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The role of the transcription factor HEY in the developing midgut of *Drosophila melanogaster*

Master thesis

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Contents

Abstract

Hey proteins are transcription factors that belong to the basic helix-loop-helix 'Orange' (bHLH-O) protein group and they are often effectors of the Notch signaling pathway. Although extensive studies about them have been done in mammalian, little is known about the role of their single orthologue in *Drosophila* so far. Briefly, *Drosophila Hey* gene has been characterized as one of the alternative neuronal fate determinants in the developing CNS. Its expression and function in the majority of neuronal lineages is dependent on Notch signaling although few case of Notch independent expression have been described. It has been, also, characterized as identity guardian of the fully differentiated enterocytes in the adult fly midgut independently of Notch. We have studied its expression and functional role in the developing midgut of *Drosophila*. Unlike its expression pattern in adult fly, we detected Hey mainly in entero-endocrine cell population of the embryonic and larval midgut. In this developmental context, *Hey* is a direct target of Notch signalling, revealing a novel Notch event in the midgut development. Hey expression seems to follow every entero- endocrine generation wave throughout embryonic and larval life. Furthermore, functional analysis has demonstrated its high association with enteroendocrine fate and maintenance, while a few indications support its putative connection with the development of adult midgut precursors, the population which gives rise to the adult intestinal stem cells. Moreover, in a regulatory analysis context, we deleted the region of the intronic enhancer located in the endogenous sequence of *Drosophila melanogaster Hey* gene. Viable flies in homozygosity for the *mutant Hey* allele have shown a redundancy of *Hey* regulatory elements.

Οι πρωτεΐνες Hey είναι μεταγραφικοί παράγοντες που ανήκουν στην ομάδα των basic helix-loop-helix 'Orange' (bHLH-O) πρωτεϊνών και είναι συχνά τελεστές του Notch σηματοδοτικού μονοπατιού. Παρά τις εκτενείς μελέτες των Hey πρωτεϊνών στα θηλαστικά, λίγα πράγματα είναι γνωστά μέχρι σήμερα για το ρόλο της ορθόλογης πρωτεΐνης στη *Δροσόφιλα*. Συνοπτικά, η πρωτεΐνη Hey στη *Δροσόφιλα* έχει χαρακτηριστεί ως καθοριστής των εναλλακτικών νευρωνικών μοιρών στο αναπτυσσόμενο ΚΝΣ. Η έκφραση και η λειτουργία της στην πλειοψηφία των νευρωνικών γενεαλογιών εξαρτάται από τη σηματοδότηση Notch, αν και έχουν περιγραφεί λίγες περιπτώσεις έκφρασης ανεξάρτητες από αυτή. Επιπλέον, ανεξάρτητη της σηματοδότησης Notch έχει χαρακτηριστεί η έκφραση της στη μεσέντερο της ενήλικης μύγας, όπου έχει δειχθεί πως συμμετέχει στη διατήρηση της ταυτότητας των πλήρως διαφοροποιημένων εντεροκυττάρων. Εμείς μελετήσαμε την έκφραση και το λειτουργικό ρόλο της Hey στο αναπτυσσόμενο μεσέντερο της *Δροσόφιλας*. Σε αντίθεση με το μοτίβο έκφρασης που έχει περιγραφεί στο ενήλικο ζώο, την εντοπίσαμε κυρίως στον πληθυσμό τον εντερο-ενδοκρινών κυττάρων στο εμβρυικό και προνυμφικό μεσέντερο. Σε αυτό το αναπτυξιακό πλαίσιο, το γονίδιο *Hey* αποτελεί άμεσο στόχο του Notch, αποκαλύπτοντας μια καινούργια συμμετοχή του Notch στην ανάπτυξη του μεσεντέρου. Η έκφραση του γονιδίου *Hey* φαίνεται να ακολουθεί κάθε κύμα γένεσης εντερο-ενδοκρινών κυττάρων κατά τη διάρκεια της εμβρυικής και προνυμφικής ζωής του ζώου. Επιπροσθέτως, η λειτουργική ανάλυση αποκάλυψε την υψηλή συσχέτιση του με την τύχη και τη διατήρηση των εντερο-ενδοκρινών κυττάρων, ενώ μερικές ενδείξεις υποστηρίζουν την πιθανή του συσχέτιση με την ανάπτυξη των προγονικών ενήλικων κυττάρων του μεσεντέρου, τον πληθυσμό δηλαδή των κυττάρων που θα δώσει γένεση στα εντερικά βλαστικά κύτταρα του ενηλίκου. Στη συνέχεια, στο πλαίσιο της ρυθμιστικής ανάλυσης του γονιδίου *Hey*, εξαλείψαμε την περιοχή του ιντρονικού ενισχυτή στην ενδογενή αλληλουχία του γονιδίου. Τα βιώσιμα ενήλικα ζώα ομόζυγα για το μεταλλαγμένο αλλήλιο του *Hey* φανερώνουν αφθονία σχετικά με τα ρυθμιστικά στοιχεία του γονιδίου.

1.Introduction

1.1 HEY: Hairy/E(spl)-related with a Y

1.1.1 Hey proteins

Hey proteins belong to the basic helix-loop-helix 'Orange' (bHLH-O) protein group and they are integral members of the Notch signaling pathway, even though these factors can transduce and integrate signal for multiple pathways as well (BMP/TGF-β, JAK-STAT, RAS and HIF signaling) (reviewed in Fischer and Gessler, 2007). BHLH-O protein group is a subfamily of bHLH transcription factors and it is distinguished by an 'Orange' protein-protein interaction domain. *Drosophila Hairy* and *Enhancer of split* (E(spl) genes, the *Drosophila* counterpart of mammalian Hes protein family, are typical members of bHLH-O protein group and closely related to Hey family, which is named after it as Hairy/E(spl) like with a Y, due to the tyrosine residue in the YRPW motif in their C-terminus substituting for tryptophan in the corresponding WRPW motif of HES. These two protein families have similar structural features (Fig. 2). Firstly, there is a basic domain, which preferentially binds to an E-box sequence (CANNTG), close to the transcriptional start site of the target gene (Heisig et al, 2012). Secondly, they share a HLH domain which serves as a platform for homo- and heterodimerization with Hey and Hes proteins as well as for interactions with other proteins. Furthermore, there are two α-helices that form the 'Orange' domain, which functions as an additional interface for protein interactions and distinguishes these families from the rest of the superfamily of bHLH transcription factors. Finally, two previously referred conserved C-terminal motifs are identified for each of these two protein groups. The YRPW motif of Hey proteins is followed, also, by a conserved TE(I/V)GAF peptide with presently unknown function(reviewed in Weber et al., 2014).

Hey bHLH-O transcription factors are conserved throughout animal evolution. Thus, there are three mammalian Hey proteins (Hey1, Hey2, HeyL) and a single Hey *Drosophila* orthologue (reviewed in Weber et al., 2014). Until recently, their function thought to be exclusively repressive (Heisig et al., 2012). However, new studies in *Drosophila* support their dual transcriptional activity, presenting them as potential activators, as well as repressors, in an enhancer context- related manner (Stampfel et al, 2015 and Brodsly et al, 2019). The latest one, also, supports an indirect/ secondary gene activation mechanism in *Drosophila* according to which Hey regulates histone tail modifications associated with gene activation in vivo (Brodsly et al, 2019).

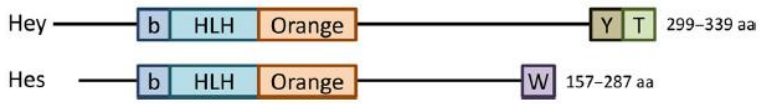


Figure 1.Domain organization of Hey and Hes proteins. Common features between mammalian and *Drosophila* are presented. Both protein families contain a basic domain (b), which bind to DNA, the helix-loop-helix (HLH) and the Orange protein domains used in protein interaction. They differ in their C-terminus motives. Hes proteins obtain a WRPW (W) motif, while Hey proteins have a YRPW (Y) motif followed by a TE(I/V)GAF (T) peptide. Amino acid(aa) numbers indicate the length of the proteins. (Weber et al., *Current Topics in Developmental Biology*, 2014)

From a developmental point of view, Hey proteins are involved in critical biological processes such as embryonic development, differentiation processes, and later tissue homeostasis, since their action varies from binary cell fate decisions to inductive processes and control of proliferation (reviewed in Weber et al., 2014). For instance, the three mammalian Hey proteins (Hey1, Hey2, and HeyL) have been identified as crucial factors in cardiac and vascular development. They are, also, involved in the control of neural, muscular and bone development, while they are deregulated in diverse malignancies (reviewed in Weber et al., 2014).

As far as its involvement in *Drosophila* development is concerned, few studies have been conducted. According to Monastirioti et al., during development of the Central Nervous System (CNS) *Hey* expression is detected in newly born post mitotic neurons, it is Notch-depended and it is implicated in the process of ganglion mother cells (GMCs) asymmetric neuron fate establishment. During both neurogenic phases of the fly life, in early embryogenesis and in larval stages, GMCs are generated from neuroblasts and after a single division, mainly asymmetric, give rise to two neurons or, less often, a neuron and a glia. Two types of neurons can be generated, type "A" and type "B", and it has been shown that unequal levels of Notch signaling determine their fates. In particular, the formation of type "A" needs high Notch signaling while the type "B" a low one. *Hey* expression is present into type "A" neurons, while further functional analysis experiments have rendered it as one of "A" fate determinant since its ectopic expression is sufficient to switch neuronal fates from the 'B' to the 'A' fate (Monastirioti et al, 2010).

In the adult fly, a recent study has rendered Hey an important factor for the proper gut homeostasis, tissue integrity and adult survival. They have characterized Hey as an identity guardian of the fully differentiated enterocytes (ECs) in the adult midgut by continuously regulating the expression of nuclear lamins, interestingly in a Notch- independent manner. They present a model in which Hey regulates EC identity partially by repressing the stem cell- related type-B lamin LamDmO and promoting the expression of EC genes signatures. The latest includes the EC founder homeobox transcription factor Pdm1, as well as, the type-A lamin LamC, which is necessary in order to prevent the expression of progenitor genes (Brodsly et al., 2019).

1.1.2 *Drosophila Hey* locus

Drosophila unique *Hey* gene is located on the 2nd chromosome and contains three exons and two introns (Fig. 2). Chip-on-chip experiments for Suppressor of Hairless (Su(H)) (a Notch co-activator) binding from larval CNSs (Zacharioudaki et al., 2016) revealed two regions containing Su(H) binding sites, required for Notch dependent activation (Bray and Furriols, 2001), indicating them as putative regulatory regions of the gene (Fig. 2). One such region is upstream of the gene promoter (3 Su(H) binding sites) and the other in the sequence of the second intron of *Hey* gene (4 Su(H) binding sites) (Fig. 2).

Previous lab members and collaborators had generated transgenic lines in which diverse sequences of the two putative *Hey* enhancer regions drive expression of marker genes as GFP and GAL4activator. These lines can be categorized in two major groups; the one of HeyUP enhancer and the other of HeyINT2 enhancer of *Hey* gene. The latter contains all the four

Su(H) binding sites mapped in the second intronic region of *Hey* (Fig. 2). HeyUP lines contain all the three Su(H) binding sites mapped in the upstream region of *Hey*, while 2+3 Su(H) lines contains only 2 of them and the NSH lines does not contain any of them (Fig. 2). Amir lines (provided by Amir Orian, Technion University, Haifa) contain a further shorter upstream region of *Hey* locus (Fig. 2). These transgenic lines are currently analyzed in the lab.

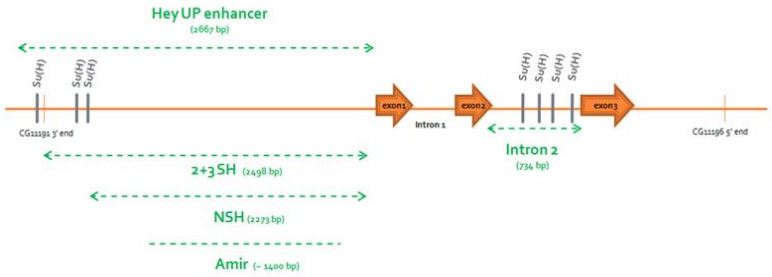


Figure 2. Scheme depicting the genetic region of Hey along with the two enhancer elements and the reporter constructs available.

1.2 *Drosophila* gastrointestinal tract

Drosophila gastrointestinal track has been for years an obscure organ in the biological research until the identification of somatic stem cells in the adult intestine a little over a decade ago. The discovery of intestinal stem cells (ISCs) by two labs simultaneously (Ohlstein and Spradling 2006;Micchelli and Perrimon (2006) was the onset of a plethora of following scientific works which have rendered this organ as a major source of signals modulating food intake, insulin secretion and energy balance. It is also a key player in immunity and, through its interaction with microbiota, can shape the physiology and behavior in complex and sometimes unexpected ways (reviewed in Miguel- Aliaga, 2018; Guo and Ohlstein, 2016).

The fly gut is subdivided into distinct regions: the ectodermally derived foregut and hindgut and the endodermally derived midgut (Fig. 3b). Anatomical specialization and regional compartmentalization can be observed along the anterior- posterior axis, both enabling sequential ingestion, storage, digestion, absorption and defecation (reviewed in Miguel-Aliaga, 2018; Guo and Ohlstein, 2016).

Foregut is a short narrow tube located at the most anterior part of the intestine, consists of the esophagus, crop and cardia (named also proventriculus) and act as food storage, regulating its passage into the midgut for further processing. The most posterior part, the hindgut is far less studied. It is subdivided into pylorus (a second valve-like structure), ileum, and rectum, where water/ion exchange may occur (reviewed in Miguel- Aliaga, 2018; Guo and Ohlstein, 2016).

Midgut is a long tube serving as the animal's principal organ for digestion and nutrient absorption. It is grossly subdivided into the anterior midgut, the middle midgut and the posterior midgut, but further morphological and molecular subdivision into 10-14 regions is often reported(Fig. 3b; colored regions). Specific histological and cellular features (villi size,

lumen width), stem cell proliferation rates, physical properties (e.g. luminal pH) and even gene expression profiles display variations along these subdivided regions (Marianes and Spradling, 2013). The end of the midgut and its junction with the hindgut is marked by the characteristic structure of the Malpighian tubules, the tubular excretory organs. We should note that midgut is the most well studied part of the fly intestine not only because its primary function in food digestion and absorption but also because it is generally assumed that it displays higher regenerative rates than the other two intestinal parts (reviewed in Miguel-Aliaga, 2018; Guo and Ohlstein, 2016)

Adult Drosophila midgut consists of a tube lined by an epithelial monolayer consisting of four cell types: intestinal stem cells (ISCs), absorptive enterocytes ECs, secretory enteroendocrine (EE cells), and enteroblasts (EBs): a post-mitotic, immature cell type which will differentiate as an EC or, possibly, as an EE (see below "The origins of *Drosophila* midgut cells" section and the review of Bardin et al., 2017) (Fig. 3c).This epithelium is protected from pathogens arriving trough lumen by the chitinious layer called peritrophic matrix (Fig.3 c-d) (reviewed in Miguel- Aliaga, 2018).

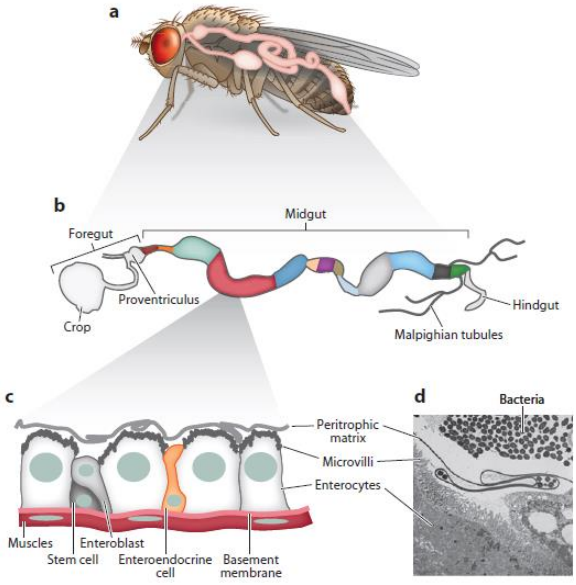


Figure 3.Schematic organization of the *Drosophila* digestive tract. (*a*) A 3D reconstruction of the digestive tract within the body cavity. (*b*) The digestive tract is divided into three discrete domains of different developmental origin: foregut, midgut, and hindgut. Each of these domains is further subdivided into genetically distinct compartments (*illustrated by different colors in the case of the midgut*). (*c*) The midgut is composed of an epithelium surrounded by two layers of visceral muscles. The midgut epithelium consists of enterocytes, enteroendocrine cells (EEC), and progenitor cells. (*d*) Electron microscopy sections of a third-instar larvalgut following infection with *Erwiniacarotovora 15*. The peritrophic matrix establishes a physical barrier that prevents contact between bacteria and the epithelial cell.*(adapted from Miguel- Aliaga et al., Genetics, 2018)*

1.2.1 Early formation of *Drosophila* midgut

As mentioned before, the three subdivisions of the Drosophila gut have different developmental origins. While foregut and hindgut come from ectoderm (Fig. 4; blue color), midgut is endodermally derived (Fig.4; red color). The endodermal midgut arises from two cell primodia, the anterior midgut primordium (AMG) and the posterior midgut primodium (PMG), which reside on the termini of the early embryo very close to the ectodermally derived primordia of the foregut and hindgut (Hartenstein, 1993).

