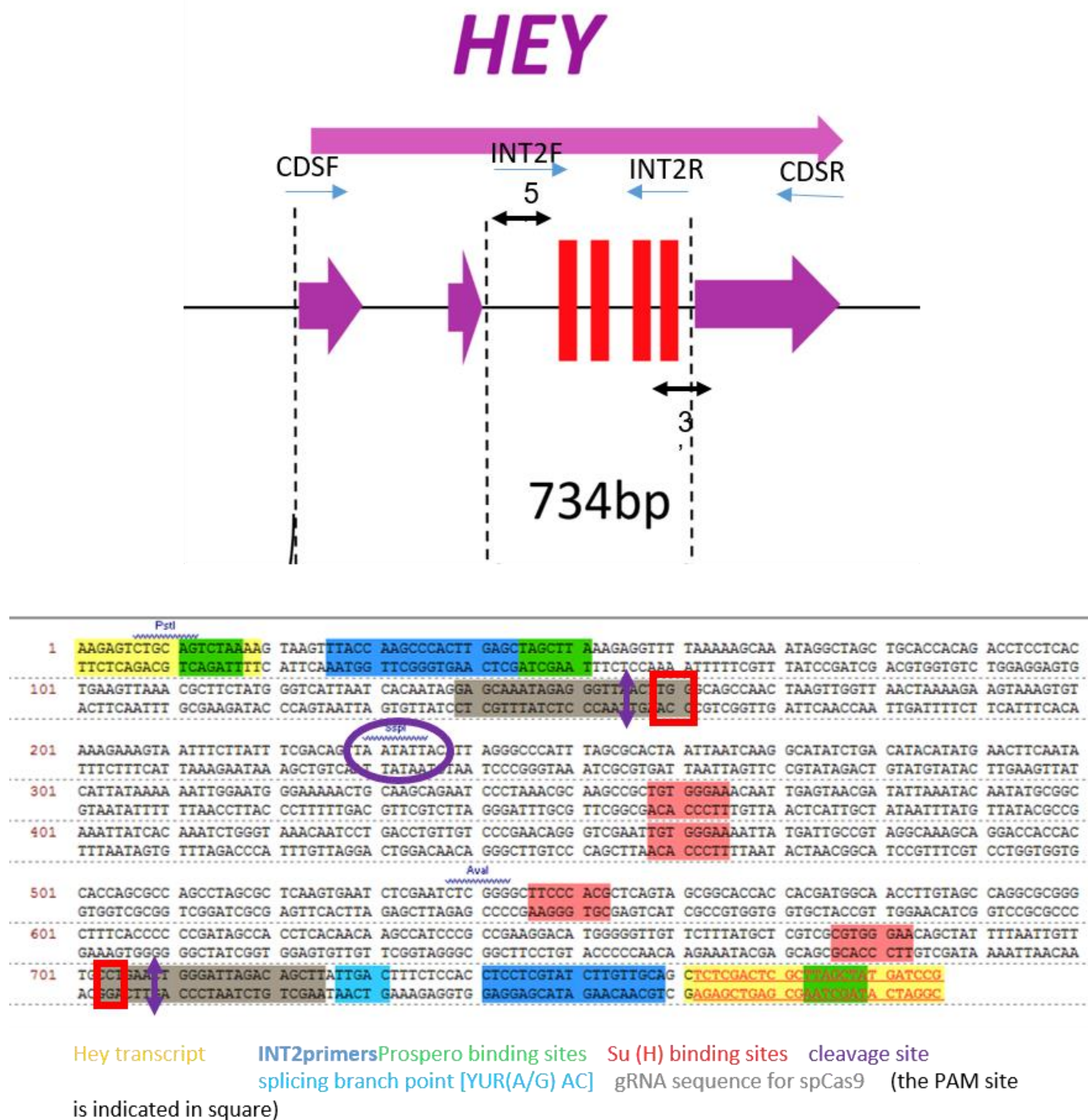

Figure 48. The two gRNAs were designed to target Cas9 at the 5' and 3' ends of the intron 2, without disturbing the splicing of the intron. The PCR primers (blue arrows) used for screening for deletion events were designed upstream and downstream of the gRNA target sites.

