UNIVERSITY OF CRETE
SCHOOL OF MEDICINE

The role of e(z) in intestinal stem cell homeostasis and tissue repair in Drosophila melanogaster

Master Dissertation

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

Ο ρόλος του επιγενετικού παράγοντα e(z) στην ομοιόσταση των εντερικών βλαστικών κυττάρων και την εντερική ανάπλαση της Drosophila melanogaster

Μεταπτυχιακή Διπλωματική

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‘Μέσα στη θλίψη της απέραντης μετριότητας, που μας πνίγει από παντού, παρηγοριέμαι ότι κάπου, σε κάποιο καμαράκι, κάποιοι πεισματάρηδες αγωνίζονται να εξουδετερώσουν τη φθορά.’

Οδυσσέας Ελύτης

ΠΡΟΛΟΓΟΣ

Η διπλωματική αυτή στάθηκε για μένα η ευκαιρία να εξερευνήσω για άλλη μια φόρα τον υπέροχα μυστήριο κόσμο της Μοριακής και Κυτταρικής Βιολογίας. Φτάνοντας στο πέρας και κοιτάζοντας πίσω, με ικανοποίηση διαπιστώνω πως τα δύο χρόνια που έζησα στην Κρήτη ήταν ίσως από τα πιο διδακτικά όχι μόνο για την πορεία μου στον επιστημονικό χώρο αλλά και τη ζωή μου.

Κατ' αρχήν θα ήθελα να ευχαριστήσω τον καθηγητή κ. Αριστείδη Ηλιόπουλο, ο οποίος ήταν ο κύριος επιβλέποντας της παρούσας εργασίας. Τον ευχαριστώ θερμά που με δέχθηκε στο εργαστήριό του και φυσικά για όλη του την βοήθεια προκειμένου να ολοκληρωθεί η μεταπτυχιακή διατριβή μου. Οι συμβουλές, οι επιστημονικές κατευθύνσεις του και οι προβληματισμοί που έθετε βοήθησαν στην ανάπτυξη της επιστημονικής μου σκέψης και θα είναι πολύτιμα εφόδια για την μετέπειτα πορεία μου. Θερμές ευχαριστίες θα ήθελα να δώσω και στον επίκουρο καθηγητή κ. Σωτήριο Καμπράνη που πρώτος μου πρότεινε να ασχοληθώ με αυτό το θέμα και για τη σημαντική βοήθεια του. Επίσης, θα ήθελα να ευχαριστήσω τον καθηγητή κ. Χρήστο Δελιδάκη για τις επιστημονικές παρεμβάσεις και συμβουλές του που διευκόλυναν κατά πολύ τη διεκπεραίωση της διπλωματικής μου.

Ένα ειλικρινές ευχαριστώ θα ήθελα, επίσης, να πω στη μεταδιδακτορική ερευνήτρια Ζωή Βενέτη, η βοήθεια της οποίας ήταν ανεκτίμητη. Την ευχαριστώ θερμά για τις συμβουλές της, την ορθή καθοδήγησή της, την εμπιστοσύνη που έδειξε στις ικανότητες μου και την ελευθερία με την οποία μου επέτρεπε να δουλεύω. Μαζί της μοιράστηκα ένα χρόνο καθημερινότητας γεμάτο εμπειρίες, ευχάριστες στιγμές, επιστημονικές επιτυχίες, αλλά και αποτυχίες, και διδακτικές συζητήσεις που θα θυμάμαι με νοσταλγία. Θα ήταν παράβλεψη μου να μην ευχαριστήσω όλα τα μέλη του εργαστηρίου, τη Δήμητρα, τη Μαρία, τη Νάντια, το Μιχάλη και την Κατερίνα καθώς πιστεύω ότι από όλους έμαθα κάτι που θα έχω να θυμάμαι.

Τέλος, ως ελάχιστη ένδειξη εκτίμησης και αγάπης, ευχαριστώ τους γονείς μου που πάντα στηρίζουν με χαρά τις προσπάθειες μου να βελτιώνομαι και να προοδεύω, και δεν παύουν να μου υπενθυμίζουν να κυνηγώ με αξιοπρέπεια αυτά που θεωρώ ουσιώδη στη ζωή.

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I. Summary

The intestinal epithelium represents the most vigorously renewing tissue in adult mammals and its proper function requires a balance between the removing of the dead cells and the producing of new cells of the tissue. Intestinal homeostasis is a procedure of high significance while impaired tissue regeneration leads to inflammatory disease and cancer. This replenishment of the lost cells is realized by intestinal cells that are responsible for both generating new enterocytes and the retaining of the stem cell pool. It has been proposed that intestinal stem cells found in the mammalian intestinal crypts are cells-of-origin of colorectal cancer. Furthermore, there is considerable evidence that genetic and environmental factors act in concert with epigenetic mechanisms to regulate stem cell dynamics. Since experimental manipulation of adult mammalian intestinal stem cells remains challenging, we use Drosophila as a model system to study the involvement of epigenetics in intestinal stem cell function. By performing a small scale in vivo RNAi screen we have identified the polycomb group gene, enhancer of zeste (e(z)), a homologue of mammalian EZH2, as a potent regulator of intestinal stem cells. Studying further its role in tissue homeostasis, we show that e(z) controls intestinal stem cell renewal capacity and may also impact the differentiation of transient progenitor cells, the enteroblasts.

Ι. ΠΕΡΙΛΗΨΗ

Το εντερικό επιθήλιο των ενήλικων θηλαστικών είναι ένας από τους ιστούς με την πιο έντονη και δυναμική αναπλαστική ικανότητα. Η διατήρηση του ισοζυγίου μεταξύ κυτταρικής απόπτωσης και αναγέννησης είναι καθοριστικής σημασίας για την ομαλή λειτουργία του εντέρου. Διαταραχές στην εντερική αναγέννηση συχνά έχουν ως αποτέλεσμα φλεγμονώδη νοσήματα, δυσπλασίες και καρκινογέννεση. Η ανανέωση των κυττάρων του εντέρου πραγματοποιείται από πολυδύναμα εντερικά κύτταρα (εντερικά βλαστικά κύτταρα) που είναι υπεύθυνα τόσο για την αντικατάσταση των νεκρών επιθηλιακών κυττάρων του εντέρου όσο και για την διατήρηση του ίδιου του πληθυσμού του των εντερικών βλαστοκυττάρων. Πρόσφατη βιβλιογραφία έχει δείξει ότι η δυναμική των βλαστικών κυττάρων ρυθμίζεται από γενετικούς και περιβαλλοντικούς παράγοντες που δρουν σε συνδυασμό με επιγενετικούς ρυθμιστές. Η χρήση του πειραματικού μοντέλου της Drosophila για την μελέτη της επιγενετικής ρύθμισης της λειτουργίας των εντερικών βλαστοκυττάρων αποφέρει πολλά πλεονεκτήματα, καθώς η απομόνωση και ο χειρισμός των εντερικών βλαστοκυττάρων στα θηλαστικά παραμένει δύσκολος. Προηγούμενη μελέτη του εργαστηρίου μας έχει δείξει ότι η μεθυλτρανσφεράση e(z), η οποία ανήκει στις πρωτεΐνες του polycomb group, ρυθμίζει την λειτουργία των εντερικών βλαστικών κυττάρων. Μελετώντας σε βάθος το ρόλο της στην ομοιόσταση του εντέρου, καταλήγουμε ότι η e(z) ελέγχει την αναγεννητική ικανότητα των βλαστικών κυττάρων του εντέρου καθώς επίσης υπάρχουν ενδείξεις ενδεχόμενης εμπλοκής της στη διαφοροποίηση των εντερικών προγονικών κυττάρων, των εντεροβλαστών.

II. Introduction

II.1 Intestinal homeostasis in mammals and Drosophila

II.1.1 The cellular organization of the adult intestinal epithelium

The organization of mammalian intestinal tissue is highly adapted to its functions, the digestion of food and the absorption of the containing nutrients. The intestinal epithelium forms finger-like structures, the villi, which maximize the digestive and absorptive surface area. Each villus is surrounded by tubular invaginations, the crypts of the Lieberkuhn, harbouring stem cells and progenitors in their base. The cellular organization slightly differs between the regions of the intestinal track with respect to the specific functional requirements. The duodenum, the proximal third of the small intestine closest to stomach, where the demands in absorptive area are increased, is characterized by long villi with high density of absorptive enterocytes that produce hydrolytic enzymes. Among the enterocytes, goblet and enteroendocrine cells also secrete mucus and hormones, respectively. In the base of the crypts along the intestinal track reside Paneth cells which produce antimicrobial substances and lysozyme. The epithelium contains at least three other cell types, cup cells, tuft cells and Peyer's patch-associated M cells the function of which is yet to be defined (Gerbe, Legraverend et al. 2012). The cellular organization is different in the colon, the posterior part of the intestine which specializes in compacting of the stool for rapid excretion. The colon is characterized by absence of villi and Paneth cells but increased number of crypts with goblet cells. All the cell types found in intestinal track are generated by multipotent stem cells and progenitors located in the base of each crypt.

II.1.2 The intestinal stem cells and regeneration

The intestinal epithelium represents the most rigorously renewing tissue in adult mammals. More than 300 million new epithelial cells must be generated daily in the small intestine in order to replace the dead cells, thus retaining the proper function of the tissue. The regeneration of the intestine occurs in the base of the crypts by small populations of adult stem cells that reside within specialized niches (Potten 1991). Analysis of genetic marker expression patterns has revealed that this type of cells exist since the first 2 weeks of life, exhibiting a rapid expan-

sion that leads to the establishment of a limited adult stem cells pool which is retained throughout life (Itzkovitz, Blat et al. 2012). These undifferentiated, mitotically active crypt base columnar cells (CBC) are distributed between Paneth cells in the base of the intestinal crypts and express specifically $Lgr5^+$ (Leu-rich repeat contain G protein-coupled receptor 5), a WNT target gene that has been established as intestinal stem cell marker (Barker, van Es et al. 2007). $Lgr5^+$ CBC cells divide to produce either cells that retain the multipotent identity or highly proliferative progenitors known as transit-amplifying (TA) cells. TA cells differentiate into various types of cells, enterocytes, globet cells, enteroendocrine cells that gradually migrate upwards the villi, exiting from the crypt (**Fig. 1**). This process which is completed in mice within 3-5 days, results in the necessary replacement of the dead cells and the proper renewal of the intestinal epithelium. It has been found that Paneth cells produce signals that regulate stem cell activity and fate, including WNT3, Notch signal in response to nutrient availability or other conditions (Sato, van Es et al. 2011, Yilmaz, Katajisto et al. 2012)

Moreover, in the region +4 of the crypt base, above the Paneth cells reside reverse cells that act upon damage conditions restoring intestinal stem cells pool (ISC) (Leblond and Walker 1956, Barker, van de Wetering et al. 2008, Potten, Gandara et al. 2009). These cells express Bmi1, a member of the Polycomb group gene family. Although $Bmi1^+$ cells have long term self renewal capacities and can give arise to all the cell types found within the intestinal epithelium similarly to $Lgr5^+$ cells, they reside only in the 10% of the intestinal crypts of the proximal 10cm murine intestinal epithelium and are absent in the ileum (Sangiorgi and Capecchi 2008, Yeung, Chia et al. 2011). Recent studies have shown that another reverse stem cells pool is present specifically in the colon. These $Krt19^+$ (keratin-19) cells are long-lived and able to restore the $Lgr5^+$ cell pool in the colonic epithelium (Asfaha, Hayakawa et al. 2015).

It has been demonstrated that Wnt and Notch signalling pathways are essential in the maintenance of the stemness and determination of the intestinal stem cell fate, respectively. Inhibition of Wnt pathway in the intestinal epithelium leads to absence of progenitors and stem cells whereas increased Wnt signal results in a tumorigenic phenotype due to accumulation of stem cells (Pinto, Gregorieff et al. 2003). Moreover, Notch acts in concert with Wnt signalling to control the fate of ISCs and their descendants (**Fig 2**). Depletion of active Notch signal in the intestine enforces stem cells to follow the secretory lineages (mainly goblet cells) whereas consecutive function of Notch pathway results in enterocyte generation (Koch, Lehal et al. 2013). It has been also found that simultaneous expression of both Wnt and Notch pathways blocks the differentiation in all the cell types of the epithelium, revealing that the appropriate

switches in the expression of these pathways are required for the proper intestinal cell fate determination (Wang and Hou 2010).

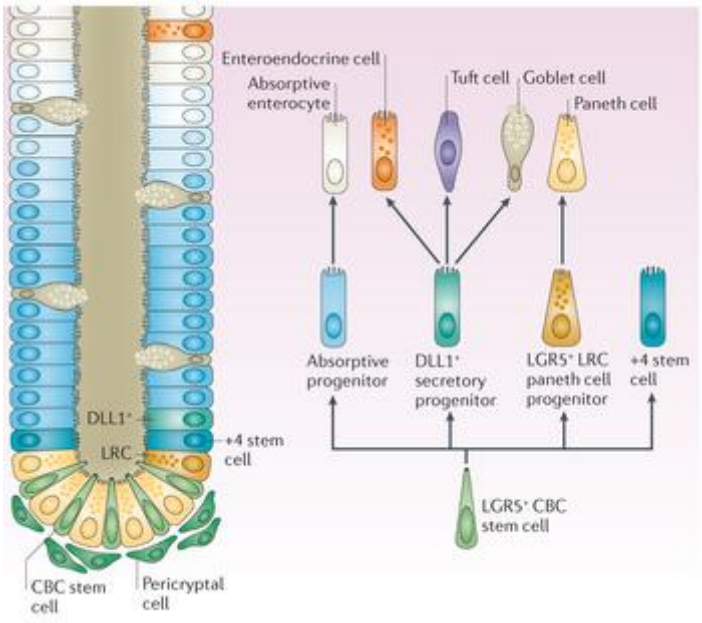

Fig 1. Cellular organization of the intestinal crypts (LEFT) and the multilineage differentiation potential of the Lgr5^{+} CBC stem cells (RIGHT). Lgr5^{+} cells reside in the base of the mammalian intestinal crypts and generate intestinal progenitor cells that differentiate into enterocytes, enteroendocrine, tuft, globlet and Paneth cells (Barker 2014).