During gastrulation the anterior and posterior midgut rudiments invaginate and they approach each other at the following stages. During stage 12, a significant reorganization of the midgut rudiments takes place. First, both AMG and PGM stretch in the longitudinal axis and become bilobate. Then during germ- band retraction, the midgut rudiments still come closer to each other and finally fuse at about the middle point of the egg (stage 13). Eventually, in the stage 15 they totally fuse in the ventral and dorsal midline, thereby forming a closed chamber that encloses the yolk (Hartenstein, 1993).

Following constrictions in the stages 15 and 16 subdivide the midgut into four chambers. During later development, all four chambers will constrict further, resulting in the long, highly convoluted tube representing the larval midgut. Later on there is an expansion of midgut, reaching a maximum length in the 96h after egg deposition (AED) (Michelli et al., 2011). During later larval stages, as well as the first two hours after pupa formation (APF), midgut length declines sharply (Michelli et al., 2011). 24 hours APF has its minimum size since the larval midgut has fully delaminated into the lumen forming the "yellow body", getting replaced by the adult midgut epithelium (Robertson, 1936).The following hours of metamorphosis midgut becomes gradually longer, while it obtains the characteristic looped configuration of the adult fly (Hartenstein, 1993).

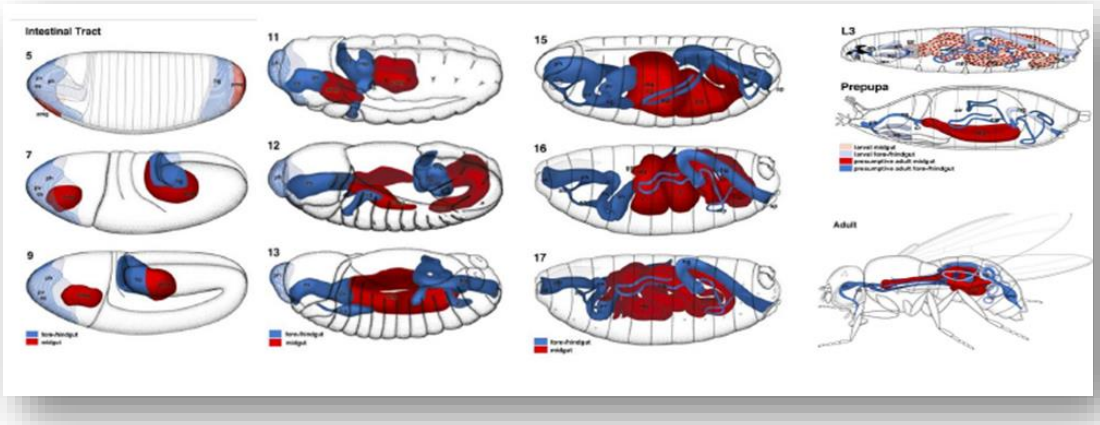


Figure 4.Depiction of early midgut development from embryo to adult fly. Blue structures; ectoderm, red structures; endoderm. Description in the main text. (Adapted from *Atlas of Drosophila Development* by Volker Hartenstein published by Cold Spring Harbor Laboratory Press, 1993)

1.2.2 The origins of *Drosophila* midgut cells

Drosophila melanogaster is a member of the holometabolous insects which undergo complete metamorphosis. After metamorphosis, the larval midgut degenerates to form meconium, which expelled soon after eclosion (Lemaitre et al., 2013). The adult midgut is generated de novo during metamorphosis by a molecularly distinct population of single cells, the adult midgut precursors (AMPs) that are generated during embryonic stages and remain undifferentiated through the larval stages (Michelli, 2012).

During embryonic stages 9-10 the epithelial cells of the endodermal rudiments lose their epithelial character, undergoing epithelial to mesenchymal transition (EMT),resulting into the formation of a mesenchymal cell mass (Tepass and Hartenstein, 1995).At these stages all the dividing endodermal cells are esg^+ cells (Takashima et al., 2001). Escargot (esg) is a member of the snail/slag superfamily of transcription factors and characteristic marker of undifferentiated cells including adult midgut precursors (AMPs) later on as well as the adult intestinal stem cells (ISCs) and enteroblasts (Michelli et al., 2013).At stage 11, endoderm has split into an outer layer and inner layer (Fig. 5A) (Tepass and Hartenstein, 1995; Takashima, 2011).The embryonic midgut epithelium starts forming from this stage on when mesenchymal cells from the outer layer gradually come into contact with visceral mesoderm and undergo a mesenchymal to epithelial transition (MET) (Tepass and Hartenstein, 1995) giving rise to Enterocytes (ECs), the characteristic post mitotic large polyploid cells of the intestine epithelium, (Tepass and Hartenstein, 1995; Takashima, 2011).Consequently, Escargot expression is lost from the outer layer but it is still maintained transiently in all cells of inner layer (Fig. 5A).

The inner layer give birth to precursors of the $Prospero^+$ ($Pros^+$) enteroendocrine(EE) cells and progenitor cells which generate the AMP pool (Tepass and Hartenstein, 1995; Takashima, 2011) (Fig. 5B). $Pros^+$ cells are present from the 10.5 embryonic stage and for 1-2 hours are, also, Esg^+ indicating that the early EE cells share certain molecular properties with the midgut progenitor population (Fig. 5B). From the end of embryonic stage 14 the expression of Prospero characterizes the EE cell population while expression of the esg marker is maintained mostly in the subset of inner cells that are pros negative and represent the AMPs. (Takashima et al., 2011) (Fig. 5C, C'). Lineage tracing experiments have shown that this $Esg^+/Pros^-$subpopulation generates the adult midgut(Michelli et al., 2011).AMPs and EEs reside to the lumen of the midgut until embryonic stage 16 and then they translocate to the epithelial niche in close proximity to the surrounding musculature and basement membrane (Michelli et al., 2011).Finally, there is evidence that a small number of $Esg^+/Pros^+$ cells of unknown fate is also detected at stage 17 during the embryonic/ larval transition (Michelli et al., 2011).

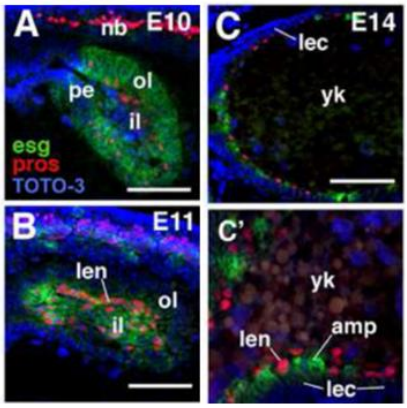


Figure 5.Emergence of larval entero-endocrine cells during the embryonic period. A–C: Z-projections of confocal sections of embryos labeled with antibody against Prospero (red) and probe against *esg*(green) mRNA. Nuclei are labeled with TOTO-3 (blue). (A) Embryonic stage 10(E10). Pros-positive endocrine precursors appear in the endoderm at the stage when it starts to split into an inner (il) and outer layer (ol). pe: posterior endoderm, nb: neuroblast. Durin gstage 11 (B), larval endocrine precursors (len) form part of the *esg*-positive inner layer. By

stage 14 (C), *esg* expression has become restricted to the adult midgut progenitor, which form a subpopulation of the inner layer separate from endocrine precursors (len in C'). lec: larval enterocyte precursor, yk yolk. (*Adapted from Takashima et al., 2011*)

During the first larval instar (L1) AMPs are scattered through the midgut as small diploid singe cells (Fig. 6A). Later on they start to proliferate and appear as doublets, until the late second/ early third larval instars (late L2) (Fig. 6B). In this stage, Larval ECs undergo several rounds of endoreplication, enlarging the larval midgut, while a slight increase of $Pros^{+}$ EE cells is also reported (Michelli et al., 2011), against the notion that all the larval EE cells have been formed in embryo (Takashima et al., 2011).In the middle third larval instar (middle L3) AMPs start forming clusters, named "AMPs islands" (Fig. 6C-D) (Jiang and Edgar, 2009) that contain 2-16 small cells (Michelli, 2012) by the end of L3. Some cells of the cluster (1-3) differentiate into flattened peripheral cells (PCs), with extended, sheet like cytoplasm, surrounding a more central group of undifferentiated AMPs (Mathur et al., 2010).Experiments have shown that PCs are required for maintaining AMPs in an undifferentiated state until the onset of metamorphosis (Mathur et al., 2010). Interestingly, In the very late L3 instar, *pros* expression appears again in a single cell or doublets of cells within some AMPs islands, but the fate of these cells has not been investigated and remains unknown (Takashima et al., 2011).

At the onset of metamorphosis, the PC-AMPs niche breaks down, resulting in islands spreading out (Fig. 6E) (Jiang and Edgar, 2009; Mathur et al., 2010). The PCs of each midgut progenitor cluster start to fuse into a continuous epithelial layer interposed between larval midgut and adult midgut progenitors (Jiang and Edgar, 2009). This layer represents the transient pupal midgut epithelium and forms a complete cellular layer around the central mass of degenerating larval midgut cells (the "yellow body"). They are both discharged from the intestinal tract with the yellow body after eclosion (Takashima et al., 2011).

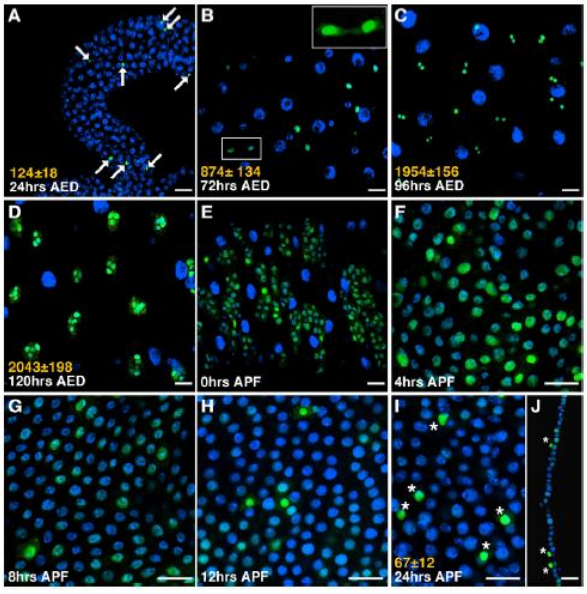


Figure 6.Development of *Drosophila* adult midgut progenitors (AMPs).AMPs were marked by GFP expression (green) driven by *esgGal4NP7397*. The numbers of GFP-positive AMPs, AMP clusters or adult intestinal stem cells (ISCs) are indicated in the appropriate panels. DNA is stained withDAPI (blue). (**A**) First instar larval midgut (24 hours AED). GFP was detected in the AMPs as individual diploid cells (arrows). (**B**) Early third instar larval midgut (72 hours AED). Larval enterocytes (ECs) undergo several rounds of endoreplication, enlarging the larval midgut. AMPs remain diploid and their numbers increase during the first two larval stages. However, they remain

mostly dispersed as individual cells. Inset shows GFP expression that is overexposed to show cell contacts between two neighboring AMPs. (**C**) Mid-third instar larval midgut (96 hours AED). AMPs form distinctive 2-to3-cell clusters. (**D**) Late third instar larval midgut (120 hours AED). AMPs continue to proliferate and enlarge the clusters. (**E**) White prepupa stage (0hours APF, ~130 hours AED). The size of the AMP clusters has increased further. (**F**) Prepupa stage (4 hours APF). The AMP clusters fuse to form a new midgut epithelium. Larval ECs (out of focal plane) are sloughed into the lumen and histolyze. (**G**, **H**) Early pupa stage (8 and 12 hours APF). The majority of the cells in the new midgut epithelium gradually lose GFP expression, except for a few scattered cells that maintain strong GFP expression. (**I**, **J**) Pupa stage (24 hours APF). The future adult ISCs are clearly identifiable by strong GFP expression (asterisks in I) and basal localization in the epithelium (asterisks in J, cross-sectional view). GFP expression is lost in the rest of the cells in the new epithelium. Scale bars: 20μm.*(Adapted from Jiang and Edgar , 2009)*

Following the PC breakdown, the majority of AMPs start to differentiate into ECs, forming the adult epithelium, while few continue to proliferate (Fig. 6F-H)(Jiang and Edgar, 2009; Mathur et al., 2010). At around 12 hour after pupa formation (APF), AMPs, now called presumptive ISCs (pISCs), are basally localized in the new epithelium(Fig. 6I-J). At 24 APF they start proliferating again to increase their number and subsequently the ISC pool, and only at 48 APF they begin to generate differentiated cells that are exclusively EE ones(Jiang and Edgar, 2009; Takashima, 2011).Enterocytes do not appear among the pISCs progeny until around the time of eclosion (Guo and Ohlstein, 2015). Interestingly, it is estimated that one AMP per island, on average, remains undifferentiated and becomes a future ISC (Mathur et al., 2010), but this issue remains controversial.

1.3 Notch signaling in the *Drosophila* midgut development

1.3.1 Notch signaling pathway at a glance

Notch signaling is a conserved, cell – cell communication pathway shared by many multicellular organisms, with a significant role in multiple developmental processes, such as cell fate decisions, proliferation and cell apoptosis (Artavanis- Tsakonas et al., 1999). Notch signaling links the fate of one cell with that of a cellular neighbor through physical interactions between the Notch receptor and the membrane-bound ligands that are expressed in anapposing cell (Hori et al., 2013).

Notch receptors are single-pass multidomain transmembrane proteins, highly conserved from invertebrates to humans. 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 and 3LNR domains. Their activation follows the binding of a Notch ligand. 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. The ligand recognition leads to two consecutive cleavage events releasing the intracellular domain (NICD), which then translocates to the nucleus. There NICD interacts with the DNA-binding protein RBPJk (also known as CBF1, Rbpsuh or Su(H) in Drosophila), which is associated with co-repressors (e.g. N-CoR, SHARP, CtBP). Interaction with NICD replaces these co-repressors and allows recruitment of co-activators like Mastermind/MAML

and p300/CBP leading to transcriptional activation of target genes (Fig. 7; Gordon, 2008 and Andreas Fischer and Manfred Gessler, 2007).

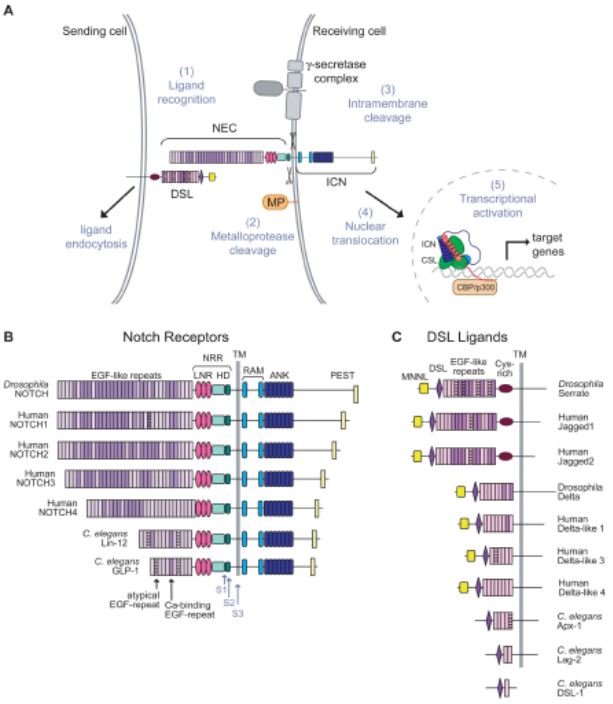


Figure 7. Notch signaling pathway and domain organization of Notch receptors and DSL ligands. (A) Model for the major events in the Notch signaling pathway. Signals initiated by the engagement of ligand (1) lead to metalloprotease cleavage (MP) at site S2 (2). This proteolytic step allows the cleavage 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;5). (B, C) The domain organization of Notch receptors (B) and DSL-family ligands(C) from fly, human and worm. (Gordon et al., *J Cell Sci*. 2008)

1.3.1 Notch signaling in the *Drosophila* midgut development

Notch signaling plays a pivotal role in the development of Drosophila midgut in all the stages of its life cycle. High Notch signaling is linked with EC generation from EBs (Ohlstein and Spradling, 2006; 2007; Micchelli and Perrimon, 2006; Takashima et al., 2011). In this context, after a typical ISC division which gives a new ISC and a EB, the ISC produce and exhibit in its membrane the Delta ligand which consequently activates its receptor Notch in EBs, resulting in their differentiation into ECs (Ohlstein and Spradling, 2006; 2007; Micchelli and Perrimon, 2006). This notion is supported by studies both in embryo and adult midgut. In embryo, lack of Notch signalling leads to an increase of the number of AMPs and EE cells at the expense of ECs (Hartenstein, 1995; Takashima et al., 2011), while in the adult midgut it has been shown that ISCs lacking Notch activity produce a strongly increased number of undifferentiated ISCs, causing the formation of Dl^{+} and $Pros^{+}$ cell tumors(Ohlstein and Spradling, 2006;Micchelli and Perrimon, 2006).