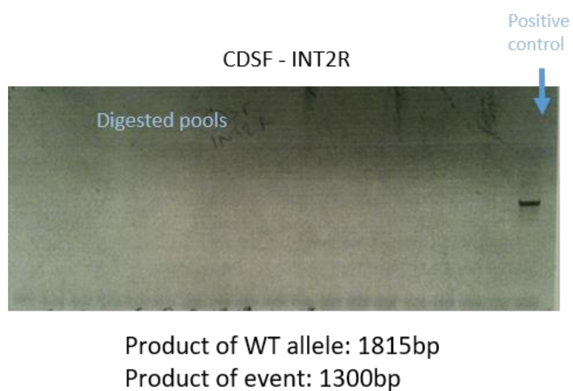

Figure 49. PCR screening on SspI digested genomic DNA of pools of CRISPR progeny flies, using the CDSF-INT2R primer set. In the case of a deletion event a PCR product of 1300bp would be evident. Positive control performed on undigested DNA template from a random pool designates the product of the wild type allele

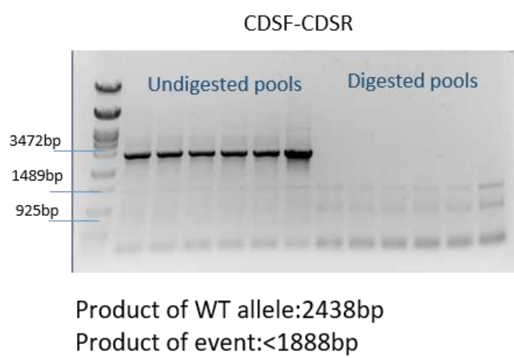

Figure 50. PCR screening on undigested and digested genomic DNA of 6 samples of pooled CRISPR progeny flies, using the CDSF-CDSR primer set.

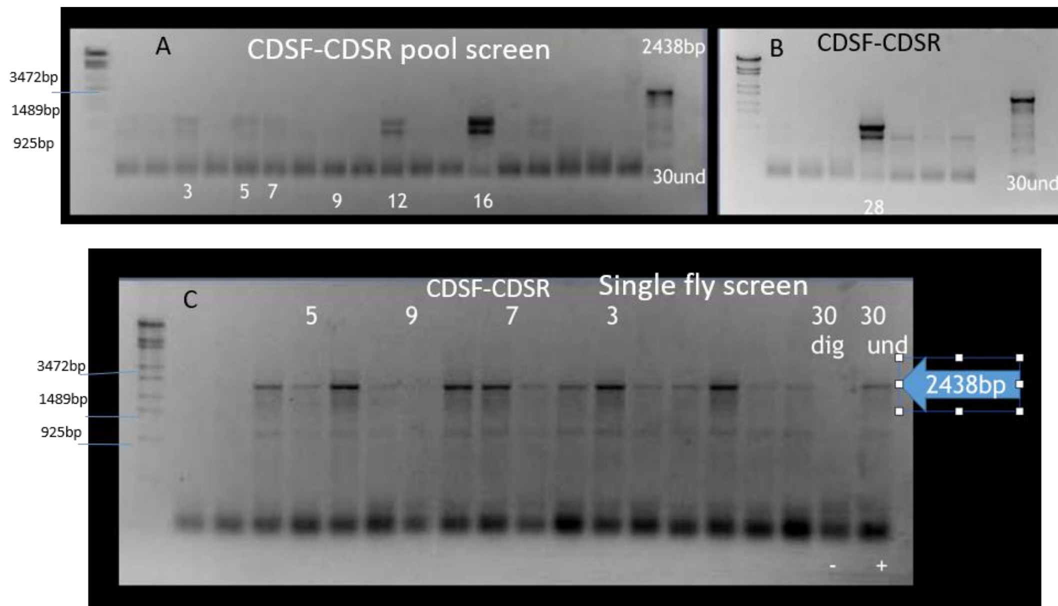

Figure 51. PCR screening on digested genomic DNA of pooled CRISPR progeny flies' samples (A, B) and on undigested genomic DNA of single fly progeny samples (C) using the CDSF-CDSR primer set. The wild type alleles after SspI digest should not lead to any product, while any deletion events should lead to a product of less than 1888bp (A, B). The undigested wild type alleles lead to a fragment of 2438 bp.