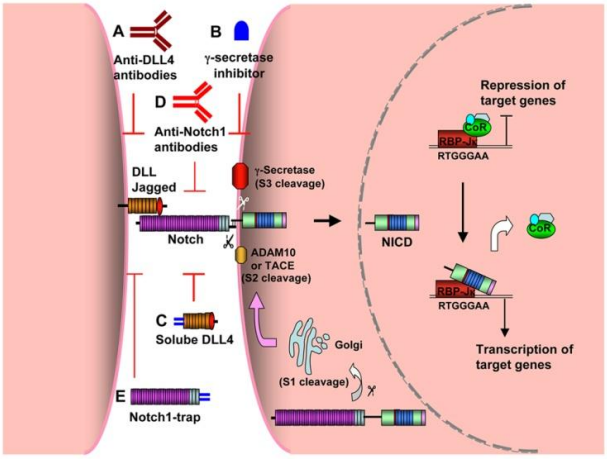

Fig 2. The Notch pathway. A precursor protein is formatted in the Golgi by cleavage (S1 cleavage) that results in the formation of the Notch receptor that is transported in cells surface, where it resides as a heterodimer. Two other proteolytic cleavages, stimulated by the Interaction of Notch receptors with Notch ligands, such as Delta-like or Jagged, S2 cleavage and S3 cleavage, release the Notch intracellular domain (NICD) from the cell membrane. NICD then translocates to the nucleus, where it interacts with the DNA-binding protein RBP-Jkappa and displaces corepressor proteins, thus activating the transcription of Notch target genes. Blockade of Notch signalling has been achieved by using different strategies, including (A) anti-DLL4 monoclonal antibodies, (B) gamma-secretase inhibitors such as DBZ and DAPT, (C) soluble DLL4-Fc, (D) anti-Notch1 neutralising antibodies, and (E) Notch1-trap (Li and Harris 2009).

II.1.3 Drosophila as a model to study intestinal homeostasis

Drosophila melanogaster is a species of fly in the family of Drosophilidae, widely known as the common fruit fly or vinegar fly. *D. melanogaster* has been extensively studied for almost 100 years while it comprises all the characteristics of an ideal model organism. First of all, Drosophila are small (2mm), easy to grow in the laboratory and their care and culture requires little equipment and space (Giacomotto and Segalat 2010). Moreover, it has short generation time approximately 10-12 days at room temperature and high fecundity, with more than 100 eggs to be laid daily from one female, thus facilitating a massive animal production (Greenspan 1997). Further, these small-sized animals require also small quantities of reagents, food and drugs, decreasing the cost of the investigation of the activity of various drugs in pathological situations (Heo, Lee et al. 2009). Based on these features, Drosophila is the most inexpensive experimental model organism compared to those of mice.

In addition, the short genome of 135.5 million bp of *D. melanogaster*, organized in only 4 chromosomes, makes this species very useful in genetic analysis. According to the sequencing of 2000, the genome of Drosophila contains around 15,682 genes with high overall similarity to those of humans. Interestingly, about 75% of the known human genes associated with disease have a recognisable match in the genome of fruit flies and 50% of fly protein sequence has mammalian homologs (Reiter, Potocki et al. 2001, Lloyd and Taylor 2010). It has also been demonstrated that crucial signalling pathways such as JNK, JAK-STAT, NFκB, Wnt/Wg, Notch, K-Ras/Ras1 that regulate immune responses and are associated with intestinal homeostasis and response to chronic inflammation, are preserved between flies and humans (Ohlstein and Spradling 2006, Ohlstein and Spradling 2007, Panayidou and Apidianakis 2013). For these reasons, Drosophila is being used as a genetic model for several human diseases like Parkinson, Alzheimer, diabetes, cancer e.g.

Furthermore, the extensive study of *D. melanogaster* over years resulted in the development of tools that allow an easy and precise gene manipulation of the organism. The generation of transgenic animals is a simple procedure in Drosophila in which the wanted genetic element is injected in gametic cells of flies' embryos and is successfully expressed in approximately 10-15% of the descendant generation. Another valuable tool established in Drosophila is the GAL4/UAS system. This two-component system allows directed gene expression in particular tissues. GAL4 is a modular protein consisting broadly of a DNA-binding domain and an activa-

tion domain and specifically binds UAS promoter. The GAL4 gene is placed under the control of a native gene promoter, or driver gene, while UAS controls the expression of the target gene. Thus, GAL4 is only expressed in cells where the driver gene is active and in turn GAL4 protein activates gene transcription where UAS has been introduced (**Fig. 3**). The expression patterns of the target genes can then be determined by fusing them with a gene encoding a visible marker like GFP (Duffy 2002, del Valle Rodriguez, Didiano et al. 2012).

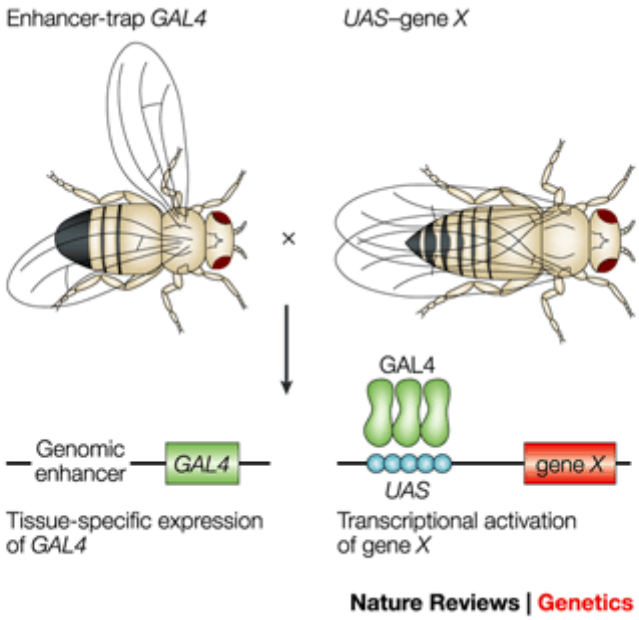

Fig 3. The two-component system GAL4-UAS consists of a transcriptional activator expressed in a specific pattern and a transgene under the control of a promoter that is silent in the absence of the activator (St Johnston 2002).

Mosaic analysis with a repressible cell marker system, MARCM, is another useful tool developed, based on GAL4/UAS system, in Drosophila. MARCM is a genetic technique for creating individually labelled homozygous cells in an otherwise heterozygous fly. It has been used το study the development of Drosophila nervous system but over the years its utility has become extended to other tissues such as the intestine for the identification of the cell lineages arising from the self renewing gastric stem cells (Lee and Luo 2001, Wu and Luo 2007). The technique relies on recombination during mitosis mediated but FLP-FRT recombination and the labelling of a small population of cells from a common progenitor using GAL4/UAS system. GAL4 is ubiquitously expressed in flies and GFP is placed under the control of UAS promoter. In addi-

tion, GAL80, an inhibitor of GAL4 is driven by strong promoter such a tubP. This tubP-GAL80 element is placed distal to an FRT site. A second FRT site is placed in trans to the GAL80 site, usually with a gene or mutation of interest distal to it. Finally, FLP recombinase is driven by an inducible promoter such as heat shock. When FLP transcription is induced, it will recombine the chromosomes at the 2 FRT sites in cells undergoing mitosis. These cells will divide into 2 homozygous daughter cells- one carrying both GAL80 elements, and one carrying none. The daughter cell lacking GAL80 will be labelled due to expression of the marker via the GAL4-UAS system. All subsequent daughter cells from this progenitor will also express the marker (del Valle Rodriguez, Didiano et al. 2012) (**Fig. 4**).

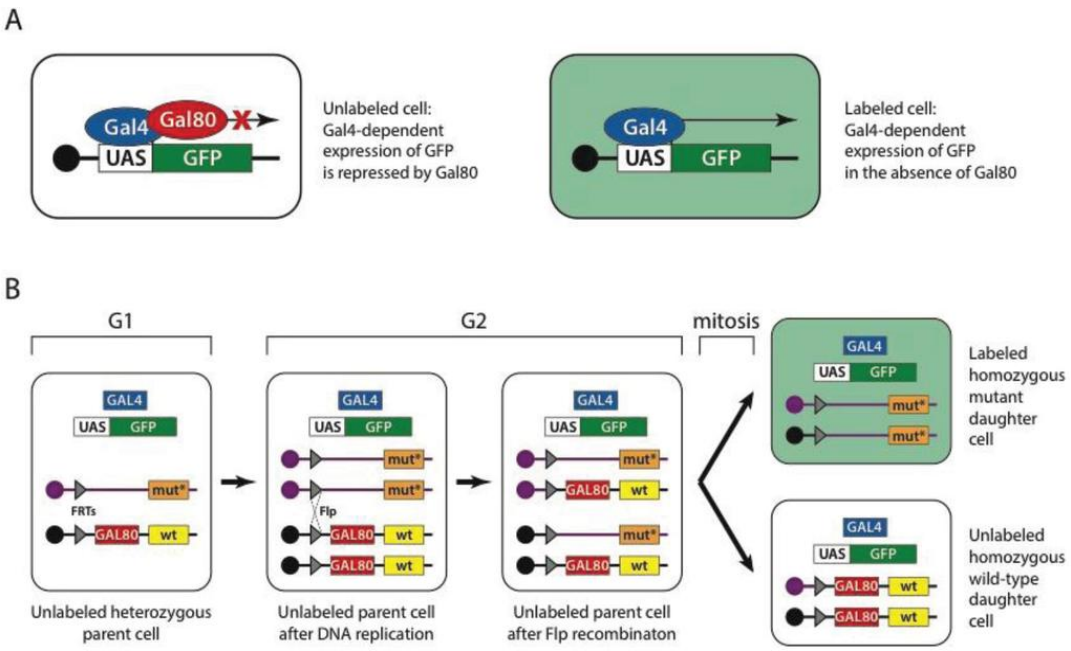

Fig 4. Mosaic analysis with a repressible cell marker (MARCM). (A) Gal4 transcription factor (blue oval) binds the activation sequence of UAS (white box), thus promoting the expression of green fluorescent protein (GFP) gene (green box). Gal80 (red oval) blocks this procedure by inactivating GAL4. As a result, when GAL80 is absent, GFP is expressed and marks the cell. (B) In MARCM, the GAL80 repressor gene (red box) is placed in a chromosome between a sequence that can be recognized by a flippase, the flippase recognition target (FRTs) (grey triangle) and the wild type sequence of the gene of interest (yellow box). The homologous chromosome lacks GAL80 but carries the FRT and a mutated form of the gene interest (orange box) in the same position as in the WT allele. These chromosomes coexist in the cells with the GAL4-UAS-GFP system. After DNA replication, in G2 phase, the flippase exhibits the recombination of the sequences that are surrounded by the FRTs, resulting in the formation of one chromatid that carries the WT allele and GAL80 and a second that has only the mutated allele. After mitosis and the separation of the chromatid alleles in the daughter cells, two different types of cells are generated in the same fly, the homozygous cells for the mutant allele that lack GAL8O and as a result express GFP and the homozygous WT cells where GAL80 blocks the expression of GFP (Ejsmont and Hassan 2014).

II.1.4 Drosophila life cycle

Drosophila melanogaster is a holometabolous insect where a larval and a pupal stage precede the adult stage of life. The adult flies live for more than 50 days in 18^{o}C and almost continuously lay 50-70 eggs daily. The eggs are laid half buried in the medium, carrying two respiratory filaments that facilitate gas exchange and hatch in 22-24h at 25^{o}C. The larval life time is divided into 3 larval stages. During the first two stages the larva are stationary in the medium while the third instar larvas climb upwards looking for more clean and dry conditions required for the pupation. The third instar larva molts into a stationary pupa after 30 hours. During the pupal stage, the larva is metamorphosing into the adult fly through a procedure that includes the lyses of the most larval structure although some organs are preserved such as the nervous system, fat bodies and gonads. The pupal stage lasts for 3-4 days, after which the adult fly emerges from the pupal case with a procedure called eclosion. Adult flies are sexually active within 6-8 hours but females do not lay eggs until 2 days after eclosion. Drosophila can live maximum 50-60 days in good conditions.

II.1.5 Cellular organisation of the intestine and ISCs in Drosophila

Although the majority of the studies of intestinal stem cells biology and gut renewal have been conducted in mammals, the mammalian intestine due to its complexity is not yet a convenient system for the investigation of gut responses under experimental or environmental challenges. From the other hand, Drosophila digestive tract characterized by flat architecture, simple cellular organization and high equivalence with the respect mammalian system, converts the fruit fly to an excellent model in the study of intestinal stem cells dynamics and tissue regeneration.

The equivalent of mammalian small intestine in Drosophila is the region of posterior midgut, defined as region R4, the main role of which is the absorption of nutrients and electrolytes. It is typified by large polyploid cells of the intestinal epithelium, the enterocytes (EC) (Buchon, Osman et al. 2013). Enterocytes comprise the most abundant cell population of Drosophila midgut and secrete digestive enzymes and produce antimicrobial peptides, resembling the Paneth cells of the mammalian intestinal epithelium (Santaolalla and Abreu 2012, Buchon, Osman et al. 2013). Apart from ECs, Drosophila midgut also carries secretory enteroendrocrine

cells (EE) that secrete hormones in response to luminal contents, precursor enteroblasts (EB) and multipotent intestinal stem cells (ISCs).