In the larval developing midgut, Notch signalling is required for the determination of the peripheral cell that acts as a niche, where the AMP and its following daughters can remain undifferentiated (Mathur et al., 2010). In this context, after the first- and its only- asymmetric division of a founder AMP, this cell expresses Delta ligand which activates Notch signalling to its first daughter cell to promote its fate into peripheral cell (Mathur et al., 2010).

As far as the EE cell fate concerned, the prevailing model argued that EE cell generation needs low or no Notch signalling (Ohlstein and Spradling, 2007; Takashima et al., 2011). However, Guo and Ohlstein, suggest that the initial EE cell fate choice does not require Notch signalling, but only an asymmetric localization during ISC division of the transcription factor Prospero, the well known EE-fate-determination factor (Guo and Ohlstein, 2015). On the other hand, *pros* expression is induced by Scute (Sc), a factor of the acheate-scute complex (AS-C) genes, whose coordinated action is regulated by negative feedback loop between Sc and Notch targets, which proves the involvement of Notch in EE fate (Chen et al., 2018).We should note that both of these studies suggest possible EE formation from an EE progenitor generated from ISC instead of deriving from EBs, a question that is still under investigation in the field (see review Bardin et al., 2017). Despite that, another study has analyzed EE cell diversity and found that Notch active EBs can give rise to class II EE cells, in addition to ECs (EE classification based on the neuropeptides produced) (Michelli and Evans, 2015).

Controversial remains, also, the issue that concerns the involvement of Notch signalling in the maintenance of ISC identity. Bardin et al. refer that transcriptional repression of Notch targets, for instance E(spl), is required for ISC maintenance (Bardin et al., 2010) . In contrast, Guo and Ohlstein support that ISCs after their division and the following ee formation need Notch activation triggered by their ee daughter in ordered to maintain their multipotency(Guo and Ohlstein, 2015).

1.4 The aim of the study

Previous studies have investigated the expression and the role of HEY throughoutneurogenesis in the developing fly (Monastirioti et al., 2010). Interestingly , *hey* expression has been also detected in the embrionic midgut(Fig.9). Thus, since it has been already known that *hey* is expressed in neurons in a Notch- depented manner we speculated that this may be the case for Hey expression in the midgut as well.Such a case would suggest a novel Notch event occuring in the developing midgut besides the one described earlier involving E(spl) genes as effectors (Tepass and Hartenstein, 1995; Bardin et al., 2010). Therefore, this study focused on elucidating *hey* expression pattern during embryo and larval midgut development as well as on investigating HEY functional role during this process Further study of the *hey* gene regulationin the fly midgut was an additional part of this project in order to elucidate the requirement of each enhancer region on the proper expression of the gene.

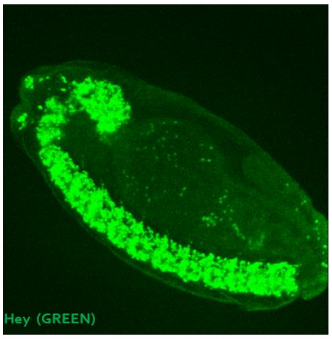


Figure 9.*Hey* expression in the *Drosophila* embryo. Hey (green) is expressed in neurons as well as in embryonic gut.

2. Results

2.1 Hey expression pattern in the developing midgut of *Drosophila*

The first aim of this study was to elucidate the expression pattern of *Hey* in the developing midgut of Drosophila. For this purpose, we performed immunostainings in collections of staged embryos as well as in dissected larval midguts. We used specific antibodies against Drosophila Hey (Monastirioti et al 2010) as well as gene markers that characterize the different cell populations of the midgut-Escargot (Esg) for undifferentiated cells /AMPs, Prospero (Pros) for enteroendocrine cells and Pdm-1 for Enterocytes. The different populations can be also distinguished by the size of their nucleus (big polyploid ECs versus small diploid EEs and AMPs) when using the nuclear marker DAPI.

2.1.1 *Hey* expression in *Drosophila* embryonic midgut

The onset of *Hey* expression in the midgut is detected in the embryonic late stage 12/ stage 13 in a subset of $Pros^{+}$ cells (Fig. 10A). In this stage the AMPs and EE cells have not formed yet two completely separated populations, as the pan-EE marker Prospero expresses in some Esg^{+} progenitors cells. Thus, in stage 13 we observed cell populations not only Esg^{-}/ $Pros^{+}$/ Hey^{+} but Esg^{+}/ $Pros^{+}$/ Hey^{+} as well (Fig. 10A pos).In few cases some Esg^{+}/ $Pros^{-}$/ Hey^{+} were also observed (Fig. 10Az ant). By the end of stage 14 and onwards that Pros expression is limited to EE cells and *Esg* expression remains in the AMPs, it is clear that Hey expression appears only in a subpopulation of $Pros^{+}$ cells (Fig. 10B) and ECs are devoid of *Hey* expression (Fig. 10D). Wondering whether Hey express in dividing cells, we stained embryos with the mitotic marker PH3. In the embryonic midgut of stage 13, low division activity could be observed only in cells devoid of Hey^{+} $Pros^{+}$ (Fig. 10C).These observations suggest that Hey appears in a subpopulation of cells that have adopted the EE fate in late embryo, but earlier it is expressed in non dividing precursors.

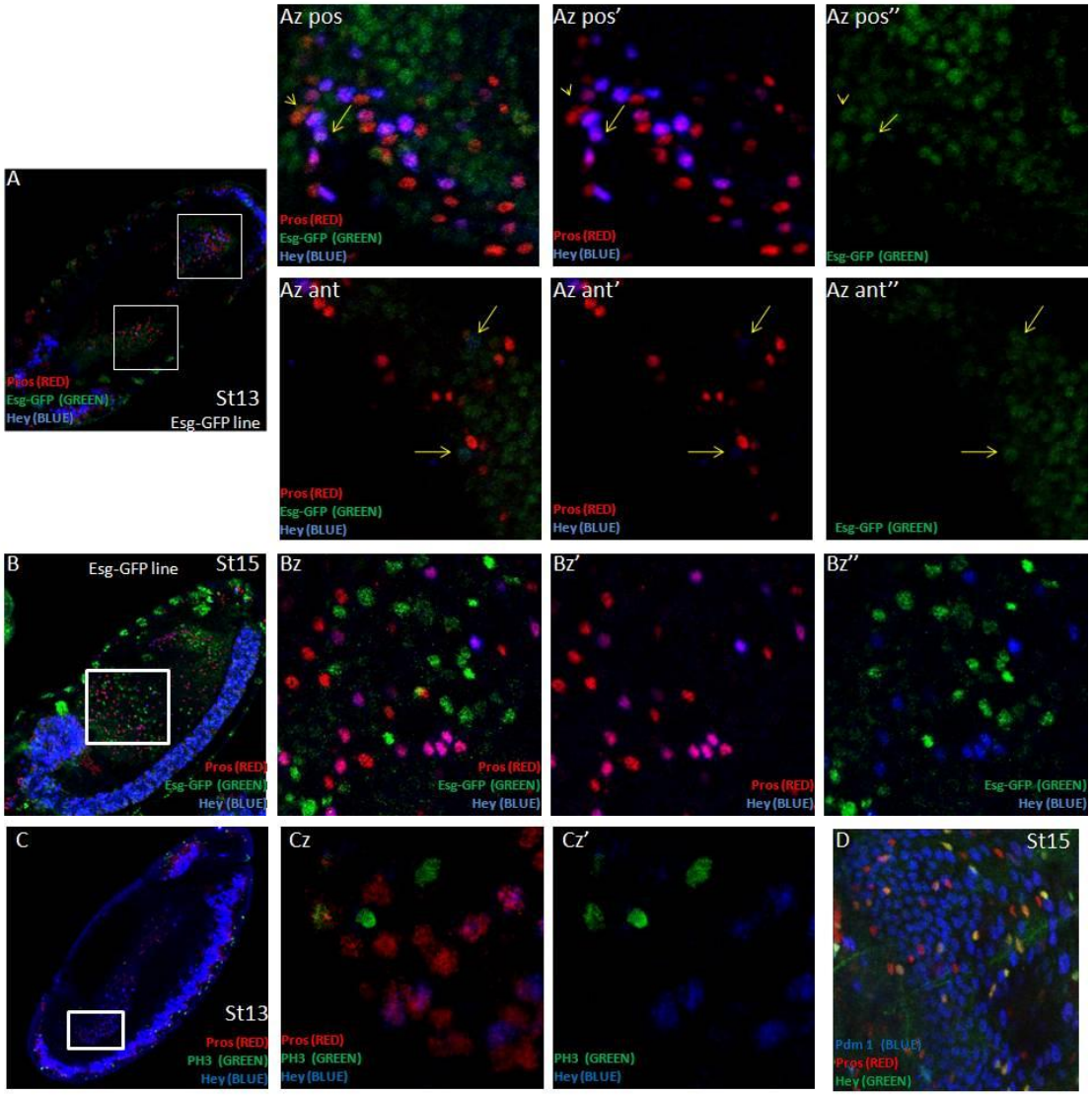


Figure 10. Hey expression in the midgut during embryogenesis. (A- B) Esg-GFP line stained for Hey (blue), Prospero (red) and Escargot (GFP) (green).**(A)** In stage 13 embryo **. Azpos, ,Az ant** are higher magnifications of the regions in squares, anterior is bottom left and posterior is top right. Hey starts expressing in a subpopulation of $Pros^{+}$ cells (Azpos, Az ant) some of which are, also Esg^{+} (Azpos' and Azpos'' arrow).Similarly, some of the Hey^{-} / Pros+ cells are Esg^{+} and(Azpos' and Azpos'' arrowhead) and some are not. In a few regions Hey^{+}/ Esg^{+}/ $Pros^{-}$ cells can be detected (Az ant,Az ant' and Az ant'' arrows). **(B)** Stage 15embryo**. (Bz)** is higher magnification of the region in square. Hey expression is limited in a subpopulation of $Pros^{+}$ cells (Bz'), which are devoid of Esg expression (Bz''). **(C)** Wild- type stage 13embryo stained for Hey (blue), Prospero (red) and PH3 (green). **(Cz)** is higher magnification. In this stage there are some $Pros^{+}$ as well as some $Pros^{-}$ dividing cells but while Hey appears in not dividing cells.(D) Wild- type stage 15 embryo stained for Hey (green), Prospero (red) and Pdm1 (blue). Pdm1+ ECs cells are devoid of *hey* expression.

As it was known that *Hey* is target of Notch in the embryonic CNS (Monastirioti et al., 2010), we used mutations that perturb Notch signalling to address whether *Hey* expression is regulated by Notch in the embryonic midgut as well. It has been known for years that mutants embryos devoid of Notch signaling (Dl^{-} Ser^{-} mutants) display an extensive number of AMPs and EE cells in expense of ECs (Hartenstein et al., 1995; Takashima et al., 2011), a phenotype recapitulated in a milder way in E(spl) deficient embryos (Hartenstein et al., 1995). Embryos from double-null mutant for *Dl* and *Ser*, the genes encoding the only two Notch ligands in *Drosophila*, were tested and no Hey expression was observed in their midgut (Fig. 11B). Given the fact that Hey is close related with E(spl) bHLH-O proteins, with which are able to

form heterodimers(Weber et al., 2014), we wanted to figure out whether they are linked in the midgut developmental context, since both families are Notch effectors. We used embryos of *Df(3R)E(spl)b32.2* strain, which is a deficiency for the entire E(spl)complex. *Df(3R)E(spl)b32.2*-homozygous embryos display a normal *hey* expression in their midgut (Fig. 11C). Thus, the expression of *hey* is Notch-dependent but E(spl) independent.

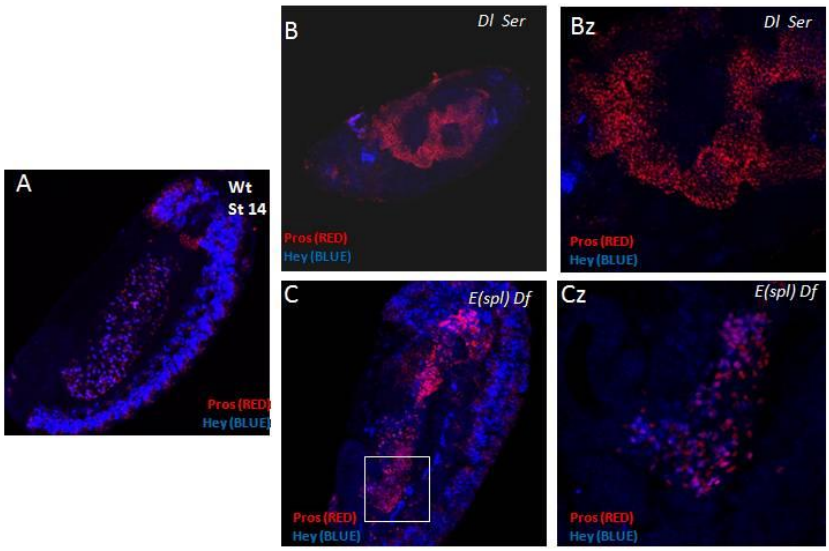


Figure 11.*Hey* expression (BLUE) in two different genetic backgrounds with deficiencies in Notch signaling at affect the Pros+ (RED) population .(A) Wild-type embryo in stage 14 **(B**)Dl^{-}Ser^{-} mutant embryo stained **(Bz)** is higher magnification. Hey expression in midgut is abolished in *Dl Ser* background. **(C)**E(spl) deficient embryos stained for Prospero (red) and Hey (blue). **(Cz)** is higher magnification. Hey expression it is not disturbed in *E(spl) Df*in the embryonic midgut. White quadrangle indicates the region presented each time as higher magnification in the corresponding figure.

2.1.2 Tracing the expression of Hey in *Drosophila* midgut

Hey expression in those mixed populations of Esg^{+}/Pros^{+} cells, present in embryonic stage 13, prompted a further investigation of their identity by conducting a series of genetic lineage tracing experiments. These were performed according to the G-Trace system (Fig. 12) developed by Evans et al. (Evans et al., 2009). G-Trace system is a combination of Gal4/UAS, FLP/FRT and fluorescent reporters to generate cell clones which can provide spatial, temporal, and genetic information about the origins of individual cells in *Drosophila*.

In brief, the Gal4 expression is regulated by the enhancer whose activity we want to study. The Gal4 activates the expression of red florescent protein (UAS-RFP) or a similar florescent protein as well as the expression of flippase (UAS- FLP). Then, in cells expressing FLP, an FRT-flanked STOP cassette separatingthe ubiquitously active Ubi-p63E promoter and the nuclear enhanced green fluorescent protein gene (nEGFP) open reading frame is excised. This modification initiates constitutive expression of EGFP in these cells as well as in their progeny. The "cell color" gradient represents the apparent color of cells subsequent to the initiation of Gal4 expression (Fig. 12).