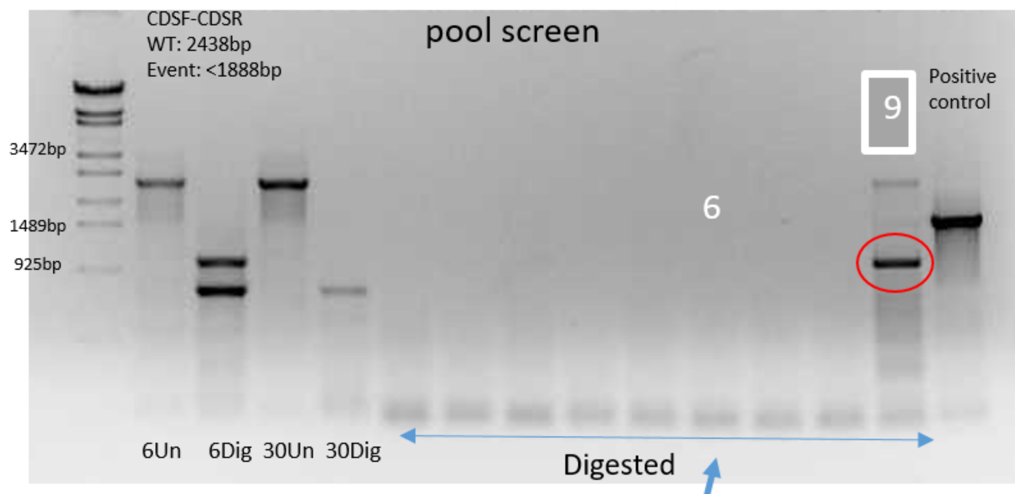

Figure 52. PCR screening on sample 6 of pooled flies' DNA template, undigested and digested compared to sample 30, using the CDSF-CDSR primer combination. On the same gel the PCR reactions of several digested samples including sample 6 and 9 were screened applying the INT2-CDSR primer combination. This primer combination leads to a product of 1767bp on the undigested wild type allele (positive control) and on approximately 1200bp product on a deletion event allele.

Discussion

Hey is a gene transiently expressed in the CNS of *Drosophila* embryos and larvae. Previous studies have shown that Hey expression in the 3rd instar larvae is driven upon Notch signaling, even though there are some regions of Notch independent expression (Monastirioti, Giagtzoglou et al. 2010). The Suppressor of hairless is a known component of the Notch singling pathway and thus, the study of its binding sites is significant in revealing the precise regulatory effect, that Notch exerts on *Hey* expression. ChIP-chip experiments performed, revealed two regions with Su(H) binding sites in the region of *Hey*, upstream of the promoter and in the second intron of the gene. Applying EvoPrinterHD, I performed conservation analysis of the Su(H) binding sites included in the intronic region of *Hey*, where high conservation was observed in all four sites among 12 species of *Drosophila* (*D.melanogaster, D.simulans, D.sechellia, D.yakuba, D.erecta, D.ananassae, D.persimilis, D.pseudoobscura, D.willistoni, D.virilis, D.mojavensis* and *D.grimshawi).* This result indicates the significance of these sites and a potential functional role in gene expression regulation.