ISCs resemble the morphology of the mammalian $Lgr5^{+}$ CBC population and are equally distributed along the adult Drosophila midgut, located basally to the mature enterocytes. In Drosophila intestine, ISCs are the only cell type with proliferation capacity, being therefore responsible either for the proper regeneration of all the other intestinal cell populations and/or for the retaining of the intestinal stem cells pool (Micchelli and Perrimon 2006, Ohlstein and Spradling 2006). After an asymmetric division, ISC gives rise to one new ISC that retains the multipotent identity and one EB that is not able to divide but undergoes differentiation into EE or EC (**Fig. 5**). In contrast, an ISC symmetric division results in the generation of either two ISCs or two EBs, leading eventually to two ECs or two EE. Available evidence demonstrates that among various regulatory factors and signalling pathways, the orientation of ISC division is also significant for the determination of stem cell fate (Ohlstein and Spradling 2007, O'Brien, Soliman et al. 2011, de Navascues, Perdigoto et al. 2012, Goulas, Conder et al. 2012). Moreover, recent studies have shown that asymmetric divisions in midgut are twofold more than symmetric and the 90% percent of the newborn enteroblasts will differentiate into enterocytes (Micchelli and Perrimon 2006, Ohlstein and Spradling 2007). The decision between a symmetric or asymmetric division as well as the EB differentiation into ECs or EE is dependent on a complex of numerous interacting signalling pathways.

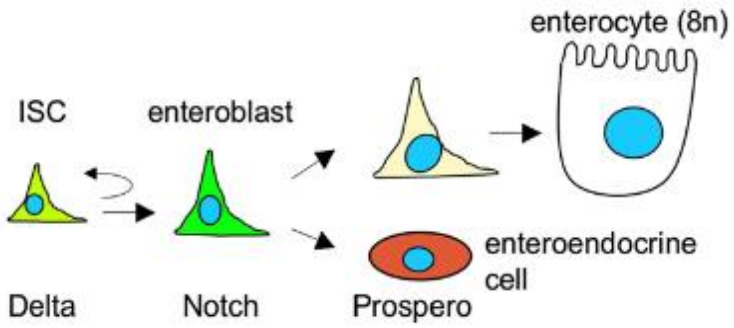

Fig 5. An illustration of the cell types and differentiation pathways in adult midgut of Drosophila. When an ISC divides asymmetrically, it generates an ISC daughter cell and an enteroblast. Enteroblasts differentiate into either enterocytes that undergo endoreplication or enteroendocrine cells (Amcheslavsky, Jiang et al. 2009).

II.1.6 Signalling pathways involved in ISC homeostasis

A variety of different signalling pathways, regulatory factors and environmental signals act in concert to control the process of initiation of ISC division to the final differentiation and the proper regeneration of the tissue.

ISC division is controlled by pathways that are activated by neighbouring cells or tissue. In particular, the enteroblasts and the enterocytes of the ISCs microenvironment stimulate ISC division by secreting ligands and cytokines which stimulate, EGFR and JAK/STAT signalling pathways. Activation of EGFR pathway results in the expression of the transcription factor, FOS, required for ISC survival (Biteau and Jasper 2011, Xu, Wang et al. 2011). It has also been found that the dividing ISC regulates its own division through Pvf2/Pvr activation (Bond and Foley 2012). Other mechanisms such as Wg/Wnt and the interaction of the proteins Integrin and Laminin A with the basement membrane are also required for the progression of ISC division (Lin, Xu et al. 2008, Lin, Zhang et al. 2013). Moreover, the neighbouring tissues and cellular structures such as the visceral mesoderm and the haemolymph are involved in the process of secreting signals to the dividing cells. In contrast, the activation of the pathways Hpo/Yki and BMP/TGFβ has an inhibitory effect on ISC division (**Fig. 6**) (Badouel and McNeill 2011, Vanuytsel, Senger et al. 2013).

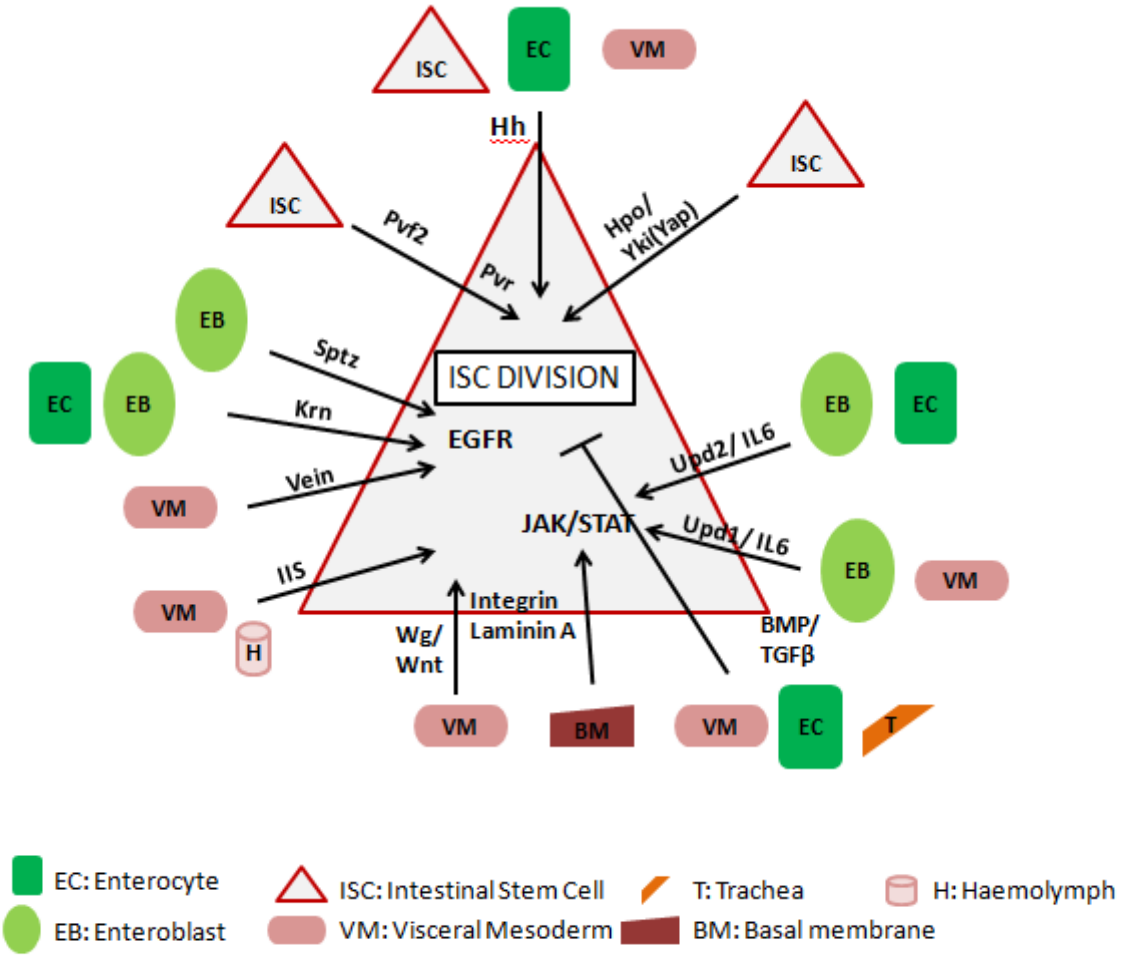

Fig 6. Control of ISC division. Various signalling pathways stimulated by many sources induce or inhibit ISC division.

Upon ISC division, the fate of the dividing cells is highly determined by the Notch signalling pathway which is active in the 'newborn' EBs. Notch acts by downregulating two transcription factors, Hairless and Daughterless required for ISC proliferation, thus inducing cell cycle arrest and promotion of differentiation into EC and EE (Bardin, Perdigoto et al. 2010). Notch is in turn regulated by delta expressed in ISCs, a transmembrane ligand of Notch receptor. Following an asymmetric division, delta expressed in ISC binds the Notch receptor of the EB and, through cleavage and nuclear mobilization, activates Notch target genes that block the expression of delta, leading the cells to an non-ISC phenotype (Perdigoto and Bardin 2013). Recently conducted studies suggest that the BMP pathway and Pvf2/Pvr, a homolog of the mammalian PDGF/VEGF in Drosophila, are also implicated in cell fate determination by acting antagonistically to Notch and sustaining stemness (Bond and Foley 2012, Tian and Jiang 2014).

The orientation of ISC division, symmetric or asymmetric, has also an important role in the determination of cell fate upon ISC division. Par complex and integrins are regulatory factors involved in this decision. During asymmetric ISC mitosis, Par complex is apically disturbed in the dividing cells, retaining the mitotic spindle vertical to the basement membrane while integrins keep the daughter ISC in touch with the membrane in the appropriate niche. Instead, the apical daughter cell moves away from the niche and commits differentiation. On the other hand, upon a symmetric division, Par complex forms parallel orientation of the spindle to the membrane by being equally distributed with the daughter cells (Goulas, Conder et al. 2012, Wolfenson, Lavelin et al. 2013, Scadden 2014).

Once the division is completed the daughter EB cell will follow the EC or EE lineage depending on the levels of Notch signalling. High levels of Notch signal are associated with differentiation into EC whereas low levels with differentiation into EE (Perdigoto and Bardin 2013). It has been proposed that the contact of EB with ISC increases Notch levels, forcing the EB to become EC. JAK/STAT signalling promotes differentiation towards EE by interacting with Notch. High levels of Notch reduce JAK/STAT whereas low levels of Notch are unable to inhibit the Upd-mediated activation of JAK/STAT, leading to EE generation (Lin, Xu et al. 2010, Liu, Singh et al. 2010). Finally, EGFR, BMP and the growth factor Dilp3 are essential for EC growth and EC endoreplication, respectively (Buchon, Broderick et al. 2010, Li, Zhang et al. 2013).

II.2 Chronic Intestinal Inflammation and Colorectal Cancer

II.2.1 Dextran Sodium Sulfate-induced gut pathology

The mammalian gastro-intestinal track is one of the most vulnerable tissues of the organism due to highly exposure to numerous bacteria and food or environmental toxins, thus increasing its susceptibility to develop inflammatory bowel disease (IBD). IBD includes a range of intestinal pathologies characterized by similar symptoms, such as diarrhea, blood in stools, abdominal pain and weight loss (Sands 2004). Ulcerative colitis (UC) and Crohn's disease (CD) are the most common IBD with their prevalence ranging from 10 to 70 per 100,000 people. UC and CD are histologically characterized by intestinal crypts reduction and deterioration, loss of tissue integrity and ulcerational infiltration of neutrophils, monocytes and lymphocytes. Although there is evidence that genetic and environmental factors are equally involved in the patho-

genesis of these diseases, the exact mechanisms underlying these pathologies remains unknown.

To date, various experimental models of colitis have been developed either by generating strains of animals susceptible to disease or by administration of chemicals that promote intestinal inflammation (Sartor, Bond et al. 1988, Morris, Beck et al. 1989, Okayasu, Hatakeyama et al. 1990, Kuhn, Lohler et al. 1993, Mombaerts, Mizoguchi et al. 1993, Sadlack, Merz et al. 1993, Mashimo, Wu et al. 1996, Bang and Lichtenberger 2016). Among of the chemical induced inflammation models, DSS (Dextran Sodium Sulfate)-induced colitis model is the most successful and widely used in the study of IBD because of its simple administration protocol (through the animals' drinking water) and its reproducibility in inducing intestinal pathology. DSS is a negatively charged, heparin-like polysaccharide with highly variable molecular weight from 5 to 1400 kD. DSS causes either acute or chronic inflammation depending on the molecular weight of DSS administered (Elson, Sartor et al. 1995).

DSS damages the colonic epithelial cells by forming nano-lipocomplexes with medium-chain length fatty acids (MCFAs), causing erosions and increased colonic epithelial permeability (Cooper, Murthy et al. 1993, Laroui, Sitaraman et al. 2012), thus allowing the infiltration of intestinal bacteria, neutrophils and mononuclear leukocytes into the underneath cellular layers, lamina propria and submucosa, similarly to human IBD. It has also been found that DSS administration provokes changes in the expression of tight junction proteins and increased expression of proinflammatory cytokines. In particular, upon DSS challenge the cytokines IL-4, IL-1a/b, IL-6, IL-8 and the major target of therapeutic approaches against IBD, TNFa, are upregulated indicating the association of DSS with colonic inflammation (Chassaing, Aitken et al. 2014). Regarding stem cells dynamics, it has been observed that in mice treated with DSS, the proliferation of progenitor cells is increased (Pull, Doherty et al. 2005).

The knowledge of the impact of DSS in the Drosophila intestine remains limited. Amcheslavsky et. al have elegantly demonstrated that Drosophila could present a simpler model of DSS-induced intestinal damage. DSS administration in Drosophila deregulates the basement membrane structure but has no additional effect on the overall gut morphology. Moreover, it has been found that upon DSS challenge the rates of intestinal stem cell divisions and the numbers of enteroblasts are increased. DSS also promotes accumulation of enteroblasts but blocks their differentiation into enterocytes (Amcheslavsky, Jiang et al. 2009).

II.2.2 Colitis associated colorectal cancer

Chronic inflammation represents a major pathological basis for tumor development and it is estimated that 25% of all cancers are associated with chronic inflammatory conditions caused by various environmental factors. It has been suggested that the link between inflammation and cancer arises from cytokines and other inflammatory mediators and reactive oxygen species that are produced in inflamed tissue which deregulate local immune responses and establish a microenviroment conductive of malignant transformation. (Mantovani, Allavena et al. 2008).