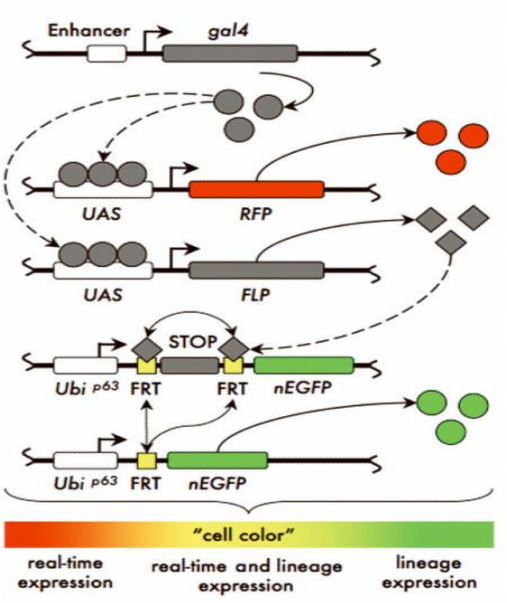


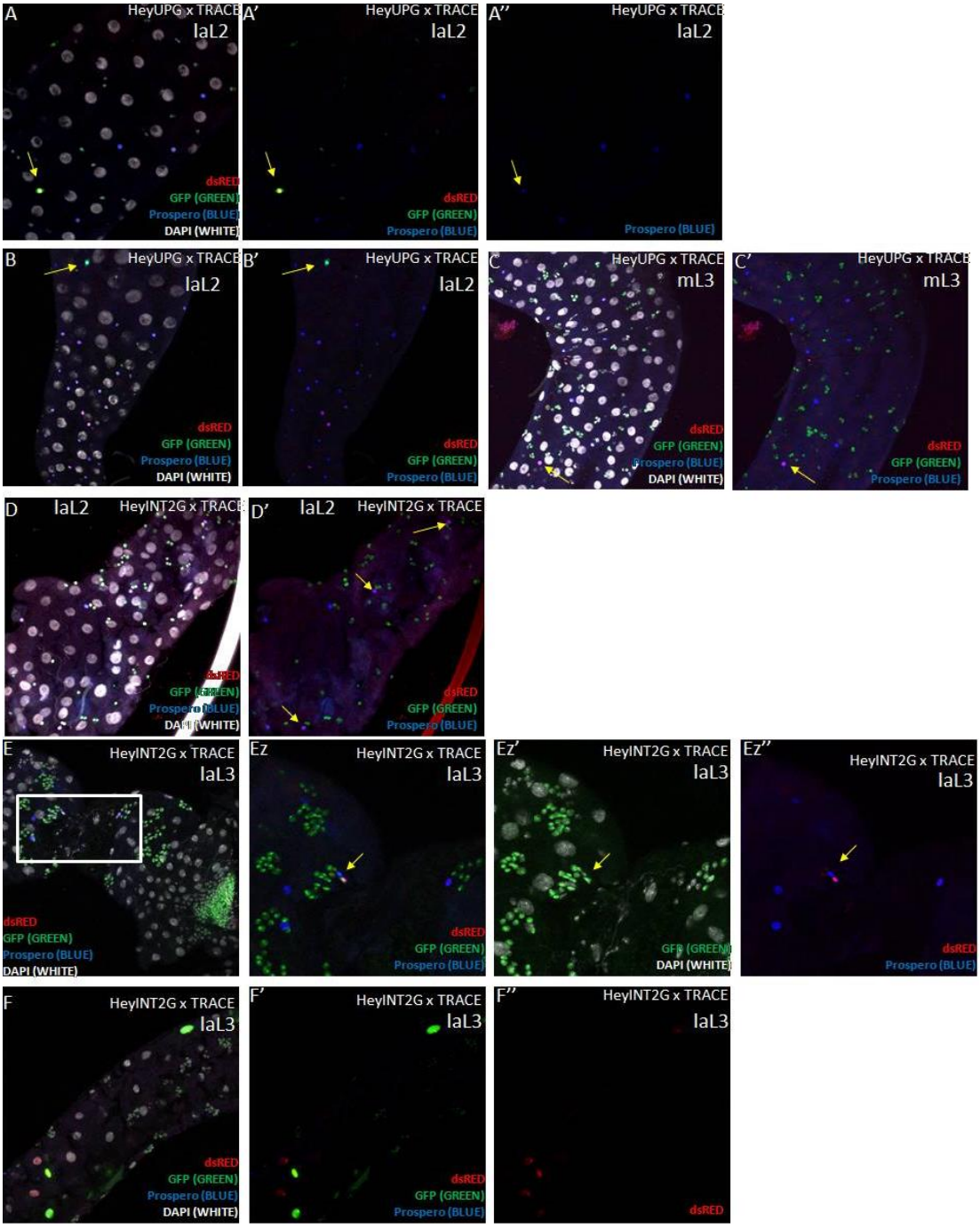
Figure 12.The G-trace analysis system. Gal4 activates the expression of RFP and FLP recombinase. Cells expressing FLP then excise the FRT-flanked STOP cassette separating the Ubi-p63E promoter and nEGFP open reading frame. This initiates expression of EGFP, which is heritably maintained in all daughter cells. The "cell color" gradient represents the apparent color of cells subsequent to the initiation of Gal4 expression. (*Evans et al, 2009*)

Using this system we can demonstrate whether the activity of an enhancer of interest exists at the time of detection (real-time expression) or it happened sometime during an earlier developmental stage (lineage expression). Therefore, we used 2 of the enhancer – GAL4 lines made in our lab, which correspond to Hey UP and INT2 enhancer elements (unpublished data) and one made by Amir Orian group named after him as Amir – GAL4 line (Brodsly et al., 2019). All of them are presented in the schematic description of hey locus in Figure 2. (see Introduction).

In our G-Trace experiments we looked at different developmental windows in larval life; late L2 (laL2) instar, middle L3 (mL3) instar and late L3 (laL3) instar. We started with the analysis based on Hey UP and INT2 GAL4 lines and we observed that they display many similarities in their expression patterns. In Figure 13 we present some indicative pictures. In late L2 and in middle L3 lineage expression was found in AMPs, the small $Pros^{-}$ cells, which sometimes had formed doublets in laL2 and they finally had formed their characteristic islands in later stages. (Fig. 13A, C, D). In addition, real time expression was observed in a subpopulation of $Pros^{+}$ EE cells (Fig. 13A-D) and only in few cases seemed that GFP had accumulated (Fig. 13A; arrow). Pros+ cells with only lineage expression were also present, consistent with Hey expression detected in embryo (Fig. 13 B). In late L3 real time expression is not only evident in a subpopulation of $Pros^{+}$ EE cells but also in some AMPs within the islands and in few cases is combined with *pros* expression (Fig. 13E). Exceptionally, Hey INT2 enhancer shows further real time expression in ECs, identified by their big polyploid nuclei, in some particular regions, in the anterior and middle part of the midgut (Fig. 13F).

As far as AmirGAL4 driver is concerned, G-TRACE analysis in late L3 larval gut, surprisingly, exhibited extensive real time expression in some AMPs islands, apart from

lineage expression(Fig. 13G). A subpopulation of Pros^{+} EE cells show real time expression, as well as, some Pros^{+} small cells within islands (Fig. 13H). We should notice here that in contrast with HeyUP-GAL4 case the real time expression of AmirGAL4 within the islands was not always combined with pros expression.



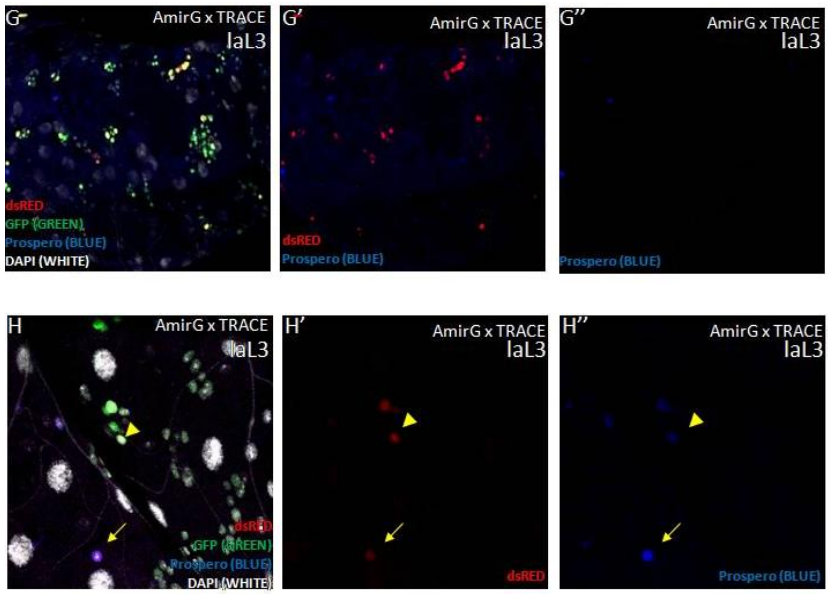


Figure 13. G- Trace analysis using Hey enhancers – GAL4 lines. G-trace system was used to identify the activity pattern of *Hey* enhancers in larval midgut. Real-time and lineage expression of Hey is observed by dsRed (red) and EGFP (green) expression, respectively. Further staining with Prospero (blue) and nuclear staining with DAPI (white). **(A-C)**Hey UP enhancer G-Trace analysis in late L2 (A-B) and middle L3 (C) larval guts. In late L2 instar, *hey* lineage expression is observed in presumptive AMPs, the small Pros$^-$ cells which, in this stage, are single cells or they have started form doublets. The slightly larger Pros$^+$ cells are EE, and a subpopulation of them shows real time expression of Hey (A-B). Some of the EE cells have already accumulated GFP (A, A'; arrow), while others no (B). Interestingly, there are EE cells with only lineage expression (B, B'; arrow). In middle L3 instar, AMPs characteristic islands show lineage expression, while a subpopulation of Pros$^+$ cells has real time expression of the enhancer (C, C'; arrow). **(D-F)** Hey INT2 enhancer G-Trace analysis in late L2 and late L3 larval guts. (Ez') is higher magnification of figure E as indicated by the white quadrangle. (D) In late L2 instar lineage expression appears in presumptive AMP islands and real time expression is present in a subpopulation of Pros$^+$ EE cells (D; arrows). (E) In late stages, real time expression starts in small cells within the islands, as one cell of Pros$^+$ doublet in them express dsRed (E; arrow).Note that there are, also dsRed$^+$/ Pros$^-$ doublets within the island (E; island pointed by the arrow). (F) The Hey INT2 enhancer shows further both real and lineage expression in ECs, indentified by their big polyploid nuclei, in some particular regions, in the anterior and middle part of the midgut **(G-H)**Hey Amir enhancer G-Trace analysis late L3 larval guts. Apart from the lineage expression , this enhancer shows extensive real time expression in AMPs within some islands, which not always have simultaneous pros expression G, G', G''. A subpopulation of Pros$^+$ EE cells show real time expression (E, E', E''; arrow), as well as, some Pros+ small cells within islands (E, E', E''; arrowhead).

Even if the tracing analysis was quite informative, unfortunately further control experiments proved that the lineage expression signal of GFP has been probably an artifact, since GFP signal was seen in AMP islands even in the absence of any Gal4 line, implying an AMP-specific leakiness of the UAS-FLP transgene, (data not shown) and so not trustful or conclusive. However, we verified *Hey* enhancer expression in a subpopulation of EE cells and specific ECs throughout larval development. Secondly, the lineage expression of *Hey* in EE cells suggests that *Hey* expressed in the past (probably in embryogenesis) for some reason but now is dispensable for one particular EE subtype, while for another still be necessary, as indicated by the real time expression in some other EE cells. In addition, its presence in AMP islands in late L3 instar when *pros* expression emerges in some of their cells implies a further putative role of Hey in EE generation. Therefore, we could assume that *Hey* performs a transient expression during developing *Drosophila* midgut associated mainly with enteroendocrine cell fate.

2.1.3 Hey expression in *Drosophila* larval midgut

Afterwards, we wanted to investigate *Hey* expression in the larval midgut. Surprisingly, in the larval midgut *Hey* expression is barely detected by immunostaining using specific antibodies against Hey protein, a fact that was not consistent with the real time expression of Hey enhancer lines observed before. . The only time window when it can be detected is in very early1st larval stage, a couple of hours after hatching. Then, it is found in Pros^{+} cells, beside to Esg^{+}/Pros^{+} located in a basal position in the midgut epithelium (Fig. 15A). This double positive cell population has been described by et al. who have shown that Esg$^{+/}$Pros^{+} cells are present in relatively low abundance throughout the course of the larval stages (Micchelli et al., 2011). At 2nd and 3rd instar larval midgut we did not detect *Hey* expression by the specific antibodies. A very recently generated transgenic line bringing GFP-tagged Hey protein (Kudron et al, 2018) was considered a good tool to overcome this problem. The locus encoding this fused protein is under the same regulation as the endogenous gene and the stability of GFP gave us the opportunity to follow *Hey* expression in the rest of the larval stages. We first checked the similarity between the expression patterns of this new Hey-GFP line with the one given by a-Hey antibody staining in the embryo. We found out that they give an absolutely co-localized signal (Fig. 14A-B) and, as expected, Hey-GFP is expressed in a subpopulation of Pros^{+} cells, (Fig. 14B’’).

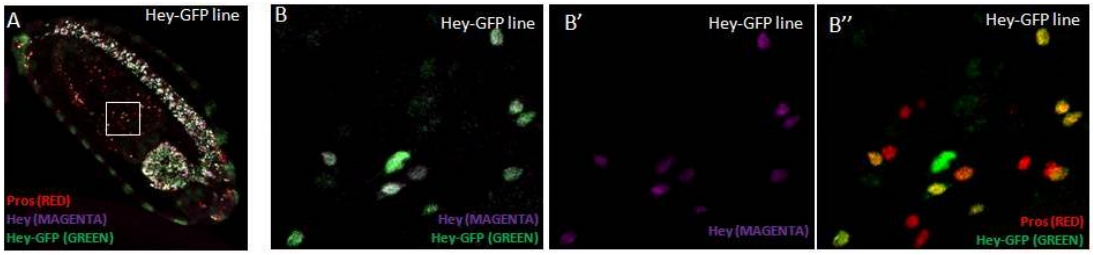


Figure 14.Testing Hey-GFP expression pattern in embryos.(A-B). Hey-GFP line stained for Hey (magenta), Prospero (red), GFP (green). Hey-GFP expression pattern is completely matched with a-Hey staining in a subpopulation of Pros^{+} cells (B’’).

Afterwards, we continued by monitoring Hey-GFP expression in the late larval midguts (Fig. 15B-F). The three cell population can be easily distinguished in this stage. AMPs have extensively proliferated, forming their characteristic islands, and have a little smaller nuclei than the Pros^{+}, usually single, EE cells. ECs are characterized by their large polyploid nuclei. Interestingly *Hey-GFP* expression is evident in a subpopulation of Pros^{+} EE cells (Fig 15B) suggesting that levels of endogenous Hey levels are low thus presumably undetectable by the a-Hey antibody. In addition, Hey-GFP is also detected in some Pros^{+} cells within AMP islands (Fig. 15C). Apart from single Pros^{+} cells, it has been reported that doublets of Pros^{+} cells can be formed within the islands (Takashima et al., 2011) and, in some cases only one of the two cells expresses Hey (Fig. 15D). Furthermore, Hey-*GFP* expression can be observed in ECs particular regions, in the anterior (Fig. 15E) and in the middle midgut (Fig. 15F). These data indicate that through larval development *Hey* is still expressed in a subpopulation of EE cells, maybe in lower levels than in embryo, while it also follows, the new Pros expression which appears within the AMP islands of the midgut.

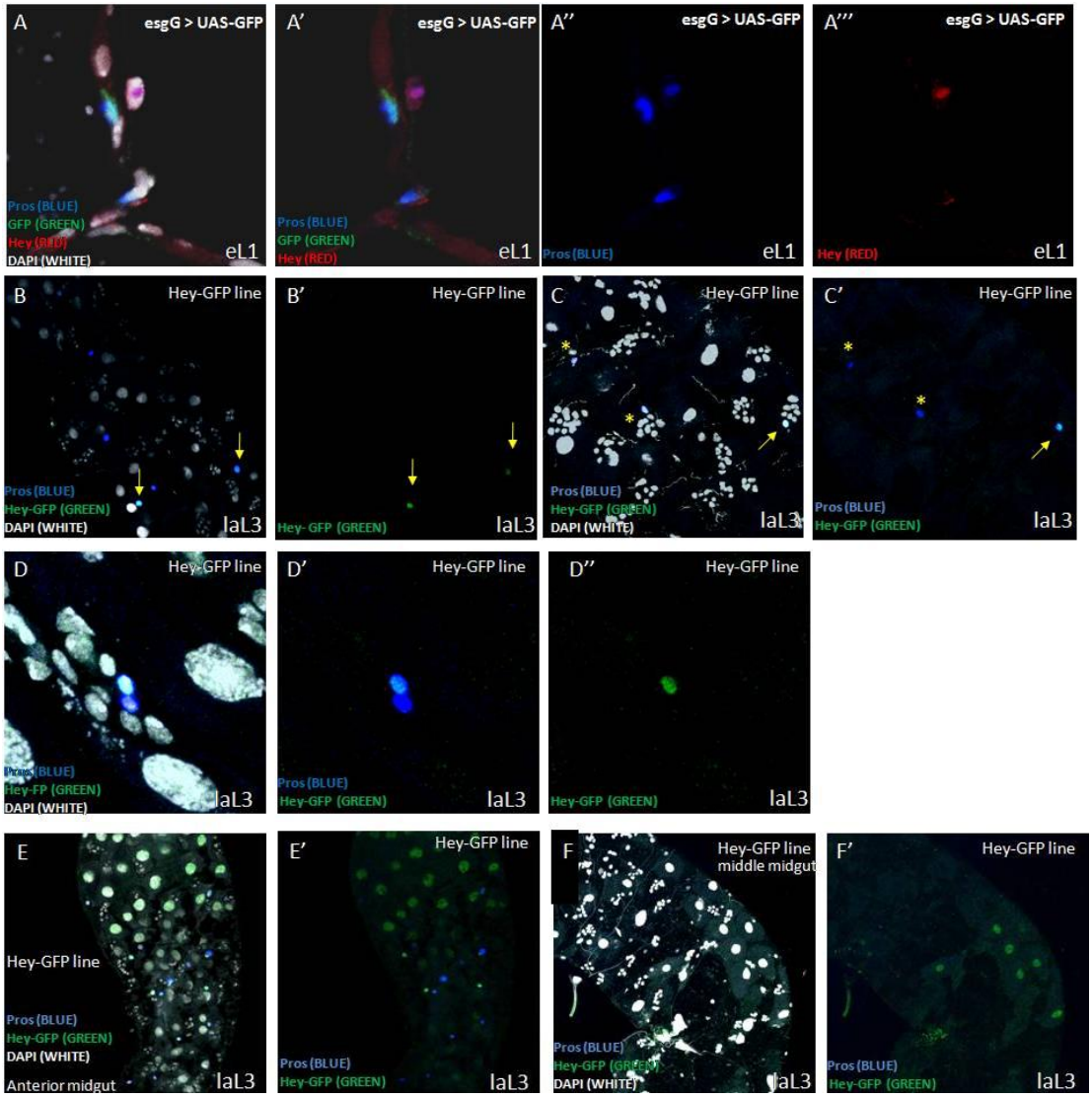


Figure 15. Hey expression in larval midgut.(A, A', A", A"') Early L1 instar larval gut of esg> UGFP line stained for Prospero (blue), Hey (red). Hey expression is detected in some Pros$^+$ cells in this early stage. **(B-F)**Hey-GFP line stained for GFP (green) and Prospero (blue) in late L3 larval stage. **(B, B')** Hey expression appears in a subpopulation of Pros$^+$ EE cells (arrows). **(C, C')** In this late stage, Pros$^+$ cells are present within AMP islands. Some of them express only Pros (asterisks), while others also express Hey (GFP) (arrow). **(D, D', D'')** Apart from single Pros+ cells, doublets of Pros+ cells can be formed, as well, within the islands. One of doublet cell expresses Hey (GFP). **(E-F)** Hey expression (GFP) can be observed in ECs (big nuclei) in particular regions, in the anterior midgut (E, E') and in the middle midgut (F, F'). DAPI stained for nuclei.