The focus of the present master thesis project is the characterization of the two enhancer elements identified in the region of *Hey* gene (I.Koltsaki). We are interested in understanding if both confer in a similar or redundant or complementary way to the expression pattern of *Hey*. Enhancer elements mediate a dynamic spatiotemporal expression and there are many cases that redundant enhancers (also mentioned as ‘’shadow’’ enhancers), confer precision and robustness to gene expression (Cannavo, Khoueiry et al. 2016). The cis-regulatory modules of *neuralized*, for instance, which is controlled by two redundant elements, could both successfully rescue the *neuralized* phenotype in lateral inhibition (Miller, Rebeiz et al. 2014). Furthermore, there are cases of partial redundancy of two enhancer elements, as well as, non-overlapping function of many such elements. Finally, shadow enhancers are believed to regulate variation during development conferring to the robust developmental pattern of animals. An example of a gene with two shadow enhancers is *Drosophila snail.* The two cis regulatory modules of snail are active at the same sites of the pre-gastrulation embryo however they exert different roles in Drosophila development with one of them being necessary for the viability of the embryos and the other being necessary upon stress conditions such as high temperature (Dunipace, Ozdemir et al. 2011). The work of the present master thesis did not include such an approach, instead characterizes the activation of the two enhancer elements in regard to the Su(H) binding sites identified in their sequence. An interesting case of cis-regulatory modules is that of *muscleblind* that present completely non redundant role, since they drive the expression of the gene in different tissues of the *Drosophila* embryo, one driving in the central nerve cord and the other in the musculature of embryos (Bargiela, Llamusi et al. 2014). In our study, even though we observe the expression of both *Hey* enhancers in two different tissues, central nerve cord and gut, no specificity in the tissue for either of the enhancer elements was observed (Fig.1,2,6,8,9,11,12).

Initially, we characterized the expression of the two enhancer elements, HeyUp and HeyINT2, during the embryonic development for the first time in *Drosophila melanogaster*. This work revealed that indeed, both of the two enhancer elements have the potential of activating transcription during the stages of neuronal development (stage 10 to stage 16).

HeyINT2 expression pattern covers partially the Hey expression pattern, but cells expressing the reporter seem to present a more uniform, strong expression (Fig. 8), compared to the reporter of the HeyUP enhancer reporter, that presents differential expression levels among neuronal lineages (Fig. 5). This is similar to the case of snail enhancer elements that because of their different functional role upon normal conditions they presented different expression levels (Dunipace, Ozdemir et al. 2011). The slightly different expression of the two *Hey* enhancers could be due to different consensus content of the two elements or differential role in expression regulation, upon a range of environmental conditions, which was not studied in the present thesis. In addition, both HeyINT2 and HeyUP reporters' expression was observed in the developing midgut (Fig.6, 12, 13), during stages 14 and 15. However, in contrast to HeyUP enhancer reporter, the intronic enhancer reporter HeyINT2 presents strong ectopic expression (Fig. 12) in the midgut, in cells of unidentified identity. Moreover, both enhancers present ectopic expression in the PNS primordia (Fig. 5,7,10,11) which were identified as glia cells from the glia marker staining, Repo, of figure 10. It is known that ectopic expression is often variable and unstable for the various reporters and depends mainly on two factors, the sequence context of the enhancer element and the insertion site (Casas-Tinto, Arnes et al. 2017). Considering the context of the two elements is different and especially HeyUP because of its extended size (>2,5kb) could include binding sites for other transcription factors, thus affecting the expression pattern. The integration site used in our studies is attP40, located on the left arm of the second chromosome. This site has been reported to have very low basal expression levels and being greatly activated upon induction in the fat body tissue and sufficiently activated in the muscle and nervous system (Markstein, Pitsouli et al. 2008) however specific reports for ectopic expression of this integration site due to position effect variegation have not been found. Finally, it would have been a possible scenario that the reporters are ectopically expressed because of the absence of regulatory elements that would suppress selectively their activation in the physiological genetic context of *Hey* locus.