A representative example of inflammation- driven cancer is Colitis Associated Cancer (CAC), a subtype of Colorectal Cancer (CRC) highly associated with inflammatory bowel diseases. More than 20% of IBD patients develop CAC within 30 years of disease onset and almost 50% of these will die from CAC (Lakatos and Lakatos 2008). The exact mechanism by which colonic inflammation leads to tumorigenesis is still to be defined, but there is considerable evidence that within the localized inflammatory microenvironment of colitis, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced by activated inflammatory cells, induce mutations and epigenetic changes that transform normal colonic epithelial cells and stem cells into cancer cells (Meira, Bugni et al. 2008, Westbrook, Wei et al. 2009). Colorectal malignant cells mainly carry mutations associated with activation of Wnt signalling pathway or β-catenin, an intracellular signal transducer in the Wnt pathway as well as inactivation of APC, a β-catenin inhibitor, thus resulting in the early formation of intestinal adenomas (Korinek, Barker et al. 1997, Morin, Sparks et al. 1997, Bienz and Clevers 2000). Moreover, colorectal tumors are usually characterized by mutations in p53 gene caused by inflammation mediated oxidative stress (Choi, Yoon et al. 2002).

Interestingly, additional genetic aberrations are required for adenoma transition to carcinoma. The activation of the oncogenes K-ras and B-raf, Cyclooxyganese 2 (COX2), an enzyme essential in prostaglandin biosynthesis, as well as the inactivation of tumor suppressor TGF-β and DNA integrity checkpoint genes are also observed in early and late stages of colorectal tumors (Oshima, Dinchuk et al. 1996, Korinek, Barker et al. 1997, Takaku, Oshima et al. 1998, Gupta and Dubois 2001, Biswas, Chytil et al. 2004, Koehne and Dubois 2004, Grady and Carethers 2008) (**Fig. 7**).

In addition, several alterations in epigenetic modifiers and microRNAs have been implicated in CAC and CRC. It has thus been proposed that epigenetic events, being capable of altering both alleles of a tumor suppressor, may serve as a more possible and successful way of gene silencing compared to genetic mutations that require the contribution of two independent alteration events. DNA methyl transferases, Dnmt1 and Dnmt3 are upregulated during colorectal inflammatory and cancer conditions, reducing the expression of numerous target genes. The expression of EZH2, a member protein of Polycomb Repressive Complex 2 (PRC2), has also been found elevated in colorectal tumors making this protein frequent target of therapeutic strategies against several types of cancer.

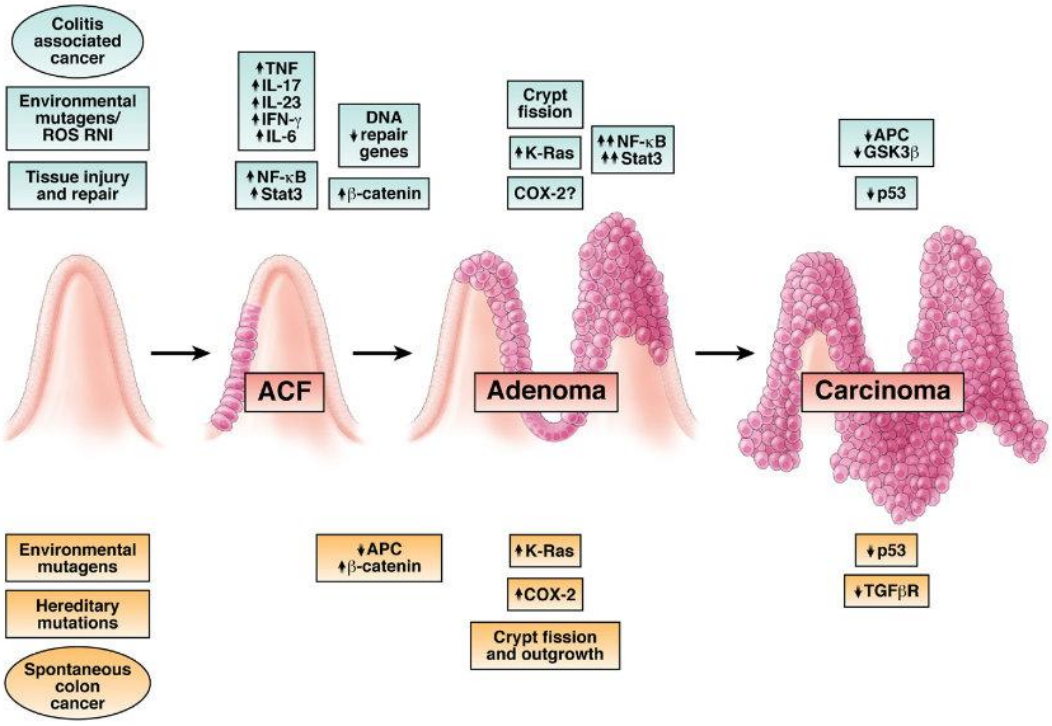

Fig 7. Mechanisms of colorectal cancer (CRC) and colitis-associated cancer (CAC) development. The hallmark of CRC development is the accumulation of mutations in tumor suppressor genes and oncogenes, resulting in abnormal function of catenin signalling pathway. Additional mutations in adenomatous polyposis coli (APC) lead to the formation of adenoma and carcinoma (Terzic, Grivennikov et al. 2010).

II.2.3 Stem cell association with colorectal cancer

Recent discoveries implicate intestinal stem cells with the development of colorectal tumors, assuming that mammalian crypt stem cells are the cells-of-origin of intestinal cancer. Barker et al. very elegantly revealed that deletion of APC in $Lgr5^+$ cells promotes their aberrant proliferation and transformation into cancer cells, resulting in formation adenomas (Barker, Ridgway et al. 2009). The upregulation of Wnt signalling pathway in $Lgr5^+$ and $Bmi1^+$ cells causes the same effects whereas its activation on progenitors and differentiated cells does not induce tumorigenesis (Sangiorgi and Capecchi 2008). Moreover, strong evidence for intestinal stem cell association with cancer development is provided by the high levels of Lgr5 in cancer stem cells and ISC that reside in colorectal tumors. In addition, Lgr5 levels are elevated in human metastatic colon cancer lines (Batlle, Henderson et al. 2002, Uchida, Yamazaki et al. 2010). All these findings support the hypothesis that colorectal cancers mainly occur due to mutations in intestinal stem cells.

II.3 Epigenetics and Polycomb repressive complex 2 in stem cells and cancer

II.3.1 Epigenetic regulation in adult stem cells

Epigenetics is the study of the heritable alterations in the gene expression that take place without changes to the underlying DNA sequence (Jaenisch and Bird 2003). These changes occur by modifications either on DNA bases and/or histones that alter the structure of the chromatin, influencing its accessibility to the transcriptional machinery. In particular for histones, this procedure is mediated mainly by methylation and acetylation of the key residues of core histone tails but also by ubiquitination, phosphorylation and sumoylation that act antagonistically or synergistically, regulating crucial biological contexts, such as X inactivation, development and cell fate determination (Kouzarides 2007). Moreover, it has been demonstrated that impaired function of the epigenetic modifiers, the enzymes that exhibit the histone and DNA modifications, results in the development of malignancies (Chen, Zhang et al. 2010).

A number of studies has uncovered important roles of epigenetic mechanisms in maintaining stem cells identity and activity. This epigenetic regulation changes the expression of several genes and the conduction of signalling pathways in multiple adult stem cell lineages.

For example, it has been revealed that wdr-5, a member of ASH-2 complex that exhibits trimethylation of histone H3 lysine 4 in *C. elegans* is required for the differentiation of germ stem cells (GSCs) and the proper gametogenesis (Li and Kelly 2011). Moreover, the H3K9 methyltransferase dG9a in Drosophila is essential for the asymmetric divisions of female GSCS and the formation of the oocytes (Lee, Yoon et al. 2010). Furthermore, deficiency of Jarid2, a recruiter of Polycomb Repressive Complex (PRC2) leads to reduced proliferation and delayed hair follicle cycling of HF-SC in mice.

Regarding intestinal stem cells dynamics, recent studies in Drosophila midgut support that several histone-modifying enzymes participate in stem cells-driven intestinal homeostasis. One of them is Scrawny (Scny) enzyme that exhibits gene silencing properties via deubiquitination of mono-ubiquitinated histone H2 (H2B). Mutations in Scny result in impaired activation of Notch pathway due to open chromatin formation, thus leading to a dramatically decreased numbers of ISCs (Lee, Yoon et al. 2010). Another epigenetic enzyme that regulates ISCs is the histone acetyltransferase HAT which causes alterations associated with gene activation. Overexpression of HAT stimulates rapid ISC differentiation whereas loss of HAT results in ISC increase (Ma, Chen et al. 2013). Moreover, SWI/SNF chromatin-remodeling complex is also required for ISC proliferation and damage-induced midgut regeneration in a lineage-specific manner (Zeng, Lin et al. 2013). Reduced histone acetylation caused by loss of the zinc-finger protein Charlatan led to severe ISC division defects (Amcheslavsky, Nie et al. 2014). Finally, expression of human methyl-CpG-binding protein-2, a bridge factor that links DNA methylation and histone modification, in Drosophila ECs, stimulated ISC proliferation (Lee, Kim et al. 2011).

II.3.2 Epigenetics and colorectal cancer

Although abundance of information about the role of gene mutations in tumorigenesis exists and the implication of genomic instability in the onset of cancer is well studied and widely accepted, the significance of epigenetics in cancer was until the past decade quite controversial. Feinberg and Vogelstein were the first 3 decades ago to reveal increased global hypomethylation in the genome of colonic cancer cells (Feinberg and Vogelstein 1983). This finding trig-

gered further investigation of the epigenetic patterns of tumors in several tissues, showing eventually that hypomethylation is associated with early genomic instability and that aberrant hypermethylation is mainly present in normally unmethylated CpG islands of tumor suppressor genes and promoters (Inamdar, Ehrlich et al. 1991). This hypermethylation takes place in the 1-10% of the CpG islands of the promoters of tumor suppressor genes such as CDKN2A, MLH1, CDH1 and VHL (**Fig. 8**) (Cunningham, Christensen et al. 1998, Grady, Willis et al. 2000, Baylin and Bestor 2002). Moreover, reduced expression of TET family DNA demethylases due to mutations are observed in a number of human cancers, including breast, liver, pancreatic cancer and leukemia, suggesting an essential role of DNA methylation in carcinogenesis (Yang, Liu et al. 2013). On the other hand, the histone demethylase LSD1 is overexpressed in pluripotent tumors such as teratocarcinoma and seminoma, revealing complex roles of epigenetic changes in cancer (Wang, Lu et al. 2011).

Regarding the contribution of epigenetic changes in the pathogenesis of colorectal cancer, numerous hypermethylated and silenced genes have been identified in adenomas as well as adenocarcinomas. Some examples are the APC gene, the proapoptotic protein RASSF1A, the Wnt antagonists SFRP1 and SFRP2 and the CRABP1 that promotes apoptosis (Lao and Grady 2011). However, there is no clear functional class of genes that seem to be more commonly affected by DNA methylation in the adenoma step of colon cancer development compared with other steps in the progression sequence (Oster, Thorsen et al. 2011). Moreover, it has been proposed that colorectal cancer is promoted by hypermethylation-mediated silencing or impaired function of DNA repair genes that leads to establishment of mutation in oncogenes such as K-RAS and in tumor suppressor genes (Estecio, Gharibyan et al. 2007). Finally, although hypermethylation is the most common epigenetic aberration in colorectal cancer, DNA hypomethylation and histone deacetylation also take place. For example, the metastasis-associated gene S100A4 and the oncogene R-RAS region both associated with colon cancer are found hypomethylated while loss of acetylation has also been reported as a hallmark of colorectal cancer (Nakamura and Takenaga 1998, Fraga, Ballestar et al. 2005, Nishigaki, Aoyagi et al. 2005).

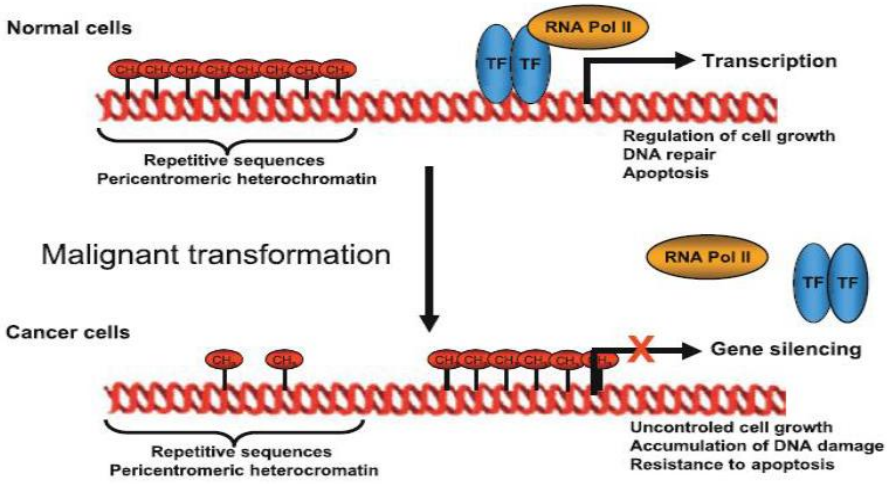

Fig 8. Epigenetic involvement in the development of cancer. Hypermethylation marks in the promoters of tumor suppressor, DNA repair, cell growth and apoptosis regulatory genes block the binding of the transcriptional machinery leading to gene silencing and malignant transformation.