Even if we had a quite complete image about *Hey* expression during larval development, we were still wondering about the unjustified extensive real time expression of Amir- GAL4 driver in previous the G-TRACE analysis (Fig. 13G). Thus, we thought to check further the expression of all the enhancer-GAL4 line available in the lab in the larval midgut. As mentioned before, previous lab members and collaborators had cloned sequences of putative *Hey* enhancer regions, differing in enhancer length, (depicted in Fig. 16A) and they had generated Hey enhancer – GFP lines.

We should point out that since both *Hey* upstream and the second intronic regions contain Su(H) binding sides, the drivers generated can be considered as putative Notch-dependent drivers. HeyINT2-GAL4 and the corresponding HeyINT2- GFP line contain all the four Su(H) binding sites mapped in the second intronic region of *Hey*. In addition, HeyUP-GAL4 and the corresponding HeyUP- GFP line contain all the three Su(H) binding sites mapped in the upstream region of *Hey*, while 2+3 Su(H) lines contains only 2 of them and the NSH lines does not contain any of them. Amir lines contain a further shorter upstream region of *Hey* locus (Fig. 16A).

Immunostaining experiments in late larval midguts of *Hey* enhancers – GFP lines revealed both similarities and important differences in their expression patterns (Fig. 16B-I). The lines of the upstream enhancer region (HeyUP-GFP, Hey 2+3Su(H)-GFP, HeyNSH-GFP and AmirG>nUGFP) show *Hey* expression in a subpopulation of $Pros^{+}$ EE cells. There is, also, expression within AMP islands, in single $Pros^{+}$ cell or in one cell of the $Pros^{+}$ doublet formed. These observations are perfectly consistent with the expression pattern of Hey-GFP. We should note that $Pros^{+}$/ GFP^{-} single cells or doublets exist within the AMP islands as well (Fig. 16B-H). Interestingly, Hey NSH-GFP line has shown a weak *Hey* expression within the islands in many of their AMP cells, which were devoid of *pros* expression (Fig 16F) and this phenomenon was much more intense in the case of AmirG>nUGFP late larval guts, as expected (Fig. 16G-H) by the G-TRACE results. These latest observations reveal a putative suppressive regulation of Hey expression in the AMP islands, which is lost when Su(H) is missing. HeyINT2-GFP line was less studied and only limited Hey not-accompanied-by-pros expression in AMP islands was detected, in those experiments. However, the more extensive G-TRACE analysis had previously revealed that the corresponding GAL4 line of INT2 enhancer region display quite similar expression pattern to Hey UP enhancer.

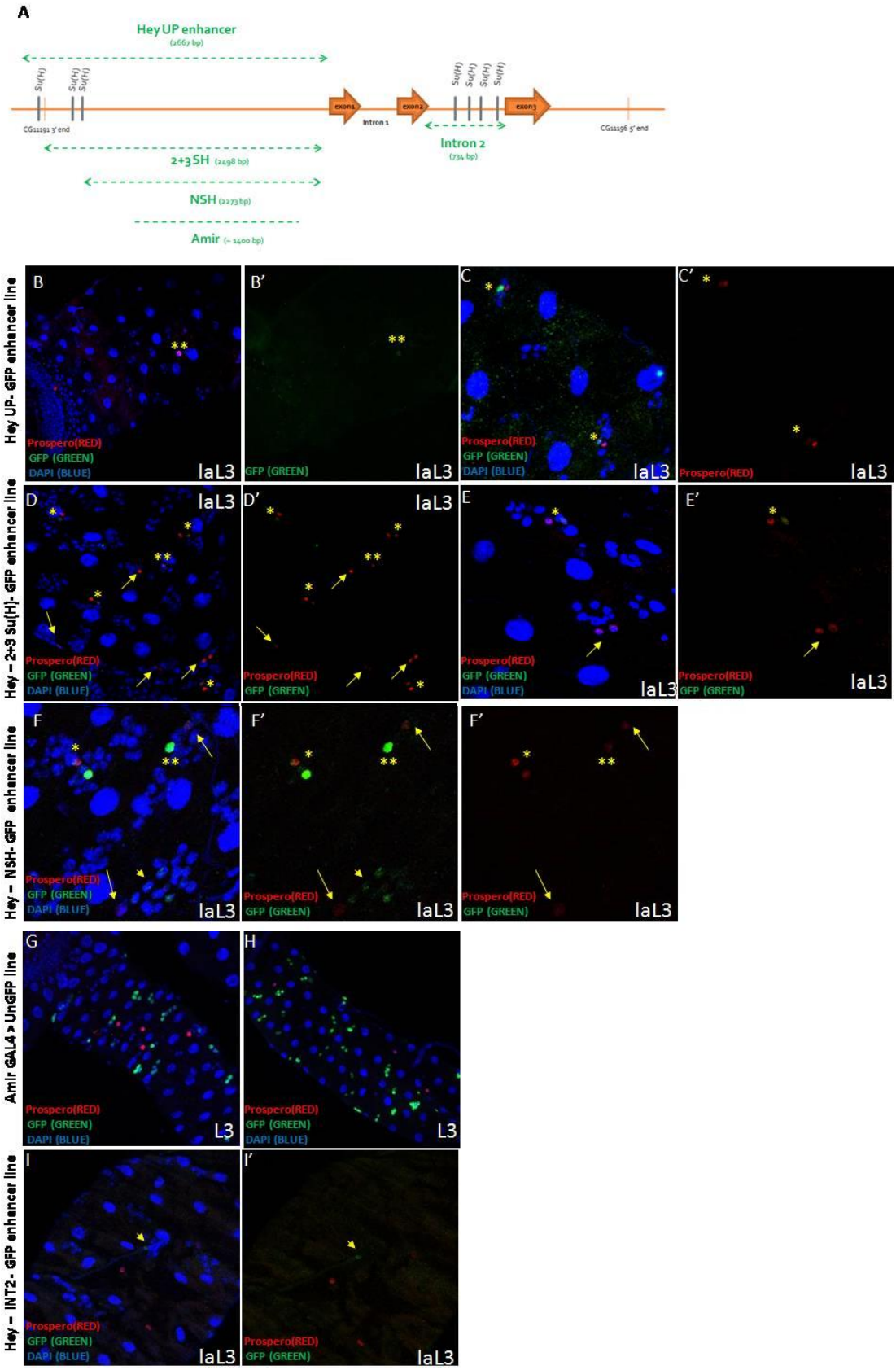


Figure 16. Hey enhancers – GFP lines' expression in the late larval midgut.(A) Schematic description of Hey locus. Green arrows indicate the extent of each of the five enhancer elements. Each of them was cloned and used for the generation of the transgenic- reporter lines with the corresponding name. Exonic and intronic regions,

Su(H) binding sites and the edges of the neighbor loci are depicted as well.**(B-I)** L3 instar larval guts from the five Hey enhancer lines stained for GFP (green) and Prospero (red). DAPI stained for nuclei. $Pros^{+}$ doublet with one green cell within AMP islands are marked with single asterisks, single $Pros^{+}/GFP^{+}$ cells are marked with double asterisks and $Pros^{+}/GFP^{-}$ single cells or doublets are marked with arrow. Arrowheads indicate $Pros^{-}/GFP^{+}$ cells within the AMPs islands.**(B-C)** Hey UP enhancer is on in a subpopulation of Pros+ EE cells (B, B') and in one of the $Pros^{+}$ doublet cell within AMP islands (C, C'). **(D-E)**Hey 2+3 Su(H) - GFP enhancer is on in a subpopulation of $Pros^{+}$ EE cells (D, D') and in one of the $Pros^{+}$ doublet cell within AMP islands. There are $pros^{+}$ but GFP^{-} single cells or doublets within AMP island, as well (D-E). **(F)**NSH - GFP enhancer is on in a subpopulation of $Pros^{+}$ EE cells and in one of the $pros^{+}$ doublet cell within AMP islands. There are $Pros^{+}$ but GFP^{-} single cells or doublets within AMP islands, as well. This enhancer line shows a further slight expression in AMP in islands which are not $Pros^{+}$. **(G-H)**AmirG>nUGFP enhancer is extensively on in AMP islands. G and H present two different gut regions. **(I)**Hey INT2 – enhancer is on in one AMP island.

2.2 Functional analysis of Hey

The second aim of this study was to shed light on the functional role of Hey in the midgut development of *Drosophila*. For this purpose we studied both *hey* mutant embryos and larvae and we conducted Hey over-expression in different developmental windows monitoring and evaluating qualitative and qualitative impacts on midgut cell populations.

2.2.1 Loss- of- function analysis of Hey

We started by studying a *Hey mutant* strain (*Hey* $^{1a/6.2A}$*/ CyO*) in which *Hey* gene has been removed (Monastirioti et al., 2010). The deletion of hey causes lethality in *Hey*$^{1a/6.2A}$ homozygous individuals and the lethal phase was determined to be in late embryonic/early larval stage. Approximately 10%of the mutant individuals hatch, even if they may hatch 2 days after egg deposition, but most die within the first instar time window. However, some of them, referred as "escapers", manage to survive for more time, they have a retarded growth (Fig. 18C; Monastirioti et al., 2010.), they are less active than their wild-type (*Hey/+*) siblings and they never become pupae. We have seen escapers reaching the age of 7-11 days after larval hatching (ALH) with size similar of late L2/ early L3 instar larvae.

We first tested for the absence of Hey protein from our *Hey*$^{1a/6.2A}$ strain. Using our antisera, we were unable to detect any Hey protein in homozygous embryos, neither with immunostaining experiments (Fig. 17A-B), comparing with their wild-type siblings, nor by western blotting of embryo protein extracts (Fig. 17C). Subsequently, we generated an esg-GFP; *Hey*$^{1a/6.2A}$/CyO strain (see Material and Methods), in order to be able to follow the AMP population (Esg^{+}) as an Esg antibody was not available. EE cells were monitored by Pros staining and big EC nuclei by DAPI. No obvious defects in the mutant embryonic midgut were detected as homozygous embryos are similar to that in wild-type embryos, at least as far as the qualitative composition of the cells is concerned (Fig. 18A-B).

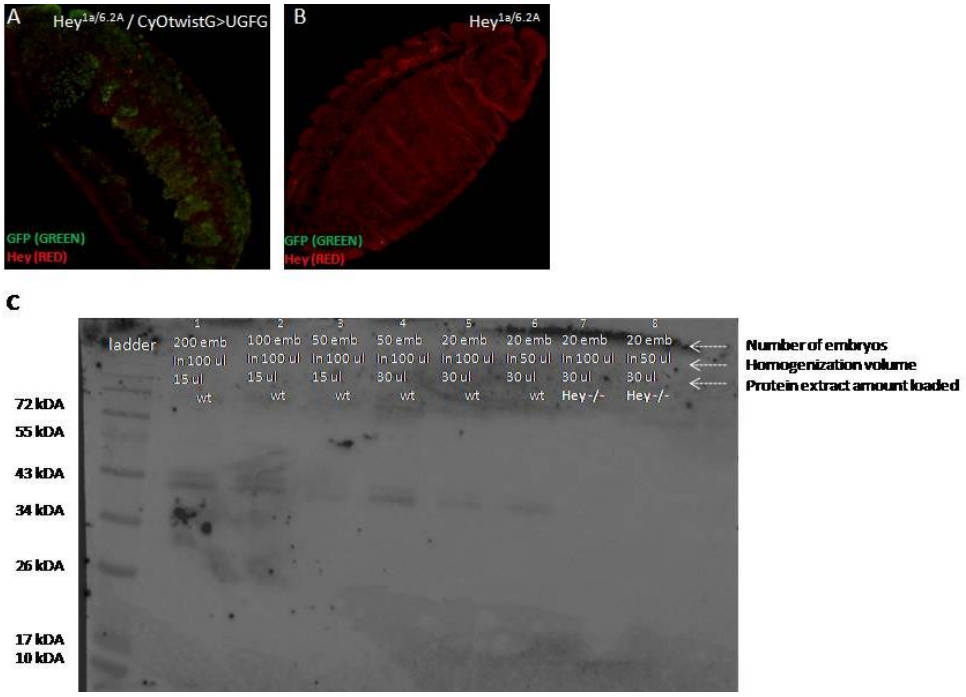
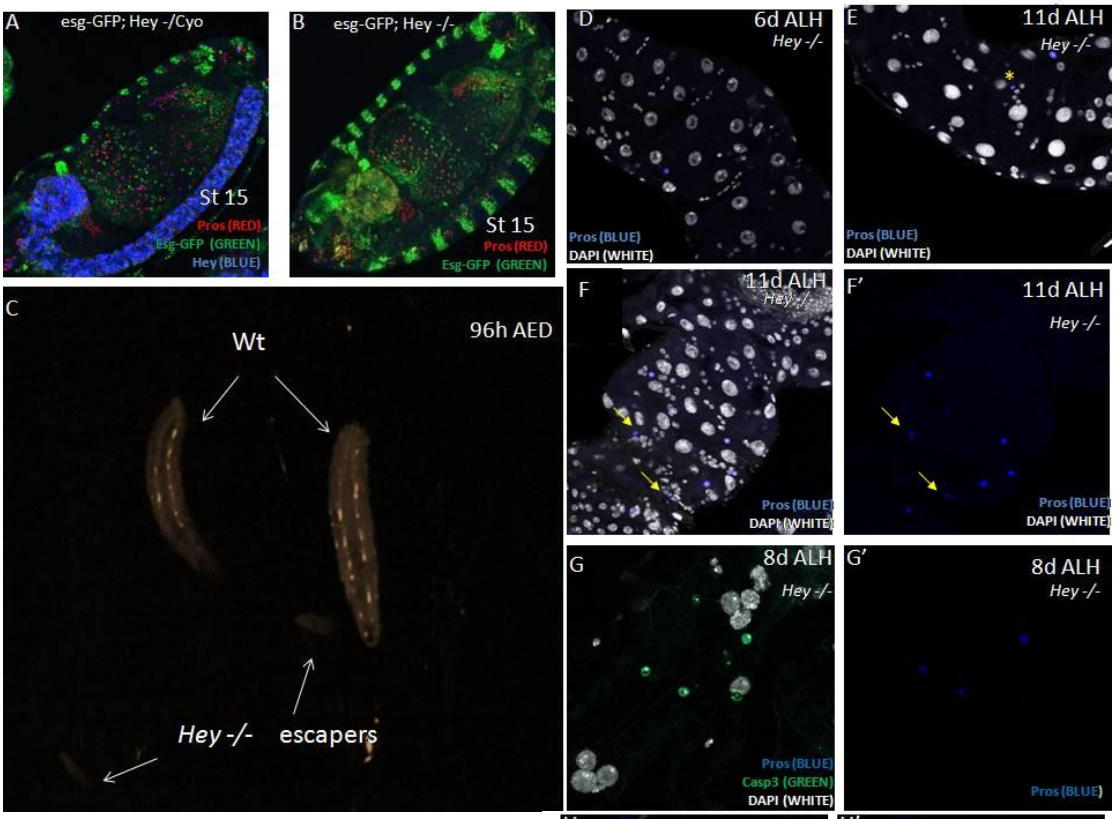


Figure 17.Verification of Hey mutant line 1a/6.2A / CyOtwistG>UGFG.(A-B) Image of two embryos stained for Hey (red) and GFP (green), which reveals *twistGAL4> UAS-GFP* from the balancer chromosome (*Hey+*) over which *Hey* $^{1.a/6.2}$is kept. The homozygous mutant embryo (B) identified by the absence of the *twistGAL4> UAS-GFP* pattern, does not express Hey in contrast to its Hey^{+} sibling (A). **(C)** Western blotting of embryo protein extracts of wild- type and *Hey* $^{1.a/6.2}$ embryos. Different embryos numbers, homogenization volumes used in protein extraction and protein extract volume loaded were tested in order to check the minimum number of embryos as well as the appropriate homogenization and loading volumes needed to detect Hey protein. In protein extracts of 20 embryos homogenized in 100 or 50 ul 1X SDS lysis buffer Hey protein can be detected (lanes 6 and 7), while in the corresponding samples of *Hey* $^{1.a/6.2}$ embryos not (lanes 7 and 8). *Hey* $^{1.a/6.2}$ embryos were selected from an embryo pool born by Hey$^{1a/6.2A}$ / CyOtwistG>UGFG strain against GFP signal. Ladder; color protein standard broad range (10-250 kDa). a-Hey antibody was use in final concentration 1:7000.