When we combined two reporter lines expressing GFP under HeyUP and HeyINT2 enhancer elements, respectively, we showed that the two enhancers recapitulate the endogenous Hey expression pattern, supporting a complementary role for these two regulatory elements. Moreover, the ectopic expression of the reporters persisted as was observed previously, highlighting the importance of the physiological genetic context in the regulation of enhancer activation.

Later, we wanted to confirm that *Hey* cis-regulatory modules expression is Notch signaling dependent and specify the Notch independent expression. In order to test that we introduced each of the two enhancer elements in a Delta/Serrate mutant background that successfully provided us with the Notch independent expression pattern of the HeyUP (Fig.15). On the contrary, HeyINT2 reporter did not present any expression upon Delta/Serrate mutant background (Fig.16). This analysis leads to the conclusion that the two enhancer elements of Hey contribute in a different manner to regulation. The upstream element, HeyUP, includes Notch dependent and independent elements driving expression even in absence of Notch signaling, while, the intronic enhancer, HeyINT2 is Notch dependent lacking completely the reporter expression upon Notch signaling absence.

Next we wanted to elucidate if all of the Su(H) binding sites included in the two *Hey* enhancer elements are crucial for *Hey expression* or if they are redundant for transcriptional regulation via Notch pathway. We proceeded into generating mutant reporter lines that would lack or carry mutated Su(H) binding sites and analyzed their expression during embryonic development and in 3rd instar larval stage. The confocal imaging analysis performed, for the two mutant lines, 2+3SH and NSH, generated for the upstream Hey enhancer reporter (Fig. 17) led to the conclusion that the first Su(H) binding site located in the 3' prime UTR of the gene CG11191, located upstream of Hey, is redundant and its deletion does not affect the expression pattern of the enhancer reporter line. However, it is interesting

how the deletion of all three Su(H) binding sites does not eliminate completely the expression of the reporter (Fig.19), indicating that there are cis-regulating elements in this enhancer region that could either be Notch responsive or not. The remaining reporter expression pattern of NSH reporter line is part of the 2+3SH reporter, expressing in a specific cell lineage of the CNS. A significant observation is that the expression pattern of NSH in figure 19A seems to present strong expression in a few cells just like the pattern of HeyUP in a Delta, Serrate mutant background (fig. 15). This is an indication that absence of the three Su(H) binding sites from HeyUP are sufficient to disrupt activation of the enhancer by Notch signaling. Moreover, the deletion of the sequence containing the three binding sites leads to ectopic expression in older neurons that do not express Hey (Fig. 26), indicating potentially the disruption of a downregulation mechanism of *Hey* expression. However, this is a speculation and further study is needed in order to elucidate this scenario.

Previous studies in the laboratory had shown that mutation of all Su(H) binding sites identified in the sequence of HeyINT2 abolished completely the expression of the reporter (mutant reporter SALL). To investigate further the significance of these binding sites we performed the mutation analysis of the intronic enhancer reporters, after construction of Sm14, Sm124, Sm134 and Sm123. The confocal image analysis suggested that the most crucial for the Notch response Su(H) binding sites are the second and the third. Abolishing these two Su(H) binding sites leads to delayed initiation of the reporter expression and to a restricted and weak pattern of expression (Fig. 31). Nevertheless, the remaining reporter expression implies an assisting role for the other two binding sites.