II.3.3 Polycomb Repressive Complex 2 (PRC2) and Enhancer of Zeste H2 (EZH2)

Polycomb repressive Complexes (PrC) are epigenetic modifiers that promote chromatin condensation and higher order chromatin organization, thus resulting in transcriptional repression of several genes (Bantignies, Roure et al. 2011). The member proteins of the complexes were firstly identified in Drosophila as developmental regulators essential for the proper formation of the body axis. One of the best characterized PrC is PRC2 which is conserved in almost all species from flies to mammals, plants and nematodes (Margueron and Reinberg 2011). In mammals, PRC2 comprises EZH2, the catalytic core of the complex which contains a SET domain that exhibits the trimethylation of lysine 27 of histone 3 (H2K27me3), EED and SUZ12 that are required for the proper function of the complex in vivo and the protein RbAp48 that has been shown to contribute to the enzymatic activity of the complex at least in vitro (Margueron and Reinberg 2011). The respective homologues in Drosophila are named e(z) (Enhancer of zeste), Esc (Extra sex combs), Su(z)12 (Suppressor of zeste 12) and Nurf-55.

Te PRC2 complex is recruited in specific DNA stretches, the Polycomb Response Elements (PRE) and CpG-rich sequences in Drosophila and mammals respectively (Ku, Koche et al. 2008) and it has been proposed that this PRC2 recruitment is mediated by interaction of the complex with other proteins such as JARID2, AEBP2 and PCL1-3 as well as transcriptional factors or lncRNAs (Zhao, Sun et al. 2008, Di Croce and Helin 2013) (**Fig. 9**). Interestingly, the methylation mark of PRC2 acts as a recruiter of PRC1 complex, another member of PrC family that also represses

gene expression via H2AK118ub1. Apart from PRC1, it has been reported that there is a physical and functional cooperation between EZH2 and DNA methyltransferases (DNMTs) and histone deacetylases (HDACs). The proposed model is that the activity of HDACs is usually required for the acetylation of K27 in order to make ε-amino group of lysine side chains available for PRC2 binding. Moreover, EZH2 cooperates with DNMTs in order to enhance the methylation status and in turn modify the chromatin structure into a more compact form (van der Vlag and Otte 1999, Tie, Furuyama et al. 2001, Vire, Brenner et al. 2006).

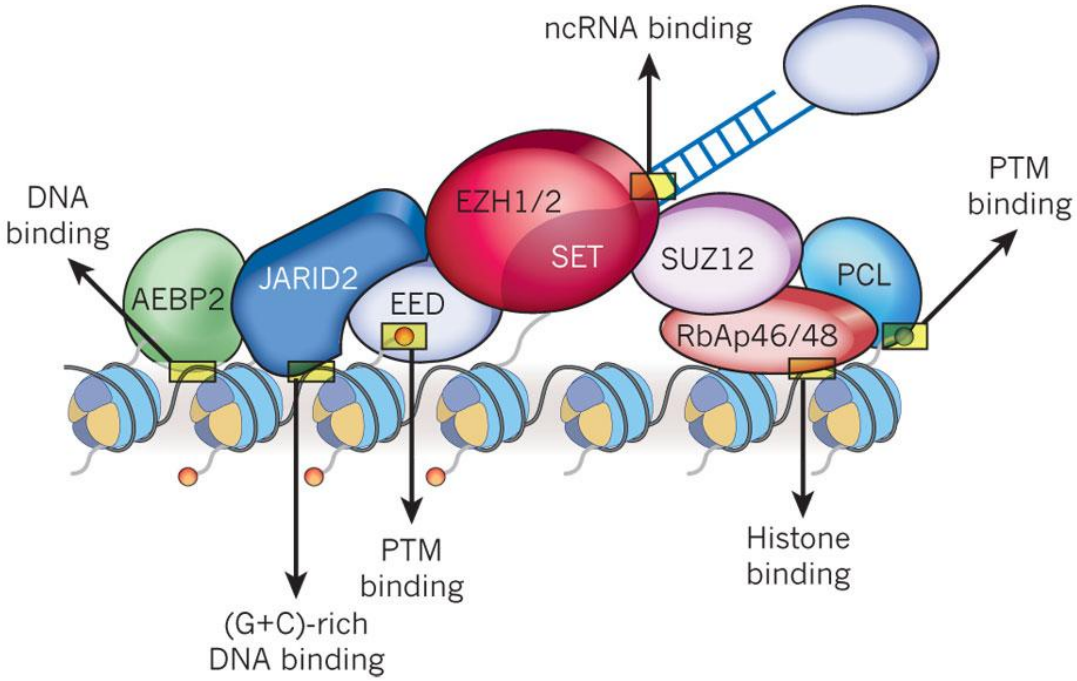

Fig 9. The structure of PRC2. PRC2 is consisted in mammals by EZH2, the catalytic core of the complex which contains a SET domain that exhibits the trimethylation of lysine 27 of histone 3 (H2K27me3), EED, SUZ12 and RbAp48. PCR2 recruitment is mediated by interaction of the complex with other proteins such as JARID2, AEBP2 AND PCL1-3 as well as transcriptional factors or lncRNAs (Margueron and Reinberg 2011).

II.3.4 PRC2 complex in stem cells and cancer

The role of PRC2 complex in retaining embryonic and tissue specific stem cell pluripotency has been widely studied for the past decades. Collectively, the plethora of these studies demonstrate important roles of chromatin repressive complexes in governing cell identity and retaining pluripotent state of ESCs. In particular, it has been revealed that mESCs knockout for PRC2 complex results in impaired function of pluripotent factors and abnormal differentiation (Pasini, Bracken et al. 2004, Chamberlain, Yee et al. 2008). The proposed explanation for this finding is that epigenetic modifiers increase the threshold of the signal required for altering the transcriptional program of the open-formatted chromatin of the mESCs, ensuring that strong signal will lead mESCs to differentiation (Lam, Steger et al. 2008). Moreover, PRC2 complex is required for the generation of iPSCs from human fibroblasts while PRC2 deficiency blocks the reprogramming of human B cells (Pereira, Piccolo et al. 2010, Onder, Kara et al. 2012). Regarding tissue stem cells, the significance of PRC2 complex was firstly identified by defects in neuronal formation of mice knockdown for JARID2, an interacting protein required for the recruitment of PRC2 in histones (Takeuchi, Yamazaki et al. 1995). Moreover, studies of PRC2 in hematopoietic system show that EZH2 is required for normal lymphopoiesis and HSC-self renewal during fetal liver hematopoiesis (Su, Basavaraj et al. 2003). In addition, PRC2 has been also found to be important for the proper function of epidermal, skeletal and cardiac stem cells as well as in the differentiation of neural stem cells to astrocytes (Hirabayashi, Suzki et al. 2009, Laugesen and Helin 2014). According to a suggested model delineating the involvement of PRC2 in lineage-committed cells, the complex regulates the chromatin switch from an open, aberrantly transcriptional active stage of stem cells to a compact, transcriptionally limited form that is required for the expression of tissue-specific genes upon differentiation.

Several studies have also been conducted regarding the role of PRC2 in intestinal stem cells. To date, the findings suggest that blockade of stem cell differentiation occur by PRC2 mediated H3K27me3 (Benoit, Lepage et al. 2012). Similarly, deficiency of PRC2 in intestinal crypt and colon cancer cells caused increased cell differentiation (Benoit, Lepage et al. 2012). However, further studies are needed for the identification of the molecular mechanisms by which PRC2 regulates cell fate determination in TA zone cells.

PrC gene expression has also been found deregulated in many types of malignancy including colorectal cancer (Benoit, Lepage et al. 2012). One described mechanism that elaborates the involvement of repressive complexes in tumorigenesis is that hypermethylation of INK4A-ARF-INK4B locus due to PrC overexpression, blocks the expression of the stress responders p14 (ARF), p15 (INK4B) and p16 (INK4A) (Bracken, Kleine-Kohlbrecher et al. 2007). Moreover, the majority of the types of cancers are characterized by EZH2 hyperactivation. This gain of function is caused by either EZH2 overexpression or mutation that increases its enzymatic activity. It has been revealed that MEK-ERK-ELK1 pathway, which frequently activated in cancers, is responsible for EZH2 overexpression by influencing its promoter (Volkel, Dupret et al. 2015). Mutations in EZH2 regulators such as mir-25, mir-30d, mir-98, mir-101, mir-137 and mir-214 also deregulate the activation of the methyltransferase (Volkel, Dupret et al. 2015). Moreover, the gain of function EZH2 mutation Y641 have been reported in the 22% of the diffuse B cell lymphoma and 7% of large follicular lymphomas (Guo, Ying et al. 2013). It has been suggested that this EZH2 overexpression promotes silencing of tumor suppressor genes and tumor angiogenesis and is associated with poor prognosis (Ohm, McGarvey et al. 2007, Lu, Han et al. 2010). In addition, cells overexpressing EZH2 form tumors when injected into mammary fat pads while overexpession of EZH2 in epithelial cells causes hyperplasia (Li, Gonzalez et al. 2009, Crea, Hurt et al. 2011). Moreover, there is evidence that polycomb repressive complexes regulate signalling pathways involved in the development of cancer stem cells (CSCs) such as Hedgehog, Wnt and Notch. CSCs derive from normal stem cells and form very aggressive and unharmed by therapeutic approaches types of cancer (**Fig. 10**). For these reasons, specific EZH2 inhibitors are attracting interest as potential anticancer drugs. Treatment with several compounds successfully decrease proliferation and increase apoptosis in lymphoma cells and rhabdoid tumors (Knutson, Wigle et al. 2012, Qi, Chan et al. 2012, Knutson, Warholic et al. 2013)

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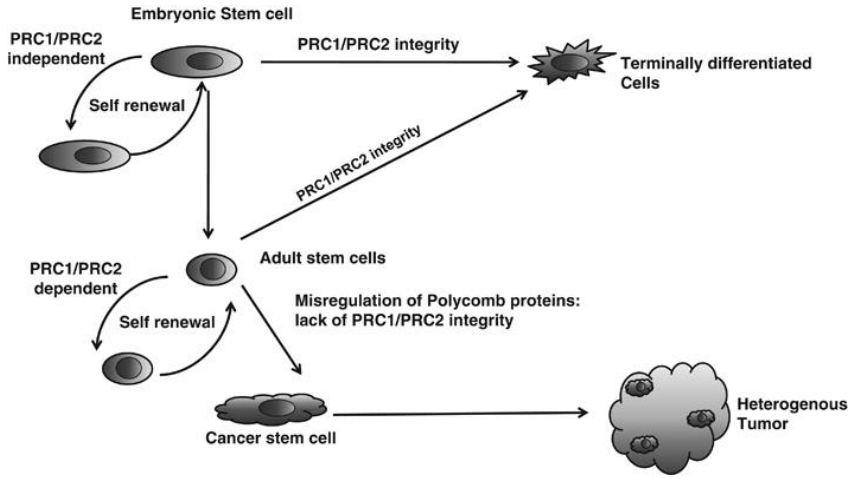

Fig 10. The role of Polycomb proteins in the development of cancer stem cells. Proper function of Polycomb complexes is essential for the self renewal and differentiation of adult stem cells. Misregulation of their levels can result in the generation of cancer stem cells (Richly, Aloia et al. 2011).

II.4 The aim of the study

The study of adult stem cell biology started almost four decades ago. The unique anatomy of the intestinal crypt epithelium makes it one of the most accessible models for the investigation of tissue stem cells dynamics. However, the stem cells that fuel the gut self-renewal process have only been recently identified. In 2007, Barker et. al. identified Lgr5 as putative marker of stem cells and provided evidence that these are the cells-of-origin of colorectal cancer. However, due to the complexity of the mammalian gut, little is still known about the roles of crypt stem cells in intestinal homeostasis and of epigenetic regulation of intestinal stem cells under normal and pathogenic conditions.

Moreover, chronic intestinal inflammation represents a major pathological basis for intestinal tumour formation and the onset of colitis-associated cancer, a type of colorectal cancer characterized by high mortality. Evidence accumulated during the past few years has indicated that epigenetic changes are strongly involved in cancer development and several genes have been found to be hypermethylated in CAC. One study suggested that polycomb group proteins might direct an aberrant inflammatory DNA methylation and histone signature that is largely maintained during tumorgenesis. In addition, previous work from our laboratory has indicated

that e(z), the catalytic core of PRC2 in Drosophila is a potential regulator of intestinal stem cells.

Drosophila is characterized by flat intestinal architecture, simple cellular organization with established markers for all the intestinal cell types and an intestinal system regulation high equivalent to that of mammals. Drosophila is thus a very convenient model for the study of epigenetic regulation of stem cell homeostasis. Moreover, Drosophila has been recently established as an organism for large-scale drug screening of stem cell tumors due to the ability to study stem cells in the context of their natural microenvironment.

On these premises, this study aims to decipher the role of e(z) in stem cell-mediated homeostasis of the gut in Drosophila and response to intestinal damage. The experimental approach will focus particularly on the investigation of the effect of e(z) on ISC proliferation, regenerative capacity and differentiation and the identification of specific e(z) target genes associated with ISCs dynamics.

III. Materials and methods

III.1 Drosophila stocks and crosses

In this study, the following fly strains were used: esg-GAL4 (w;esgGal4UASGFPGaL80^{ts} II), UAS-w1118:GFP (TransID w1118), UAS-e(z)RNAi 1(TransID 27645), UAS-e(z)RNAi 2(TransID 27646), that were obtained by Vienna Drosophila Research and MARCM2A (FTG/FM7;tubGal80FRT2A/TM6B), e(z)^{731}(w;e(z)^{731}FRT2A/TM6C,Sb,Tb) and 13:54 (yw,hsFLP,tubGal4, UAS-GFP) that were kind gifts from Christos Delidakis lab. All the stocks were remaining in 18 ^{O}C to decelerate aging and to restrict GAL4 activity. In order the binary GAL4/UAS expression system to be used, virgin female esg-GAL4 flies were collected and crossed with males, UAS-w1118:GFP (F1 esg-w1118), UAS-e(z):RNAi1 (F1 esg-e(z):RNAi 1) and UAS-e(z)RNAi 2 (F1 esg-e(z):RNAi 2). The crosses set up and cultured in 18 ^{O}C. F1 adult flies were then shifted to 29 ^{O}C to induce the transgene expression. esg-e(z)RNAi lines were used to drive GFP expression and RNAi mediated knockdown of e(z) specifically in ISCs and EBs, where esg driver is only activated, in the presence of a temperature-sensitive Gal4 repressor, tub-GAL8. Adult esg-GAL4 flies grown at the permissive temperature do not express GFP or RNAi in ISCs and EBs. Once shifted to the non-permissive temperature, RNAi is induced in these types of cells. UAS-w1118:GFP flies were used as controls and do not express the RNAi. All the flies were maintained on cornmeal-yeast-molasse-agar media (**Table 1**). Since the F1 was appeared, the flies were shifted in new bottles avoiding overpopulation.