Since the mutant strain gives larva escapers we decided to study their developing midguts (Fig. 18D-H). Their midgut development was delayed but normal comparing the 6 days old larva (Fig.18D) with the 11 old one (Fig. 18E-F); all the three cell population existed, and the formation and proliferation of AMP islands proceeded normally through growing. Despite that, we should note that in the older escapers the EC nuclei look highly disorganized (Fig. 18G-H). We should point out, also, that even *Pros* expression emerge within the islands in the older mutant larvae (Fig. 18E), where Pros^{+} doublet are, also, able to be formed (Fig. 18F).

However, further counting of AMP islands and EE cells has shown important differences in the numbers of AMP islands and EE cells between *Hey*$^{-/-}$escapers compared to wild-type larval midguts (Fig. 18I). In wild type mL3 larval guts the number of AMP islands (representing the number of AMP cells generated during embryonic midgut development) were estimated 827 +- 55 (n= 4 guts), similar to what it has been previously published (Mathur et al., 2010) and the EE cells 112+- 20 (n= 4 guts). However, The AMP cell population of an 11-days old escaper has a 20% decline (653, n= 1 gut) while the EE population shows a tremendous decrease of 63% (41,5 , n= 2 guts), despite the fact that ee cell of a 2-days escaper were estimated 135 (AMPs in this stage were difficult to be distinguished). The staining with the apoptotic marker Caspase-3 revealed cell death in Pros^{+} EE cells (Fig. 18G), as well as, in AMPs (Fig. 18H), which could explain those defects. These

preliminary data imply a possible functional role of Hey in the maintenance and survival of AMPs and enteroendocrine cells. Further investigation is needed.



I

Larvae	AMP islands	EE cells
Wt (mL3)	827 ± 55	112 ± 20
2d escaper	-	135
6 d escaper	696	38
11 d escaper	653	45

Figure 18. The midgut of Hey mutants: embryos and "escapers" larvae. (A-B) Esg-GFP; Hey -/CyO line used for comparison between wild-type and Hey mutant embryos in stage 15, after staining for Escargot (GFP) (green), Prospero (red) and Hey (blue). No qualitative differences can be observed between wt (A) and mutant (B), as both $Esg^{+}/Pros^{-}$AMP and $Esg^{-}/Pros^{+}$ EE cells population exist and the formation of the midgut seems normal in the mutant. (C) Phenotypic comparison of wild type and *Hey -/-* "escapers" larvae. Even if only 10% of the Hey mutant larvae manage to born and hatch, they show great growth retardation and they never become pupae. **(D-H)** Escaper larval midgut of diverse ages stained for Prospero (blue), Casp3 (green). Nuclear staining with DAPI (white). Delayed but normal formation and proliferation of AMP islands through growing, comparing the 6 days old larva (D) with the 11 old one (E, F, F'). The populations of both $Pros^{+}$ EE cells and big polyploid ECs are present. Prospero expression turns on in AMPs island (E; asterisk), where Pros+ doublets are, also, able to be formed (F, F'; arrows). In an 8 old mutant larval gut, the apoptotic marker caspase-3 can stain some $Pros^{+}$EE cells

(G, G') and some small AMPs, singles or in doublets (H, H'; arrows).**(I)**The table presents the numbers of AMP islands and EE cells counted in wild-type mL3 larvae and in escaper larvae, indicating decreases of 20% and 63% in each population, respectively.

2.2.2 Gain- of- function analysis of Hey

For this type of analysis we used GAL4 lines to ectopically express and/or over-express Hey and study the effects in larval midguts. We first used aseGAL4 line crossed to a UAS-Hey line to drive over-expression of Hey. Asense (ase) is a bHLH transcription factor of the achaete-scute complex and a well-studied proneural gene in terms of nervous system development. Ase is, also expressed in the midgut in early embryonic stages 10-12 and particularly in AMP/EE progenitors to regulate their fate (Tepass and Hartenstein, 1995). More recent studies support the idea that *ase* expression promotes ee differentiation in the adult midgut (Bardin et al., 2010).

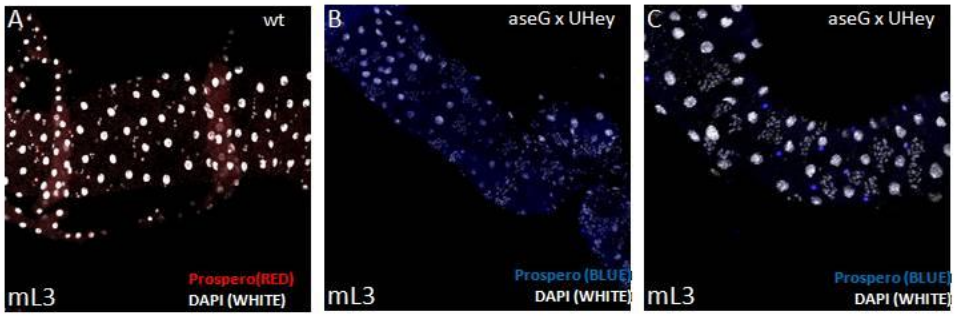
By expression of UAS-Hey by aseGAL4 driver and monitoring mL3 midguts we observed an extensive cell proliferation within the AMP islands(Fig. 19B-C), which is not consistent neither with the normal view of a wild type mL3 AMP island that includes 2-4 cells (Fig. 19A) nor with the dispersal observed around pupariation. Furthermore, by counting AMP islands (corresponding to AMP cells produced in embryo) and single EE cells we found an increase of 12.8% in AMPs (1,051, n= 1 gut) and of 63% in EE cells (183 cells, n= 1 gut) (Fig. 10D).

Furthermore, over-expression of H*ey* was conducted by using esgGAL4 > UGFP, $Gal80^{ts}$ driver.GAL4/$Gal80^{ts}$ system is based on a thermosensitive (ts) form of Gal80 inhibitor of GAL4 TF and it is used for spatiotemporal expression of genes of interest. Briefly, the Gal80 protein blocks the Gal4 function at 18 °C thus any UAS-dependent gene transcription. At 28 °C the Gal80 is unstable and the Gal4-dependent transcription can occur (McGuire et al., 2003). Escargot (esg) is a Snail-type transcription factor which is expressed in the early endoderm as it invaginates during embryogenesis (Takashima et al., 2011) and its expression is retained in AMPs during both larvae and pupal stages (Jiang and Edgar, 2009; Micchelli et al., 2011). In adult midgut Esg is expressed in intestinal stem cells (ISCs) and their transient undifferentiated daughters, enteroblasts (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Recent studies have proved that it is a critical stem cell determinant that maintains stemness by repressing differentiation promoting factors (Korzelius et al., 2014).

We first try to over-express hey in early embryonic stages 10-14 by shifting the temperature from 18 °C to 28°C but that caused lethality of the embryos, while few larvae that hatched, died in first/ second instar. Since *Esg* is expressed, also, in neuroectoderm derivates during this embryonic phase it is possible that the ectopic expression of Hey is deleterious. Thus, we decided to force *Hey* expression in mL3 larval stage, when AMPs have already formed islands consisting of 2-4 cells and a further proliferation would follow. Notably, a growth retardation effect was observed as pupae and wandering larvae appeared 5 days after the fly transfer in the permissive temperature which was investigated further. The gut of a wandering larva (laL3 stage) is presented in Figure 19E. The AMPs in which the driver is active are marked with GFP expression and surprisingly every Hey^{+} cell in AMP islands expresses

Prospero. Hence, this observation suggests that forced hey expression in the undifferentiated AMPs could promote their differentiation into EE.

Overall, the above functional analysis of *Hey* suggests that Hey involves with the stem cell and EE cells maintenance and survival, while it could be possibly sufficient to promote EE differentiation. However, since our data are from small number of samples have been supported individual observations further experiments should be performed so we can come to a safe conclusion.



D

Larvae	AMP islands	EE cells
Wt (mL3)	827 ± 55	112 ±20
aseGAL4 x UAS-Hey	1051	183

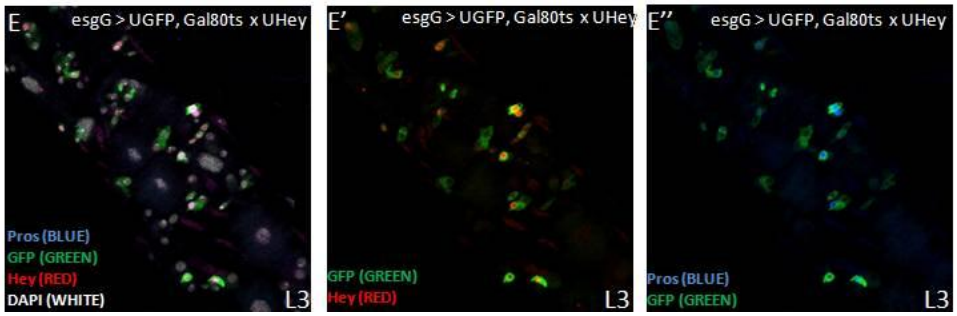


Figure 19.Gain-of-function analysis of Hey. (A-C) Comparison of mL3 larval guts between wild-type strain (A) and over-expression of Hey driven by aseGAL4 driver (B,C). In (A) Prospero (RED) staining and nuclear staining with DAPI (white).(B, C) Prospero (BLUE) staining and nuclear staining with DAPI (white). An excessive proliferation of AMPs within the islands is observed, which does not correspond with the mL3 instar (B, C).**(D)** The table presents the numbers of AMP islands and EE cells counted in wild-type mL3 larvae and in aseG x UAS-Hey larvae, indicating increases of 12.8% and 63% in each population respectively. **(E)** Ectopic/over-expression of Hey driven by esg> UGFP, Gal80ts system. The progeny were transferred in the permissive temperature, in early mL3 stage. The gut presented belongs to wandering larva and stained for the driver expression reporter GFP (green), Hey (red) and Prospero (blue). Every Hey^+ cell in AMP islands expresses, also, Prospero.

2.3 Deletion of the intronic enhancer region of *Hey* gene with CRISPR-Cas9 system

Previous lab members had initiated studying the regulation of *hey* expression. As mentioned before, two enhancers have been identified, one in the upstream region and another in the second intronic region of *Hey* gene. They showed that both enhancer lines generated are

expressed in nervous system (data not shown) as well as in midgut (Fig. 13A-F; Fig. 16B,I), recapitulating *Hey* expression pattern. Therefore, it has been decided to delete either /both of these regions in order to decipher the importance or a potential redundancy of the two enhancers

We first focus in the second intronic region of *hey* gene. 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. Ioanna Pitsidianaki, a former master student in our lab, had designed the two guide RNAs (Fig. 11) to target the 5' and 3' sites of the Hey second intronic sequence such as to maintain the branching point of the intron, without disturbing the intronic splicing process. A mix of DNA vectors containing the two gRNAs under U6 promoter was injected in fly embryos of the transgenic strain y M{nos-Cas9.P}ZH-2A w*, which expresses Cas9 during oogenesis, under control of nanos regulatory sequences (Port et al., 2014).

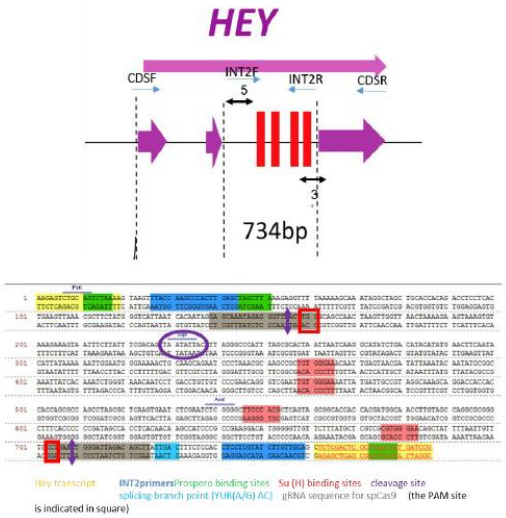


Figure 20. The two gRNAs were designed to target Cas9 at the 5' and 3' ends of the intron 2, without disturbing the splicing of the intron. The PCR primers (purple arrows) used for screening for deletion events were designed upstream and downstream of the gRNA target sites. *(Ioanna Pitsidianaki, MSc thesis)*

In order to establish a CRISPR line without the second intronic regions we followed the pipeline presented:

1. Injection of plasmids carrying gRNAs in very early embryos (0-3h) of nanosCas9 strain.
2. Transfer hatched larvae in vial with fly medium.
3. Cross injected adult flies individually with wild-type (yw67c23) flies
4. PCR screen a pool of 30 progeny (F1) flies and/or pupae of each cross of step3 for CRISPR PCR product.
5. Cross individually with yw, 10-15 F1 animals from each cross of step 3 that has given positive CRISPR event.
6. PCR Screen on F1 individuals after they have generated adequate number of offspring (F2)
7. Cross individually F2 progeny animals of CRISPR positive F1 (CR/+) with yw ; *Hey*$^{1a/6.2A}$ (w^{+})/ CyOwglacZ(w-) strain

[yw ; CR/+ (or yw; +/+) **x** yw ; *Hey*$^{1a/6.2A}$ (w^{+})/ CyOwglacZ(w-)

8. Screen F2 (yw ; CR/+ or +/+) individuals after generating adequate number of offspring (F3)
9. Selection against *Hey*$^{1a/6.2A}$ (w^{+}) phenotype and cross individually F3 (w-/CyO) animals from F2 CRISPR positive parent with yw ; *Hey*$^{1a/6.2A}$ (w^{+})/ CyOwglacZ(w-) strain.
   [yw ; CR/CyOwglacZ**x**yw ; *Hey*$^{1a/6.2A}$ (w^{+})/ CyOwglacZ **OR** yw ; +/CyOwglacZ **x** yw; *Hey*$^{1a/6.2A}$ (w^{+})/ CyOwglacZ]
10. Screen F3 individuals after generating adequate number of offspring (F4)
11. Selection of yw; CR/ CyOwglacZ F4 siblings and cross them in order to establish a CRISPR line.

Two sets of primers were used for the screenings. The first primer set used, CDSF (located at the start of *hey* coding region) and INT2Ron the 3' prime of intron 2, was upstream and downstream of the gRNA-targeting sites. This set gives a wild-type PCR product of 1650 bp and a shorter 1110 bp PCR product indicating CRISPR event (Fig. 20). The second primer set used was INT2 forward primer (INT2F), designed at the 5'end of the second intron of *Hey*, with the INT2 reverse primer (INT2R) which gives a wild-type PCR product of 734 bp and a shorter 200 bp PCR product indicating CRISPR event (Fig. 20).

We conducted two sequential injections of the plasmids carrying gRNAs. Totally, 199 larvae were finally born and only 58 of them gave adult flies, while 48 of them were fertile. Thus, 48 pools were first screened and 3 positive CRISPR events were detected, named as 15A, 21A, 10B (Fig. 21). CRISPR positive F1 individuals were detected in two of them (21A, 10B) (Fig. 13), with whom we continued the crosses in order to make HeyInt2-deleted lines (Fig. 14 and 15). The samples which were positive for Int2- mutated (CR) allele during the CRISPR line establishment procedure are summarized in Table 1. By now, we have established one line, named #21A.3.1.3, verifying the deletion event by both set of primers available (Fig 25). Sequencing of the second intronic region of *Hey* gene for further verification will follow. These flies have no viability problems and they are fertile in homozygosity. Interestingly, they can, also, complement the *Hey* null mutation. That suggests a redundancy of *Hey* enhancer elements. Therefore, we will investigate possible quality differences in *Drosophila* tissues in the near future.