In more detail, the reporter Sm123 is presenting the weakest expression compared to all the mutated reporter constructs (Fig.31J-L). the expression of this reporter is timely delayed (Fig.30J-L) and once initiated is restricted to a subset of Hey expression cells showing weak expression. This result indicates that Su(H) binding site 4, that is wild type in this reporter, is not sufficient to respond on the right developmental stage to Notch signaling and initiate sufficient expression on time. Slightly different expression presents the Sm124 reporter, showing very weak expression throughout all embryonic developmental stages (Fig. 30 D-F, 31 D-F). This reporter maintains the same expression pattern with HeyINT2 reporter, although it is much weaker in intensity. Therefore, Su(H) binding site 3 is sufficient to initiate transcription at the right developmental point but not to induce high expression levels. Similar to the wild type HeyINT2, ectopic expression is observed in the PNS of Sm124 embryos, which is weak as is the rest of the expression pattern (Fig. 30F, 31F). On the other hand, images of the Sm134 support that the 2nd Su(H) binding site is not only adequate for initiation of expression at the right developmental stage but also for inducing sufficient expression levels from the early stages of *Hey* expression (Fig. 30G-I, 31G-I). At this point I should mention that as with the HeyINT2, Sm134 is not expressed in all Hey positive cells and during late embryonic stages enhanced expression is noticed even in non Hey positive cells, similar to HeyINT2 (Fig. 30). Finally, the strongest reporter expression is observed in the line Sm14 (Fig.30A-C, 31A-C). This line includes two wild type Su(H) binding sites, the 2nd and 3rd, that combined are sufficient for strong expression, initiating at the right developmental stage. Hence, the combination of these two binding sites is necessary for the most efficient response of *Hey* intronic enhancer to Notch signaling.

Considering the results of the previous experiment, the expression of SALL (unpublished data) and after observing that the expression of the intronic enhancer reporter,

was consistent with the endogenous Hey expression, we decided to target Hey intronic region for deletion by CRISPR/Cas9 (Bassett, Tibbit et al. 2013, Gratz, Cummings et al. 2013, Yu, Ren et al. 2013, Ren, Yang et al. 2014). This experimental approach would reveal the biological role of the intronic enhancer element, within a physiological genetic context and would allow the study of the endogenous Hey expression in absence of this cis regulatory module. Unfortunately, the desired result was not reached and this could be attributed to a number of reasons. First of all, because of the intronic sequence and intending not to disrupt the process of intron splicing, a single pair of guide RNAs was applied, that did not guarantee the efficiency of the application. In most of these experimental approaches more than one sets of gRNAs are applied (Kondo and Ueda 2013, Port, Chen et al. 2014). Second, the two guide RNAs were introduced in the transgenic Cas9 flies in a separate expression vector which does not ensure the simultaneous expression of the two gRNAs and this can potentially lead to offspring carrying a single target mutation instead of a deletion. Since the screening methodology was designed to detect only deletions in the targeted area, it was not possible to detect any single gRNA small mutations, insertions or deletions. Such single site small mutations could be detected by T7 endonuclease I (T7EI) assay, since they are heterozygous after crossing the injected animals with yw flies. T7EI recognizes and cleaves DNA heteroduplexes at mismatch sites revealing small heterozygous mutations and is an ideal tool for CRISPR/Cas9 mutagenesis screening in *Drosophila* (Kondo and Ueda 2013). Finally, I need to point out that even if *Hey* intron2 deletion was lethal, we should be able to detect it, since the screening was performed on the progeny of single fly crosses of the injected animals, crossed to flies with wild type *Hey* alleles.

Conclusion and future direction

Hey is vital for the proper function of the nervous system since individual flies carrying a homozygous Hey deletion present high embryonic lethality and rare escapers present motor function impairments, as they are sluggish and seem malnourished, according to survival assays performed in the laboratory. Moreover, *Hey* could be involved in the physiological development of the midgut, since it is expressed in a subpopulation of the early midgut, however, further investigation is needed to confirm this speculation. To gain a deeper understanding of how *Hey* is being regulated, another attempt of deletion of each enhancer element should be performed. This time more than one target sites could be chosen to increase the possibility of obtaining a mutant. Moreover, it would be significant to clone both guide RNAs in a single expression vector such as PCFD1-3 ((Port, Chen et al. 2014), for optimized expression. Finally, applying a target prediction tool for miRNA target sites, indicated a potential site on the 3'UTR of Hey transcript which could be evaluated by mutation of the respective site and analysis of the effects on *Hey* expression pattern. We could even search for potential sites within the coding region of the gene as it seems to be a widespread phenomenon (Schnall-Levin, Zhao et al. 2010). This could potentially elucidate the ectopic expression observed from the isolated enhancer element reporters.

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