For the MARCM analysis MARCM2A virgin females were crossed with 13:54 (F1 FRT2A) and e(z)^{731} males (F1 e(z)^{731}FRT2A. F1 flies were heat-shocked at 37°C for 45 min 7 and 12 days after eclosion. Midguts of heat-shocked flies were dissected and prepared for confocal microscopy.

Table1. The ingredients of Drosophila regular food

H_2O	50L
Agar	360g
Corn	3600g
Malt	3600g
Molasse	1200ml
Soya	440g
Yeast	32g
Acidic mix (propionic acid and orthophosphoric acid)	280ml (500ml+32ml)

III.2 Viability/Survival tests

For viability tests, we consistently used 20-30 young flies per viral. For phenotype induction, flies were kept for 4-7days in 29 OC. After RNAi induction, mixed sex adult flies aged 2-5 days were selected and cultured in an empty viral containing a piece of 2.5cm x 3.75cm chromatography paper (Whatman). 500µl of 5% sucrose (Fluka Analytical) solution in water was used to wet the paper with a syringe as feeding medium. The chemical that used in the feeding medium for induction of colitis phenotype was 3% of fresh Dextran sulphate sodium (Chembiotin CAS 9011-18-1) solution in 5% sucrose solution. Every day the dead flies of each viral where measured and the papers were doused with fresh sucrose or DSS solutions until the death of all the flies. The analysis of the survival curves was performed with the Graphpad Prism using the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test.

III.3 Immunostaining and Fluorescence Microscopy

For gut dissection, female flies were used routinely because of their bigger sizes but male flies were also used to check the phenotypes. The entire gastrointestinal track was pulled the posterior end in 1x PBS (Sigma-Aldrich cat.no P-3813) using pinches under stereoscope. The intestines were directly placed into fixation medium contained 4% Formaldehyde solution in 1x PBS (Polysciences). Guts were fixed in this medium for 30 min and then rinsed three times with 1 x

PBS. Blocking and permeabilization of the guts were then performed by incubating in PBT medium containing 1x PBS, 0,2% Triton X-100 (Merck4Biosciences Art11869), 0,5% BSA (Sigma-Aldrich A4503) for at least 20 min in room temperature without shaking. The following primary antibodies were used: anti-phospho-histone (PH3) (rabbit 1:1500 dilution) (Millipore 06-570), anti-Delta (monoclonal mouse 1:50 solution) (gift from C. Delidakis), anti-pdm1 (monoclonal mouse 1:5 solution) (gift from M. Monastirioti), anti-prospero (monoclonal mouse 1: 50 solution) (DSHB), anti-armadillo 1: 50 (DSHB) diluted in PBT. The incubation with the primary antibodies was performed by retaining the guts in 4 ^{O}C overnight. After incubation the guts were washed three times for 5-10min each with PT solution containing 1x PBS and 0,2% Triton X-100 to remove the excess antibody. Secondary antibodies were used in 1:2000 dilution in PT solution, anti-rabbit IgG (Cell Signaling Technology 7074S) conjugated to Alexa Flyor dye 555 and anti-mouse Alexa Flyor dye 555 (Invitrogen A21424), and incubated with the guts for 2 hours in room temperature in the dark. Guts were then again washed three times 5-10min each with PT and placed carefully in slides covered by mounting medium with DAPI (VectashieId H-1200). In some cases, before this step guts were incubated for 5 min in TO-PRO solution (dilution 1:1500) (Thermofisher, T3605) (gift from D. Karagogeos). The slides were stabilized with nail polish and observed in Nikon Spinning Disk confocal microscope where the images were also taken. The slide can be maintained in 4 ^{O}C for about 6 months.

III.4 Apoptosis staining

We used the ApopTag Red in situ apoptosis detection kit (EMD Millipore S7165) to detect the occurrence of cell death in female guts. We used flies that had been maintained at least 2 days in 29 ^{O}C for the transgene induction and fed 2 days with sucrose solution in order their guts to be empty of food. The dissections were performed in 1x PBS and the intestines were fixed in 1 x PBS and 4% Formaldehyde solution for 30 min. After fixation, they were rinsed three times with 1x PBS and washed with PT containing PBS; 10 mM NaH_2PO_4/$NaHPO_4$, 175 mM NaCl, pH 7.4, and 0.1% Triton X-100) for 30 min at room temperature. Intestines were washed twice for 5 min at room temperature in equilibration buffer and then incubated for 1h at 37 ^{O}C in 110µl of TdT solution, according to the manufacturer's recommendations (77µl Reaction Buffer; 33µl TdT enzyme). Reactions were stopped by washing 10 min in stop/wash solution in water (1:35 dilution), and intestines were rinsed with PBT and incubated in warmed working strength Anti-

digoxigenin Conjugate (rhodamine) solution (45µl Blocking solution; 41µl Anti-Digoxigenin) for 30min at room temperature in the dark. Then the intestines were washed four times with PBS 2 min each and placed carefully in slides covered by mounting medium with DAPI. The slides were viewed in fluorescence microscope using rhodamine excitation and emission filter.

III.5 RNA extraction

For the RNAi extraction the Arcturus Picopure RNA isolation kit (Applied Biosystems 12204-01) was used in combination with the protocol established by Dutta et al. (Dutta, Xiang et al. 2013). 60-100 midguts of flies about 7 days in 29 ^{O}C for knockdown induction were dissected in 1x PBS- DEPC 0,1% v/v for avoiding of contamination and resuspended in 100 µl elastase (Sigma E0258) solution of dissociation Buffer (Sigma C5914) (1:4 dilution). Incubation was followed for 1h in 27 ^{O}C until the total dissolution of the tissue into cells. The cells were then centrifuged at 0.8xg for 20min and the pellet was resuspended in 500µl 1x PBS-DEPC 0,1% v/v. The GFP+ cells were then isolated with Fluorescence-activated cell sorting assay (FACS) immediately 500µl of extraction buffer and incubated in 42 ^{O}C for 1h. Centrifuge was followed at 3000xg for 4 min in 4 ^{O}C and the supernatant was added in new tube with equal volume of 70% ethanol. Precondition of the column was performed by adding 250µl Conditioning buffer onto the column and incubation for 5 min in RT and centrifuge at 16000xg for 1 min. The mix of the RNA/Ethanol was added in the preconditioned column and centrifuged at 100xg for 2 min and directly after at 16000xg for 30 sec. Then 100µl Wash buffer 1 was added in the column and centrifuge at 8000xg for 1 min took place and 100µl Wash buffer 2 following by the same centrifuge. Another 100µl of Wash buffer 2 were added in the column and 2 centrifuges at 16000xg for 2min were followed. After that the column was placed in a new tube provided by the kit, 12 µl of Elution buffer were added and incubated for 1min in RT and centrifuged at 16000xg for 1min. The isolated RNA was measured in Nanodrop Spectrophotometer and stored at -80 ^{O}C.

III.6 cDNA synthesis and Real Time PCR

A High-Capacity cDNA Reverse Transcription kit was used to synthesize cDNA from 120ng RNA, according to the manufacturer's protocol (**Table 2 and 3**) using the High-Capacity cDNA Archive kit (Applied Biosystems Invitrogen 4374967).

Table 2.Volume of components needed for the cDNA archive protocol

Component	Volume (µl)/ Reaction
10x RT buffer	2
25x dNTP Mix (100mM)	0.8
10x RT Random primers	2
Reverse Transcriptase	1
Nuclease free H_20	4.2
RNA (120ng)	10
Total Volume	20

Table 3.Program the thermal cycler conditions

Step	1	2	3
Temperature	25 ^oC	37 ^oC	85 ^oC
Time	10 min	120min	5min

Applied Biosystems TaqMan Universal PCR Mastermix and TaqMan gene expression probes for Drosophila e(z) (Dm01822553_gl), Delta (Dm02134951_m1) and RPII140 (Dm02134593_g1) as endogenous control (VIC-labeled) were obtained from Applied Biosystems and used on an Applied Biosystems ViiA Real-Time PCR Instrument. All assays were run in duplicates on an Applied Biosystems ViiA Real-Time PCR system, according to the manufacturer's instructions (**Table 4**), and the mean value was used for the analysis. mRNA levels were expressed as relative quantification (RQ) values, which were calculated as $RQ = 2^{(-\Delta\Delta Ct)}$, where ΔCt is (Ct [gene of interest] - Ct [housekeeping gene]).

Table 4. Volume of components for the Real Time PCR protocol

Component	Volume (µl)/ Reaction
Taqman Mix	7.5
Primers	0.75
Water	4.25
cDNA (120ng)	2.5

IV. Results

IV.1 Confirmation of the e(z) knockdown

Intestines were dissected from esg-w1118 and esg-e(z):RNAi line 1 flies incubated at 29 ^{O}C for transgene expression. Then intestinal GFP+ cells were isolated by FACS and RNA extraction and Real Time PCR were performed for confirmation of e(z) knockdown **(Fig 11)**. Efficient knock-down was verified in two RNAi lines, esg-e(z)-RNAi line 1 and esg-e(z)-RNAi line 2, which showed approximately 60% and 80% down-regulation of e(z) expression in their ISCs and EBs, respectively. Most of the following experiments were performed with the esg-e(z)-RNAi 1 line.

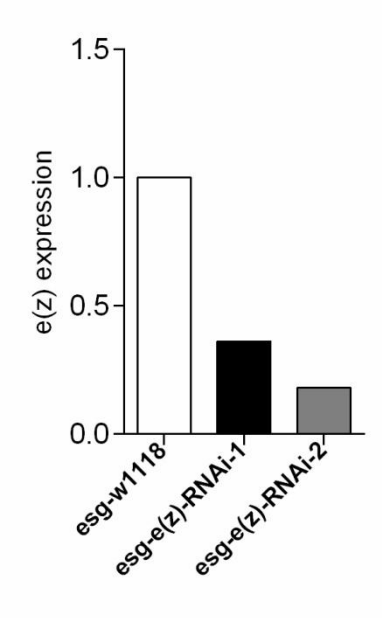

Fig 11. Verification of e(z) knockdown from FACS-isolated ISCs and EBS

IV.2 The impact of e(z) knockdown on fly survival

Esg-w1118 and esg-e(z):RNAi 1 were maintained at 29 ^{O}C for RNAi induction, and tested for survival under sucrose and DSS feeding. The survival curves showed that e(z) knockdown reduces the median survival of the flies approximately 30% and 25% under sucrose feeding **(Fig 12)** and sucrose in the presence of DSS **(Fig 13)**, respectively. Moreover, both control (w1118) and flies deficient for e(z) in their ISCs and EBs revealed reduced survival upon DSS treatment, implying that DSS-mediated intestinal damage was successfully induced.

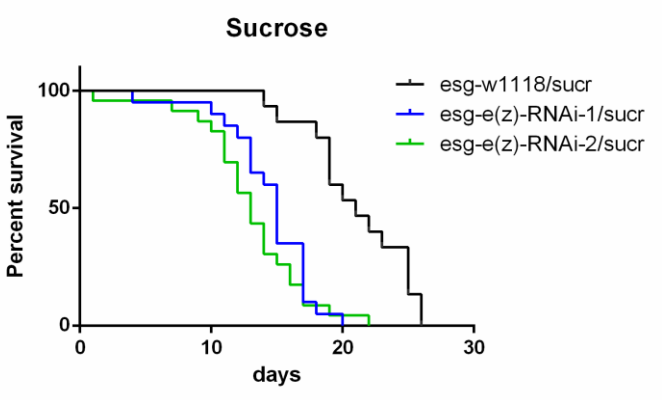

Fig 12. E(z) knockdown reduces fly viability under sucrose feeding

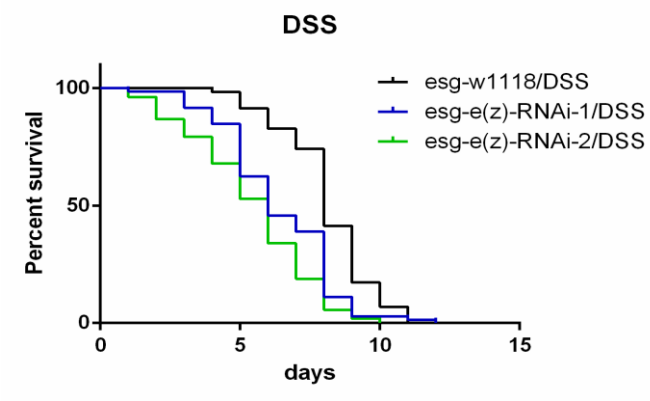

Fig 13. E(z) knockdown reduced fly survival under DSS-induced stress conditions

IV.3 e(z) knockdown reduces ISCs proliferation

Esg-w1118 and esg-e(z):RNAi 1, were maintained at 29 ^{O}C for RNAi induction and fed either with sucrose or sucrose and DSS. Guts were dissected and stained with anti-Phospho-histone 3 (PH3). PH3 is widely used as a marker of mitosis since it stains the condensed chromatin just before chromosomal segregation. In Drosophila intestine, ISCs are the only type of cells capable of division. Therefore, PH3 reacts only with dividing ISCs.