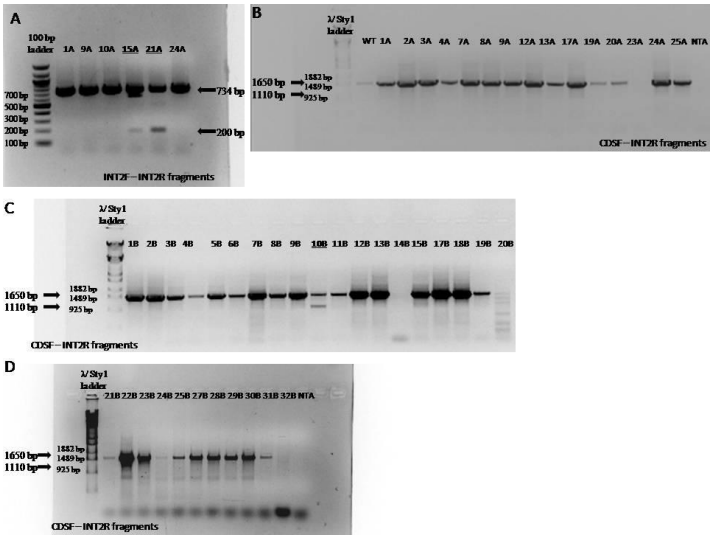


Figure 21.First screening of injected adult flies progeny pools [step 4].PCR of 18 samples of the first injection **(A-B) and of** 30 samples of the second injection round**(C-D)**. CRISPR event is evident in 15A, 21A samples in 10B sample. Screening using INT2F- INT2R set of primers gives a 734 bp PCR product of the wt allele and a 200 bp PCR product of the mutated allele. Screening using CDSF- INT2R set of primers gives a 1650 bp PCR product of the wt allele and a 1110 bp PCR product of the mutated allele. λ/ Sty1 ladder as marker. NTA: no template added.

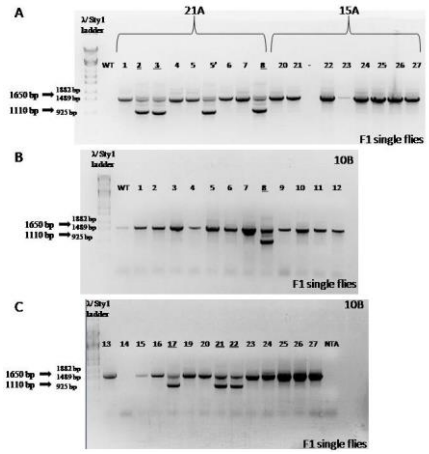


Figure 22. Screening of F1 individuals [step 6]. (A) F1 individuals of 21A and 15A (indicative) samples. **(B-C)** F1 individuals of 10B sample. Screening using CDSF- INT2R set of primers gives a 1650 bp PCR product of the wt allele and a 1110 bp PCR product of the mutated allele. λ/ Sty1 ladder as marker. NTA: no template added.

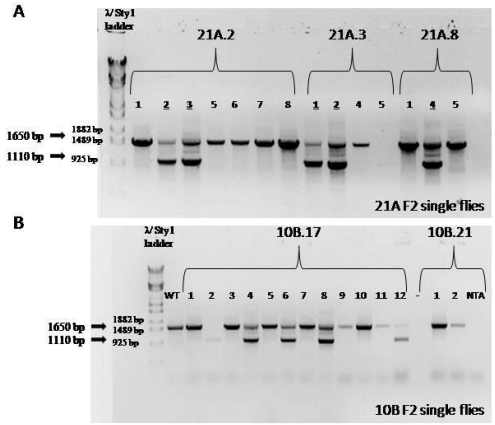


Figure 23. Screening of F2 individuals [step 8]. (A) F2 individuals of 21A sample. **(B)** F2 individuals of 10B sample. Screening using CDSF- INT2R set of primers gives a 1650 bp PCR product of the wt allele and a 1110 bp PCR product of the mutated allele. λ/ Sty1 ladder as marker. NTA: no template added.

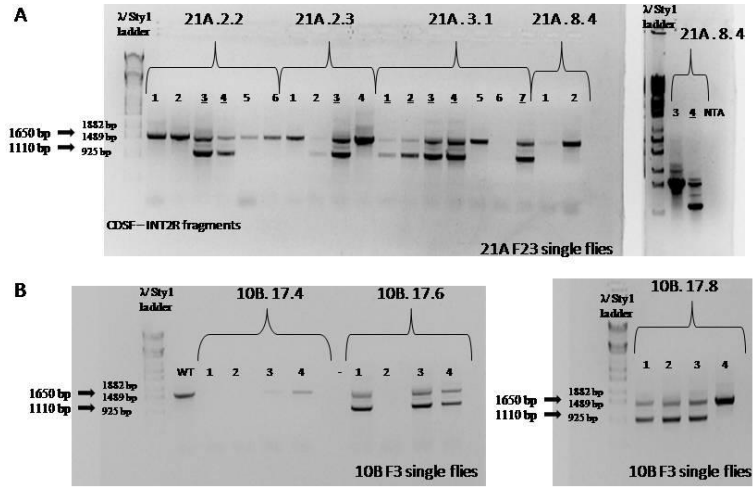
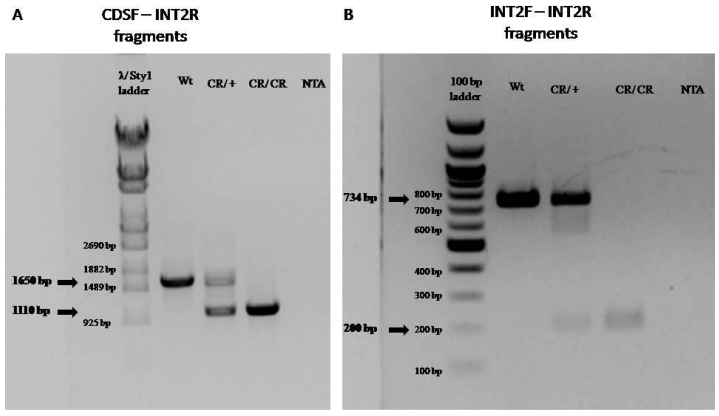


Figure 24. Screening of F3 individuals [step 10]. (A) F3 individuals of 21A sample. **(B)** F3 individuals of 10B sample. NTA: no template added.



CRISPR line – HeyInt2 deletion #21A.3.1.3 final screening

Figure 25.Final screening of #21A.3.1.3 HeyInt2-CRISPR line.(A) Screening using CDSF- INT2R set of primers. PCR product of the wt allele is 1650 bp, while the PCR product of the mutated allele (CR) is 1110 bp.**(B)** Screening using INT2F- INT2R set of primers. PCR product of the wt allele is 734 bp, while the PCR product of the mutated allele (CR) is 200 bp. NTA: no template added.

Table 1. Positive samples for Int2- mutated (CR) allele of the sequential screenings.

GENERATION	CRISPR positive samples				
P	21A 15A 10B				
F1	21A.2 21A.3 21A.8			10B.8 10B.17 10B.21	
F2	21A.2.2 21A.2.3	21A.3.1 21A.3.2	21A.8.4	10B.17.2 10B.17.4 10B.17.6 10B.17.8 10B.17.12	
F3	21A.2.2.3 21A.2.2.4 21A.2.3.2 21A.2.3.3	21A.3.1.1 21A.3.1.2 21A.3.1.3 21A.3.1.4 21A.3.1.7	21A.8.4.4	10B.17.6.1 10B.17.6.3 10B.17.6.4	10B.17.8.1 10B.17.8.2 10B.17.8.3

3. Discussion

In this study we analyzed the expression of the transcription factor Hey in the developing midgut of *Drosophila* and we performed experiments towards elucidation of its functional role in this tissue. In addition we applied CRISPR technology in order to delete one of the two putative regulatory regions of hey gene aiming to dissect the regulation of its expression in this context.

Hey proteins belong to the basic helix-loop-helix 'Orange' (bHLH-O) protein group and they are often effectors of the Notch signaling pathway. Although extensive studies about them have been done in mammalian (reviewed in Weber et al., 2014), little is known about the role of their single orthologue in *Drosophila* so far. More specifically, *Drosophila Hey* gene has been characterized as one of the alternative neuronal fate determinants in the developing CNS. Its expression and function in the majority of neuronal lineages is dependent on Notch signaling although few case of Notch independent expression have been described (Monastirioti et al., 2010). In the adult fly midgut, Hey has been described as guardian of the fully differentiated enterocytes maintenance independently of Notch (Brodsly et al., 2019).

Evidence from this study suggests that *Hey* expression in the developing midgut is Notch-dependent in accordance with its expression in a Notch- depended manner in the most areas of the developing CNS (Monastirioti et al., 2010). In embryos of a genetic background (Dl^- Ser^-) that abolishes Notch signaling *Hey* expression was not detected in the midgut primordia. This implies that its dependence on Notch signalling is developmental context- independent, although its roles in each context may be different. This could explain why we detect Hey mainly in EE population in embryonic and larval stages, while in adult fly Hey appears in ECs (Brodsly et al., 2019).

We detected Hey in the midgut primordia from around embryonic stage 13, few hours later than its first appearance in the CNS (Monastirioti et al., 2010), until the end of embryogenesis. Its expression appears mainly in a subpopulation of $Pros^+$ cells. Although Prospero is a transcription factor known as EE- determination factor in adult, larva and pupal fly midgut (Biteau et al., 2014; Zeng et al., 2015; Wang et al., 2015, Guo and Ohlstein, 2015), its precise role in the early formation of the midgut is not well-studied. Its expression starts at embryonic stage 11, when AMPs and EE cells have not formed yet two completely separated populations and that is why it is often referred as AMP marker, underlining the common origin of these two lineages (Oliver et al., 1993; Spana and Doe, 1995; Hiruta et al., 1995; Takashima et al., 2011). Thus, at the onset of Hey expression some of $Pros^+$ cells expressing Hey still express Esg as well (the undifferentiated state marker). Given that after stage 14 Hey gets restricted in a subpopulation of $Pros^+$ cells, now separated from Esg^+/ $Pros^-$AMPs, we can assume that its expression begins in a subpopulation of cells possibly committed to finally differentiate into EEs. However, it some rare cases Hey protein was also detected in Esg^+/ $Pros^-$cell sduring early embryogenesis, implying its involvement in AMP population development as well. Although we tried to follow the fate of these cells by the G-Trace experiment, our results were not conclusive and we need to reevaluate this hypothesis.

During larval life, Hey is detected again in a subpopulation of $Pros^+$ cells all over the midgut and in few ECs in some specific anterior and middle regions, reminiscent of its Notch-independent expression in the adult ECs (Brodsly et al., 2019). We should note that we could detect Hey protein with our serum only a few hours after hatching. Afterwards it can only be

detected as Hey-GFP fused protein form. This maybe is due to low expression levels of Hey in the larval stages, undetectable by the antibody used, but detectable thanks to the stability and robustness of GFP as a protein. Interestingly, in the very late larval stages *Hey* expression is also detected within some AMP islands in one of two $Pros^{+}$ cells. Doublets of $Pros^{+}$ AMP cells during late 3rd instar larva midgut has been described once before as "de novo generation of EE cells" at this developmental stage that also requires low levels of Notch signalling (Takashima et al., 2011). Our observations could render Hey as a possible player in this process.

Thus, considering *Hey* expression pattern, we can speculate that Hey is present in every wave of new EE generation but only in a subpopulation of them. Firstly, during embryonic stage 13 when EE cells are about to be fully separated from their Esg^{+} progenitor. Secondly, in the onset of larval life when according to Micchelli et al (2011)., a small Esg^{+}/ $Pros^{+}$ cell population is evident (Fig. 26A, B) possibly giving birth to newly formed EEs, since during L1 instar EE population shows a small further increase compared to embryonic/larval transition phase (Fig. 26C; Micchelli et al., 2011). The third wave of EE generation takes place in the late larval stages, when $Pros^{+}$ cells appear within AMP islands and Hey is present once again. Taking this into account, we could conclude that Hey is highly associated with the earliest steps of commitment to the EE fate in the developing midgut of *Drosophila*.

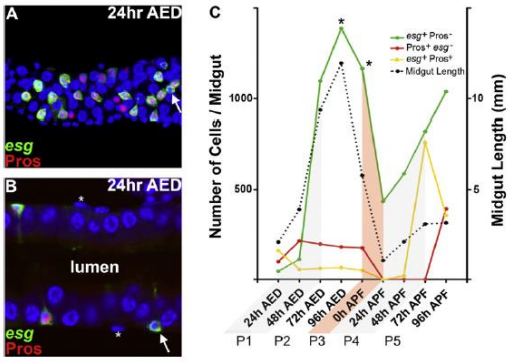


Figure 26 . Molecular markers define larval and pupal midgut cell types. **(A)** Three distinct populations of small cells can be defined in early first instar (24 h AED) esg> GFP midguts (anti-GFP, green; anti-Pros, red; DAPI, blue); $Esg^{+}Pros^{-}$, $Pros^{+}Esg^{-}$, $Esg^{+}Pros^{+}$. Arrow indicates $Esg^{+}Pros^{-}$ cell. Original magnification, 160_.**(B)** Small cells occupy a basal position in the early first instar midgut (arrow). Surrounding visceral muscle nuclei indicated with asterisk. Original magnification, 252_.**(C)** The dynamics of small cell subtypes from embryogenesis to adulthood in the midgut. Five phases (P1–5; shading) can be distinguished. Asterisks indicate the number of clusters, not single cells. AED; after egg deposition; APF; after puparium formation.*(Adapted from Micchelli et al., 2011).*

Data from our Hey functional analysis can support further this notion. *Mutant* escaper larvae, devoid of Hey protein, a couple of days after their hatching appear to have normal number of EE, consistent with the no obvious defect in the midgut of the corresponding mutant embryos .This could be explained by redundancy of Hey function in embryogenesis. However, older escapers display huge decrease in EE population caused by apoptosis. The developmental stage of these escapers, even with retarded growth rates, does not match with one close to histolysis and moreover given that even in the case of histolysis during pupariation the degeneration of the larval midgut requires activation of autophagy rather apoptosis (Denton et

al. 2009) we speculate a Hey requirement for the maintenance of this population. This hypothesis is further supported by the fact that Hey is expressed in a subpopulation of EE cells -probably in low levels- through larval life. A smaller decrease is observed at AMP population as well, giving a further indication of Hey involvement with this lineage too. However, we should note that the cause of the *Hey mutant*s high lethality rates is not known, thus the midgut defects of the escapers could be due to a systemic collapse. The more refined and informative experiment about possible defects in the midgut cell populations due to the absence of Hey would be the generation of labeled homozygous *Hey mutant* clones, coming from a single progenitor, within an otherwise unlabeled heterozygous animal, according to the genetic technique of Mosaic Analysis with a Repressible Cell Marker (MARCM) system (Tzumin Lee and Liqun Luo, 1999; Joy S Wu and Liqun Luo, 2006).

Our gain of function analysis supports further Hey association with EE and AMP lineages. Ectopic expression of *Hey* driven by an *asense (*ase*GAL4)* driver that labels AMP/EE progenitors causes a huge increase of EE population, a more mild increase of AMPs (counting them as respective islands in larval stage) and a remarkably early and extensive AMP cell proliferation within the AMP island. Even if it has been defined that *ase* is expressed in embryonic stages 11-12 in the AMP-/EE mixed population, its expression pattern in larva has not been studied (Brand et al., 1993; Tepass and Hartenstein, 1995). Thus, we should check the expression pattern of this driver through larval stages in order to take into account additional Hey over/ectopic-expression events. However, based on this first indication we could assume that expressing Hey earlier in embryogenesis favors these two fates.

Over-expressing *Hey* within the island has indicated an additional and more direct role of it. Ectopic expression of *Hey* in the AMP islands when they are in a proliferation phase (mL3), results in extended $Pros^{+}$ cell formation within the islands, reinforcing the idea that Hey could be a putative EE determinant. Since its involvement as fate determinant between two neuronal fate decisions (Monastirioti et al., 2010) has been already known, we suspect that Hey could have similar role in the midgut development as well. In the adult midgut it has been demonstrated that Hey represses the expression of the stem cell – related transcription factor Escargot via its M5-4 enhancer (Brodsly et al., 2019). Hence, we think our observations in the developing midgut context are worth of further investigation, since forced Hey expression seems to disturb the maintenance of stemness and to drive differentiation.