The staining and the quantification of PH3^{+} cells showed that e(z) knockdown reduced the number of dividing ISCs both in sucrose and DSS fed flies **(Fig 15).** Moreover, while DSS causes dramatical increase in the number of GFP^{+} and PH3^{+} cells in control flies, these were markedly reduced in DSS fed e(z) knockdown flies **(Fig 14)**.

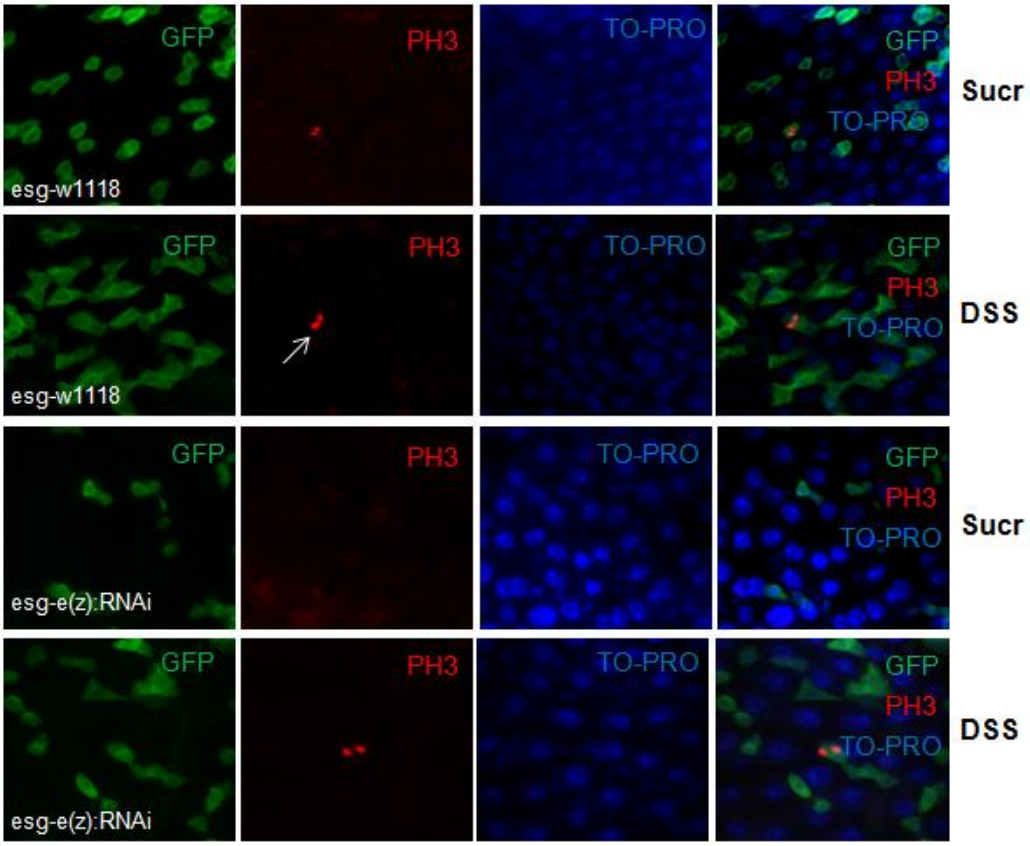

Fig 14. Immunofluorescence staining using antibody against phospho-H3 is shown (red), in flies fed with sucrose or DSS. DSS increases the number of PH3-positive cells in control but not in e(z)-RNAi lines. ISCs and EBs are simultaneously marked with GFP, red staining is anti-PH3 and blue staining is TO-PRO for DNA.

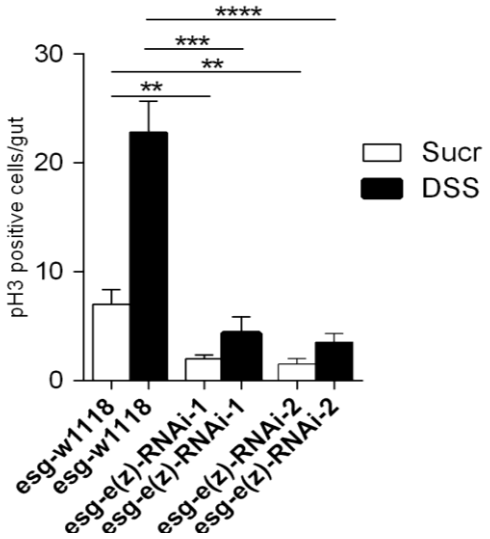

Fig 15. The average number of PH3-positive cells per gut in control and the two e(z)-RNAi lines is shown. Error bars represent standard deviation.

IV.4 e(z) knockdown does not induce ISCs apoptosis

In order to test whether the reduced number of dividing ISCs in e(z) knockdown flies is associated with increased ISCs apoptosis, midguts dissected from control and e(z) RNAi flies were analysed for apoptosis using a TUNEL labelling assay (ApopTag). The staining revealed that only a small number of GFP^+ cells were also stained with ApopTag (1-2 cells per gut) in e(z) knockdown flies, a finding similar with the results of ApopTag staining in control flies **(Fig 16)**. Quantification of the double GFP/ApopTag-positive cells in control and e(z) RNAi flies showed no significant difference, demonstrating that the reduction of PH3-positive cells was not due to apoptosis **(Fig 17)**.

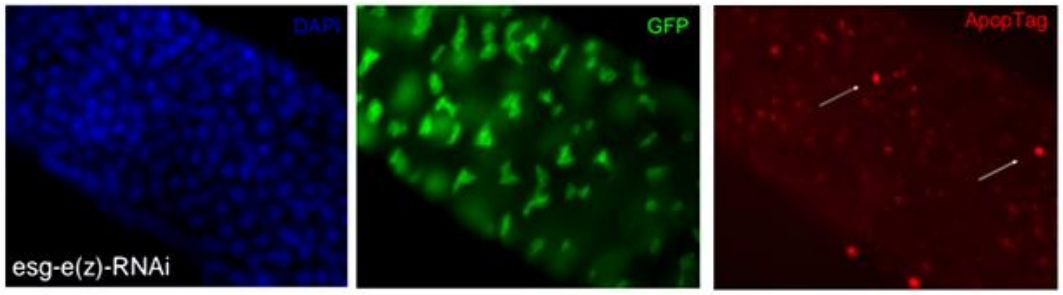

Fig 16. ApoTag staining of e(z) RNAi midguts. Only 1-2 cells per gut were GFP/ApoTag positive both in control and e(z) RNAi lines. ISCs and EBs are simultaneously marked with GFP, red staining is ApopTAg and blue staining is DAPI for DNA.

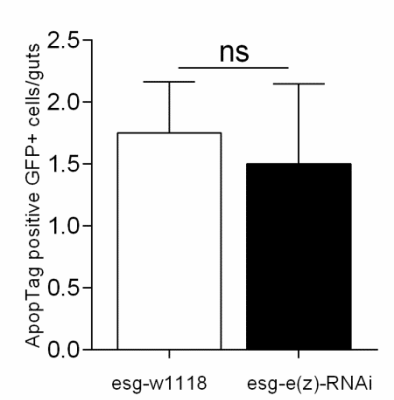

Fig 17. Average number of apoptotic GFP-positive cells per posterior midgut is presented in the graph. E(z) knockdown does not increase significantly apoptotic ISC death in sucrose fed flies. Black error bars represent standard deviation.

IV.5 Expression of mutated, catalytically inactive e(z) results in impaired ISCs driven intestinal regeneration

In order to further our knowledge of the functional outcome of e(z) in Drosophila intestine, MARCM clonal analysis system was utilized. MARCM allows the generation of e(z) homozygous mutated cells that are also labelled with GFP in an otherwise wild type for e(z), non-GFP labelled fly. Importantly, all the labelled cells are descendants of a common progenitor cell (ISC or EB) that was also e(z) mutated.

Intestines were dissected from control MARCM (FRT2A) and e(z) mutated MARCM ($e(z)^{731}$ FRT2A) flies, 7 and 12 days after clonal induction. By monitoring these time points, the regeneration capacity of the e(z) mutated clones could be tested. We observed that the multiple cell clones formed in control flies, were totally absent from e(z) mutated flies where 90% of the clones comprised of single cells and the remaining 10% contained less than 5 cells. Moreover, high regeneration defects were observed in e(z) mutated clones whereas in control flies the clones were expanded through 7 to 12 days of regeneration (**Fig 18, Fig 19**). Clones were considered groups of cells in contact to each other, as determined by staining with armadillo, a marker of cell membrane (**Fig 20**).

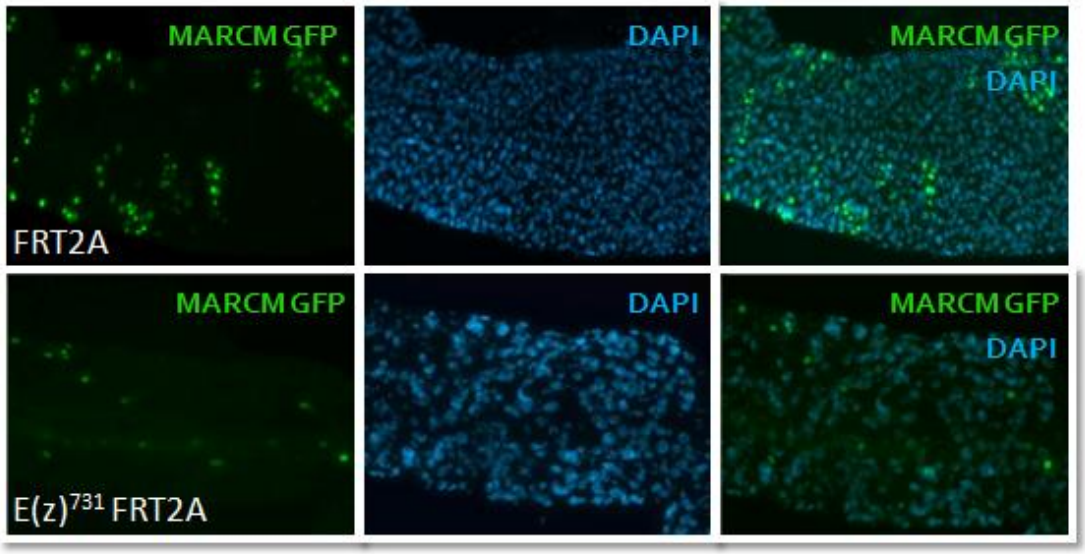

Fig 18 Each cluster of GFP-positive represents one lineage originated from one parental ISC after MARCM. The multiple cell clones of control (FRT2A) flies are almost absent from e(z) mutated (e(z)^{731}FRT2A).

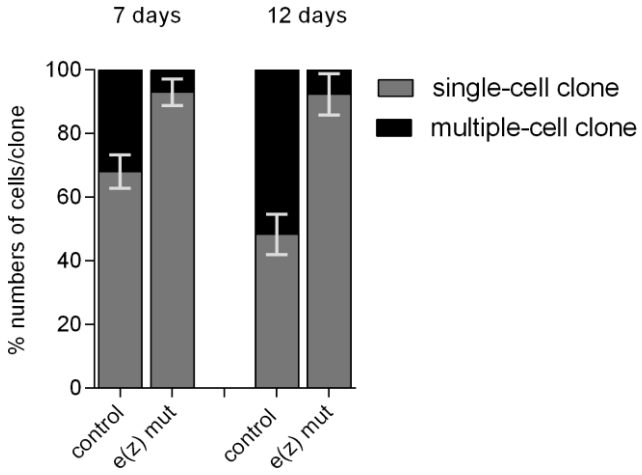

Fig 19. Quantification of the percentage of the number of cells per clone in control and e(z) mutated MARCM flies in 7 days and 12 after clonal induction. White error bars represent standard errors.

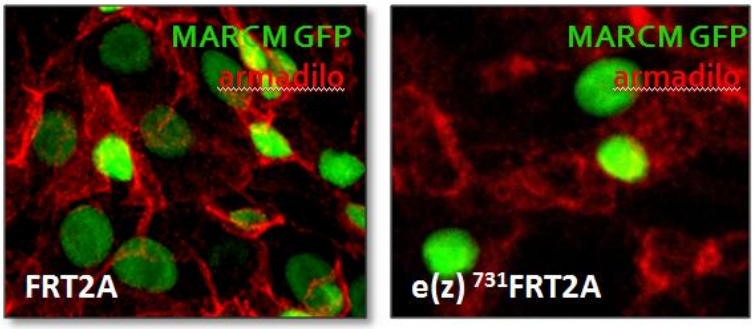

Fig 20. Immunofluorescence staining using antibody against armadillo is shown (red). FRT2A: control MARCM flies, e(z)^{731} FRT2A: e(z) mutated MARCM flies.

IV.6 Mutated e(z) affects delta-positive cells

To determine the identity of the cells in e(z) mutated clones, intestines were dissected from control MARCM (FRT2A) and e(z) mutated MARCM (e(z)^{731} FRT2A) flies and stained for delta, a marker of ISCs. Interestingly, although most of the clones in control flies had delta-positive cells as expected, those 90% of the e(z) mutated single cells were delta negative. The percentage only slightly changed in e(z) mutated multiple cells clones **(Fig 21, Fig 22)**.