Another interesting question, raised by our gain of function experiments relates to the possible role of Hey as fate determinant downstream of Notch signaling. Does the increase of AMP and EE numbers when we use the ase-GAL4 driver happens at the expense of EC population during embryogenesis? It has been known that the Hey close related bHLH-O proteins coded by E(spl) complex via high Notch signaling have a major role in EC differentiation against AMP and EE one (Tepass and Hartenstein, 1995; Takashima et al., 2011). Therefore, we wonder whether Hey, as direct Notch effector, could have a corresponding role as determinant of AMP and EE fate via low Notch signaling, which is known to be required for the generation of those populations (Ohlstein and Spradling, 2007; Takashima et al., 201; Guo and Ohlstein; 2015).

An additional aspect of Notch involvement in EE development concerns their specification in subtypes that express different combinations of neuropeptides (Hartenstein et al., 2017; Micchelli and Evans, 2015). 10 different neuropeptides that belong to distinct families, such

as allatostatins, diuretic hormones and tachykinins have been described to be secreted from EE cells, in patterns characterized by regional and local diversity along the A/P axis of *Drosophila* midgut (Hartenstein et al., 2017; Micchelli and Evans, 2015). The "choice" of peptide is made, mostly, in the post- mitotic EE cells and it has been shown that among other factors bHLH transcription factors control this specification as well. Their balanced expression in different cells types depends notably on Notch pathway (Hartenstein et al., 2017). Therefore, given that Hey is a Notch effector and appears in a subtype of EE cells, it would be interesting to check whether its expression characterized one or more specific subtypes. Notably, we have detected tachykinin antibody staining in some of the larval Hey^{+} EE cells in one of our G-TRACE experiments (Fig. 27A) however additional experiments involving abs for additional neuropeptides will have to be performed

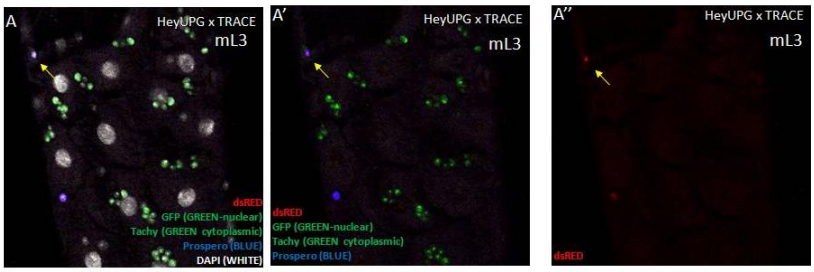


Figure 27.Tachykinin expression in mL3 larval midgut.(A) G-trace system was used to identify the activity pattern of Hey- UP enhancer in larval midgut. Real-time and lineage expression of Hey is observed by dsRed (red) and EGFP (green- nuclear) expression, respectively. Further staining with Prospero (blue) and Tachykinin (green-cytoplasmic) and nuclear staining with DAPI (white). Some of the Pros^{+} EE cells with real time expression of Hey express also the neuropeptide Tachykinin (arrow).

Overall, this study was the initiation of a project investigating possible new Notch events in the developing *Drosophila* midgut. Our observations support that Hey is associated with EE and probably AMP fates in both embryonic and larval life of the fly. Further functional analysis is needed in order to gain a better insight into its specific role in this context.

4 .Materials and Methods

4.1 Immunostaining of *Drosophila* tissue - GUT

Materials

PBT	1X PBS, 0.2% Triton, 0.5% BSA
PT	1X PBS, 0.2% Triton
DAPI	100 mg/ml in PBS (1000X) (here used dilution 1:100)
NPG- Glycerol (mounting medium)	2% n-propyl-gallate 49% glycerol and 49% PBS / pH=7.4 / store at 2-8 ˚C

Primary antibodies

Antibody	Final concentration
Rabbit a-GFP	1:10000
Mouse a-Prospero	1:100
Guinea pig a-Hey (GP1)	1:1000
Rabbit a- Caspase3 (Casp3)	1:500
Rabbit a- Tachykinin (Tk)	1:500

Secondary antibodies

Antibody	Final concentration
Goat a-mouse A555	1:2000
Donkey/ goat a-mouse A647	1:1000
Donkey a-rabbit A488	1:2000
Donkey- gp Cy5	1:400
Goat- gp A647	1:2000
Goat- gp A555	1:2000
Goat- gp A488	1:2000

1. Dissect tissues in glass plate in 1X PBS. Try to perform the dissection in a short amount of time (less than 20 min suggested) and collect dissected tissues on ice. Once you have dissected all material you need, proceed to step 2.
2. Fix with 4% FA in 1X PBS at RT for 20 min. You can perform the fix and all subsequent steps in glass plates or eppendorf tubes depending on the tissue. I use glass plates.

3. Remove fix and wash 3X with 1X PBS for 10 min.
4. Block and permeabilize tissue by incubating in PBT for at least 30 min at RT with mild shaking. My incubation usually lasts 1-2 hours. I cover the glass plates with a glass slide or parafilm in order to avoid evaporation.
5. Add primary antibody diluted in PBT. Incubate O/N at 4 ˚C. Keep it in a place in which the moisture can be maintained.
6. Wash 3X for 20 min with PT (1X PBS, 0.2% Triton) to remove excess antibody.
7. Incubate with appropriate secondary antibody diluted in PBT for 1-2 hours at RT or O/N at 4 ˚C in the dark to avoid photo bleaching.
8. Wash 3X with PT for 20 min. In the last wash add DAPI (here dilution 1:100, weak signal).
9. Quick wash with 1X PBS.

10. Mounting:

    a) Take the guts with a cut tip and place them on the slide.
    b) Lay them out carefully and remove any extra tissue attached.
    c) Remove the extra solution without letting them dry (!).
    d) Apply NPG.
    e) Cover with cover slip and seal with nail polish.

11. You can store your samples at 4 ˚C if you plan to take pictures in the next month. For long- term storage, store them at -20 ˚C.

4.2 Immunostaining of *Drosophila* embryos

PBT	1X PBS, 0.2% Triton, 0.5% BSA
PT	1X PBS, 0.2% Triton
NPG- Glycerol (mounting medium)	2% n-propyl-gallate 49% glycerol and 49% PBS / pH=7.4 / store at 2-8 ˚C

Primary antibodies

Antibody	Final concentration
Rabbit a-GFP	1:80000
Mouse a-Prospero	1:80
Guinea pig a-Hey (GP1)	1:8000
Mouse a- nubbin (Pdm1)	1:5
Rabbit a- βgal	1:8000

Secondary antibodies

Antibody	Final concentration
Goat a-mouse A555	1:2000
Donkey/ goat a-mouse A647	1:1000
Donkey a-rabbit A488	1:2000
Donkey- gp Cy5	1:400
Goat- gp A647	1:2000
Goat- gp A555	1:2000
Goat- gp A488	1:2000

STEP A. Embryo collection

1. Collect embryos on nytex. Wash well with distilled water till the yeast is washed off.
2. Dechorionate in 50% bleach for 3min. Wash well with distilled water.
3. Fix in 1:1 4%formaldehyde: Heptane in total volume 4ml at RT, performing 20 min vigorous shaking.
4. Remove aqueous layer (fix) LOWER. Important to remove as much fix as you can. Remove and discard embryos that have fallen into the aqueous layer.
5. Devitelinize: Add equal volume of MetOH to organic phase (heptane). Frappe for 45 sec min. Embryos should pop out of their vitelline membranes and fall to the bottom of the MeOH. The vitelline membranes themselves should remain at the interface.
6. Remove methanol and repeat steps 5 and 6 till the majority of the embryos are in the bottom.
7. Wash with MetOH (X3) until fix/heptane is removed (cleared).
8. Store in MetOH at -20˚C.

STEP B. Embryo Staining

1. Rehydration: Remove MetOH. Perform 3 gravity washes. The first one with PBS dropwise. Next two with PT. Perform one more wash 15 min, rocking.
2. Blocking in PBT or elsewhere for at least 1h, at RT, rocking.
3. Incubation with primary antibody diluted in PBT, O/N at 4 ˚C.
4. Washes 30 min in PBT (X3)
5. Incubation with secondary antibody diluted in PBT in dark at RT for 2-4h OR O/N at 4 ˚C.
6. Washes 30min in PBT (x3). If the incubation was O/N perform washes 1h each.
7. Final wash in PBS.
8. Leave the embryos in NPG for 30 min at RT OR O/N at 4 ˚C.
9. Mounting in NPG.

10. You can store your samples at 4 ◦C if you plan to take pictures in the next month. For long- term storage, store them at -20 ◦C.

4.3 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.

4.4 Larval Staging

Larvae of specified stage were obtained in the following manner.60mm petri dishes filled up with fly food and extra yeast extract were used for 2-3 hours egg collections of the desirable crossings. Then, the embryos were kept in 25 ◦C. The late L1 instar larvae were obtained after 48h AED. The late L2larvae were obtained after 72h AED. The middle L3 instar larvae were obtained after 96h AED. The late L3 instar "wandering" larvae were obtained after 108-119h AED.AED; After Egg Deposition.

4.5 Fly strains

Mutant strains:*eDf(3R)E(spl)b32.2/TM3* (Bloomington stock), *Hey* $^{1a.\ 6.2A}$*/CyO* (Monastirioti unpublished), *Dl e Ser 82B/ TM3 twistG> UGFP*(Bloomington stock)
For G-Trace analysis: Bloomington stock 28281-
For Over- expression experiments*: UAS- Hey 17A* (Monastirioti et al., 2010), *ase-GAL4, w; esgG> UGFP; Gal80*^{ts}
Hey reporter lines: *HeyUP-GFP* (M4m3) and *HeyINT2-GFP* (Ioanna Koltsaki, unpublished data), *Hey2+3Su(H)* lines and *HeyNSH* lines (Ioanna Pitsidianaki, unpublished data), *AmirG> UnGFP* (Brodsly et al., 2019).
For CRISPR: nos.Cas9 (Bloomington stock)
More:*w1118*(Bloomington stock), *Hey-GFP* (Kudron et al., 2018), esg-Gal4; UAS-mCD8GFP (Micchelli and Perriomon, 2006), *esg-GFP/ TM3 sb* (Sarov et al., 2016)

We generated *w; Hey* $^{1a.\ 6.2A}$*(w*$^{+}$*)/CyOwglacZ; esg-GFP/ TM3 sb* from *w; Hey* $^{1a.\ 6.2A}$*(w*$^{+}$*)/CyOwglacZ; MKRS sb/TM6 tb Hu* and *esg-GFP/ TM3 sb*

4.6 Genomic DNA extraction using DNAzol reagent

1. Grind flies in DNAzol. For more than 5 flies use 200 ul DNAzol. For single fly extraction use 50 ul DNAzol.
2. Centrifuge at 10000 rpm, 10 min at RT
3. Transfer supernatant into a new 1.5 ml eppendorf.
4. Add 250 ul 100% ethanol for more than 5 flies' extract OR 65 ul for 1 fly extract and mix well by repeatedly inverting tube.
5. Centrifuge at 13000 rpm, 20 min at RT
6. Discard supernatant.

7. Wash DNA pellet with around 1 ml 75% ethanol.
8. Centrifuge at 13000 rpm, 5 min at RT.
9. Discard supernatant, spin down.
10. Air- dry pellet.
11. Resuspend in 30-50 ul in TE buffer OR H_2O.
12. Store at -20 ◦C.

4.7 PCR protocols

	Reagents	Primers	Program	Product
PCRfor INT2 region (1)	DNA ~100 ng 10X Taq buffer with $MgCl_2$ (Minotech) 200 µM dNTPs (Invitrogen) 100 ng from each primer 0.2 µl Taq DNA polymerase (stock: 5u/µl) (Minotech) Final volume 20 µl	**CDS- Forward:** 5' CT GAGCTCA TGG ATC ACA ACA TGC ACG TCA ATG 3' **INT2- Reverse:** 5' GC GGTACC CTG CAA CAA GAT ACG AGG AGG 3'	1.94ºC, 2:30 min 2. 94ºC, 1:00 min 3. 60ºC, 1:00 min 4. 72 ºC, 2:00 sec 5. GoTo 2, 30 cycles 6. 72 ºC, 5 min 7. 12ºC, for ever	1650 bp
PCR for INT2 region (2)	DNA ~ 100 ng KAPA HiFi Buffer mix 5X KAPA dNTP Mix (10 mM each) 0.3 µM from each primer 0.5 µl KAPA HiFi DNA Polymerase (1 U/µL) Final volume 20 µl	**INT2F:** 5' CGC TCTAGA ATT ACC AAG CCC ACT TGA GC 3' **INT2- Reverse:** 5' GC GGTACC CTG CAA CAA GAT ACG AGG AGG 3'	1.95ºC, 5:00 min 2. 98ºC, 20 sec 3. 65ºC 15 sec 4. 72 ºC, 15 sec 5. GoTo 2, 30 cycles 6. 72 ºC, 5 min 7. 12ºC, for ever	734 bp

4.8 Gel extraction from agarose gel

Gel extraction from agarose gel was conducted according to **NucleoSpin®** Gel and PCR Clean-up protocol with the corresponding kits.

1. Excise DNA fragment / solubilize gel slice

Take a clean scalpel to excise the DNA fragment from
an agarose gel. Remove all excess agarose.

Determine the weight of the gel slice and transfer it to a clean tube. For each 100 mg of agarose gel < 2 % add200 µLBuffer NTI. For gels containing > 2 % agarose, double the volume of Buffer NTI.

Incubate sample for 5–10 min at 50 °C. Vortex the sample briefly every 2–3 min until the gel slice is completely dissolved!

2. Bind DNA

Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µLsample.

Centrifuge for 30 s at 11,000 x *g*. Discard flow-through and place the column back into the collection tube.

Load remaining sample if necessary and repeat thecentrifugation step.

3. Wash silica membrane

Add 700 µL Buffer NT3 to the NucleoSpin® Gel andPCR Clean-up Column. Centrifuge for 30 s at 11,000 x *g*.Discard flow-through and place the column back into thecollection tube.

4. Dry silica membrane

Centrifuge for 1 min at 11,000 x *g* to remove Buffer NT3completely. Make sure the spin column does not come incontact with the flow-through while removing it from thecentrifuge and the collection tube.

5. Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube. Add 15–30 µL Buffer NE and incubate at roomtemperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x *g*.

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer,heating to 70 °C and incubation for 5 min.

4.9 Proteins extraction from embryos

1. Collect dechorionated embryosin heptane.
2. Remove heptane and let embryos dry.
3. Freeze embryos in dry icefor few minutes.
4. Add 1X SDS lysis buffer and smash them using the pestle machine.
5. Boiling for 5 min 95°C.
6. Use an insulin needle to apply an extra mechanic force to shear the DNA and reduce the viscosity of the sample
7. Centrifuge for 15 min at full speed at 4°C.
8. Collect the supernatant and store it at -20°C.

4.10 Western Blot

1X Running buffer	25mM Tris 122 ml Glycine 0.1% SDS
1X Transfer buffer	25mM Tris 192 ml Glycine 0.01% SDS + 20% Methanol
1X TBS-T	25 mM Tris- HCl pH= 7.5 150 mM NaCl 0.05% Tween - 20
1st antibody	a-gp- Hey 1:7000
2nd antibody	Goat a-gp- HRP 1:10000

1. Gels were prepared by mixing acrylamide (30%) with the appropriate amount of separating/stacking gel buffers and polymerization was induced after the addition of 10% ammonium persulfate (APS) and TEMED® reagents. 1.5M Tris (pH 8.8) and 1M Tris (pH 6.8) were used as the buffers for the separating and the stacking phase of the gel, respectively.
2. Different volumes from protein samplesthat we wanted to test were used for western blot analysis (15-30 μl). Before samples' loading they were heated at 96ºC for 5 minutes, then spanned down and loaded onto polyacrilamide gel. For the indication of molecular weight of proteins, 7uL of color protein standard broad range (10-250 kDa)were used.
3. Electrophoresis of samples was performed at 80V while proteins moved through the stacking phase, and then at 100-120V in the separating phase of the gel. After approximately an hour, electrophoresis was stopped.
4. Proteins were transferred from gels to activated PVDF membranes (soaked previously in methanol for 1-2 minutes), in transfer buffer at a constant current of 400mA.
5. After transfer process, membranes were briefly washed in TBST buffer then blocked in 5% skimmed milk diluted in TBST for 1 hour.
6. Membranes were washed again in TBST and incubated with the primary antibodies overnight at 4 ºC. We used only gp-a-Hey antibody in final concentration 1:7000. Samples should be normalized using antibodies for housekeeping genes (e.g.β-tubulin).
7. The day after, membranes were washed X3 briefly in TBST (10 min each) and incubated at room temperature with the secondary antibody for 1 hour.
8. 3 brief washes (10min each) with TBST were performed to remove unbound secondary antibody molecules.
9. Incubation with chemiluminescent (ECL) peroxidase substrate for 1-3 min before detection.

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