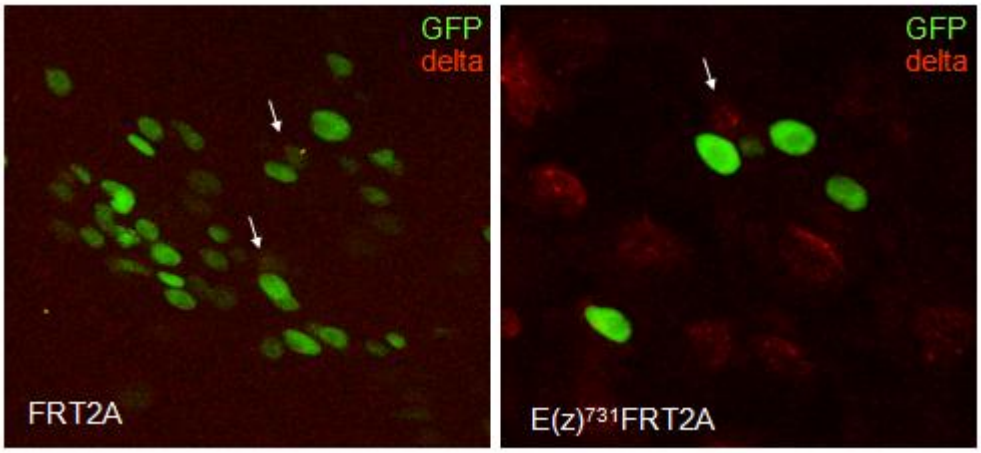

Fig 21. Immunofluorescence staining using antibody against Delta. Control clones contain delta-positive cells (Left). Most of the single-cell e(z) mutated clones were negative for delta staining although delta positive cells were observed in the adjacent tissue area (Right) (arrow).

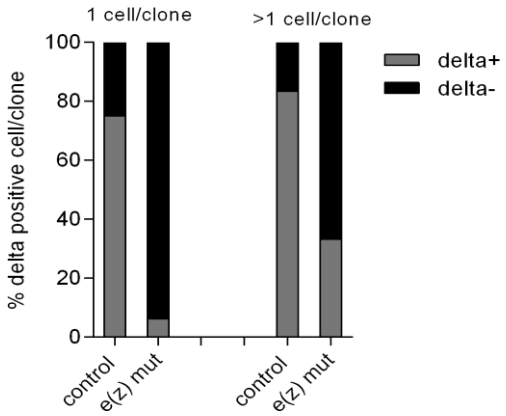

Fig 22. Quantification of the percentage of delta-positive cells in single-cell and multiple-cells clone observed in control MARCM and e(z) mutated MARCM flies.

IV.7 Mutated e(z) does not promote ISC/EBs differentiation into EE or inhibits differentiation into EC

The finding that deficient e(z) affects the clonogenic capacity of ISCs prompted us to address whether it also interferes with the differentiation process. For this reason, intestines were dissected from control MARCM (FRT2A) and mutated e(z)-expressing MARCM ($e(z)^{731}$ FRT2A) flies and stained with prospero and pdm1, markers of enteroendocrine and mature enterocytes, respectively. Regarding EE, the staining showed that all the e(z) mutated clones were prospero-negative while prospero-positive cells were observed in the adjacent tissue area and in some of the control clones **(Fig 23)**. However, since the clones of e(z) mutated MARCM flies contain few cells and the differentiation into EE is normally infrequent (10%), a clear conclusion cannot be reached. The clear finding is that e(z) mutation does not promote EB differentiation into EE.

Immunostaining with anti-pdm1 revealed that most of the cells in the control MARCM were pdm1-positive whereas in e(z) mutated clones 50% of the cells were pdm1-positive. This observation suggests that most of the cells in mutated e(z) clones are not mature enterocytes in contrast to wild-type flies **(Fig 24)**.

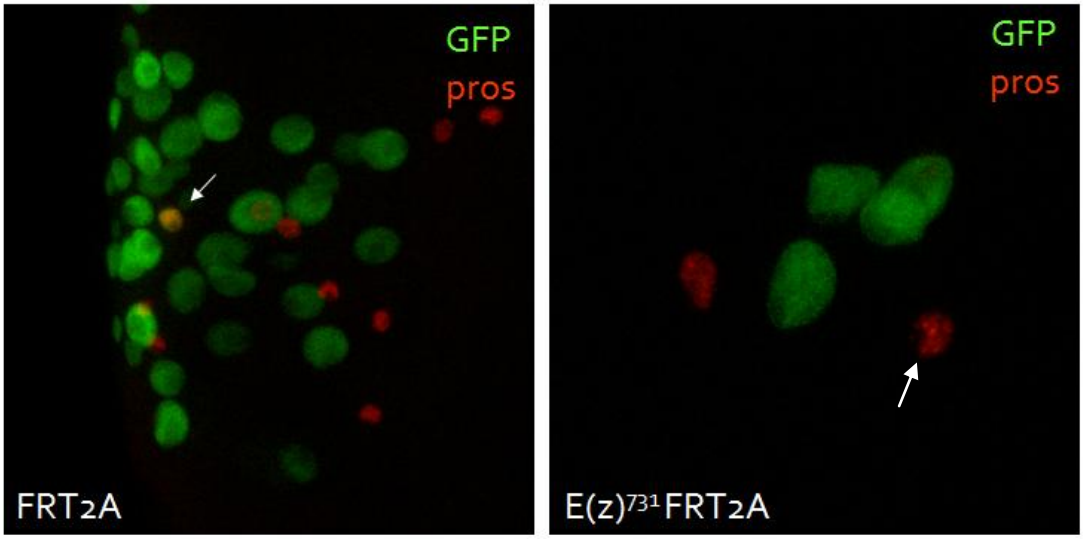

Fig 23. Immunofluorescence staining using antibody against prospero (red). Control clones contain prospero-positive cells (Left) (arrowhead). Most of the single-cell e(z) mutated clones were negative for prospero staining although prospero positive cells were observed in the adjacent tissue area (Right) (arrow).

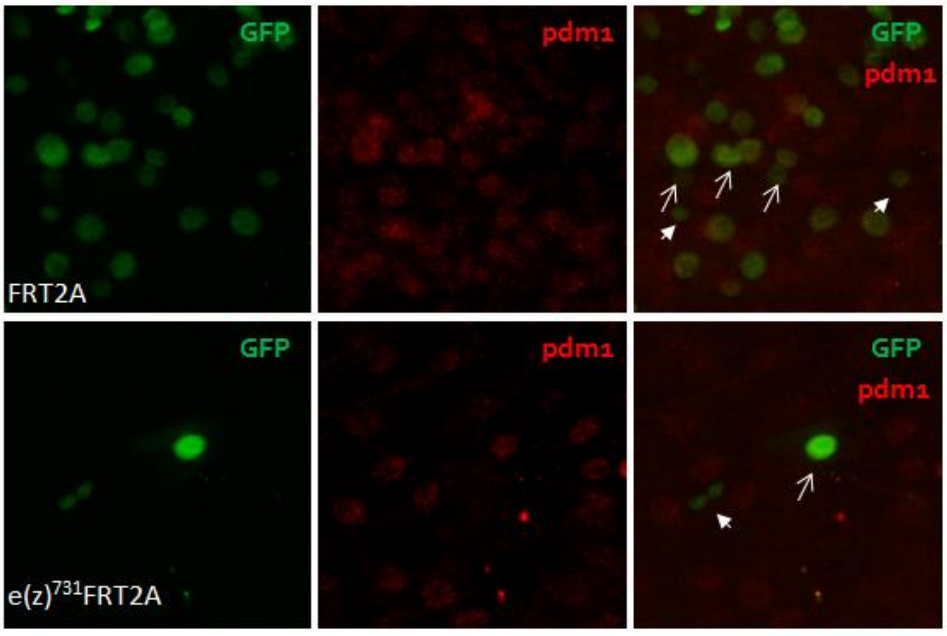

Fig 24. Immunofluorescence staining using antibody against pdm1 (red). Control clones contain mostly pdm1-positive cells while most of the half of single-cell e(z) mutated clones were positive for pdm1 staining (arrow).

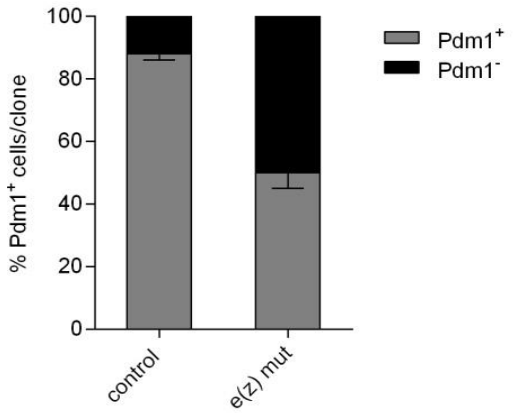

Fig 24. Quantification of the percentage of pdm1-positive and negative MARCM cells in control and e(z) mutated flies. Black error bar represents standard error.

V. Discussion

The aim of this study was the investigation of the impact of the epigenetic modifier e(z) on the intestinal tissue homeostasis and stem cell function, under normal conditions and upon DSS-induced tissue damage.

Drosophila melanogaster is widely used as experimental model for the study of the intestinal system as the pathways that regulate intestinal function, regeneration and stress response are highly equivalent to those of mammals (EGFR, JNK, JAK-STAT, Hippo). Moreover, the variety of experimental tools that have been developed in Drosophila converts this fruit fly into a powerful model for the investigation of principles that govern the intestinal system. Dextran Sodium Sulfate (DSS) is used as an intestinal stress factor that causes inflammation and abnormal regeneration in the intestine, resulting in epithelial cell apoptosis and extensive stem cell-mediated replenishment of the lost cells. For this reason, this model resembles colitis-associated intestinal disorder, a hallmark of colorectal cancer, and is ideal for the identification of the crosstalk between the environmental conditions and the epigenetics in the context of chronic inflammation and cancer.

First, the survival assays that were conducted under normal conditions (sucrose feeding) and upon tissue damage (DSS feeding) revealed that e(z) silencing in ISCs and EBs, reduces the fly median survival in both conditions, indicating a detrimental role of e(z) in the physiology and function of the intestine. This finding is supported by the additional evaluation of the numbers of ISCs and EBs and analysis of the ISC mitosis. Under normal conditions, we observed that e(z) deficiency reduces the number of ISCs or/and EBs and the rates of ISC division, as detected by pH3 staining. These observations are most likely interconnected as reduced ISC mitosis could result in lower generation of ISCs and EBs. Moreover, these findings are in line with previous studies where is has been proposed that PrC complexes are implicated in cell proliferation and maintenance of stem cell identity (Martinez and Cavalli 2006, Sparmann and van Lohuizen 2006). Since the observed numbers of the dividing ISCs were not reduced due to extensive apoptosis under e(z) silencing, e(z) may targets signalling pathways associated with cell cycle and, as a result, absence of functional e(z) may cause ISC cell cycle arrest. This hypothesis is consistent with previous findings that PCR2 controls cell cycle genes (Iovino, Ciabrelli et al. 2013). Moreover, Iovino et al. elegantly demonstrated that Drosophila ovarian cells in which e(z) has been knocked-down undergo ectopic S phases and endoreplication, as detected by

EdU incorporation, in the absence of mitotic markers (Iovino, Ciabrelli et al. 2013). Therefore, EdU analysis should be performed in order to investigate whether the same applies for e(z)-knocked down ISCs.

Regarding the functional outcome of e(z) in ISCs, using MARCM clonal analysis we found that the expression of a catalytically inactive e(z) mutant dramatically reduces the number of cells in ISCs-derived clones and these clones were also characterized by impaired expansion through time. This finding indicates that e(z) deficiency affects intestinal regeneration driven by ISCs. Moreover, only 10% of the mutated clones contained stem cells, as detected by delta staining, revealing that e(z) may target genes that regulate the maintenance of stemmness. With respect to EB differentiation, we observed a reduction in enterocytes derived from e(z) mutated EBs in contrast to normal EBs but no obvious change was indicated for enteroendocrine cells. We hypothesize that e(z) deficiency specifically affects a pathway that leads to EB differentiation into ECs. Clearly, more analysis is needed to confirm (or exclude) this hypothesis.

Nevertheless, delta^{+} and pdm1^{+} cells were still present in some of the mutated clones. This may be the result of the e(z) mutant being partly functional or because some functional e(z) was inherited from the first normal dividing ISC to the first daughter cells and co-existed with the mutant e(z), fading its effects. In all cases more analysis of EBs is required in order to be investigated whether e(z) deficiency affects their identity, impairs their differentiation or reduces their numbers. Moreover, analysis of EBs under conditions of absence of functional e(z) will define which type of division, asymmetric or symmetric, is mostly affected by e(z).

Regarding intestinal inflammation, we observed that DSS administration reduces the median survival and, results in increased GFP-positive cells (ISCs and EBs) in wild type flies. This finding is consistent with previous published studies of Amcheslavsky et. al. who proposed that the intestinal inflammation leads to accumulation of enteroblasts (Amcheslavsky, Jiang et al. 2009). Moreover, in our study, this observation was correlated with an increase in the number of the dividing ISCs. These findings could be explained by the fact that DSS-caused epithelial damage stimulates an extensive division of ISCs in order for the lost cells to be replenished. This hypothesis is also supported by similar findings in mice, where upon damage of the intestinal epithelium, the surviving crypt stem cells divide to increase their number and subsequently restore sufficient numbers of crypts by crypt fission, to maintain epithelial homeostasis (Potten 1990).

In contrast to WT flies, the DSS-fed flies that expressed e(z)-RNAi in ISCs and EBs, showed dramatic reduction in ISC proliferation and absence of EBs/ISCs accumulation. This could be also the reason of the dramatic reduction in the survival of the flies which lived less than 6 days. These findings indicate a crucial role of e(z) in intestinal regeneration capacity and response to inflammatory factors. Previous studies have also proposed that EZH2, the mammalian homologue of e(z) is overexpressed in pancreatic stem cells and that it has a synergistic role with Myc oncogene, accelerating lymphomas formation (Berg, Thoene et al. 2014, Pethe, Nagvenkar et al. 2014). These findings are in line with our hypothesis that e(z) has a crucial role in tumorigenesis and therefore its deficiency may causes an anti-tumorigenic effect upon DSS treatment, characterized by low levels of cell proliferation and precursor cell generation.

Our ongoing studies aim to further investigate the role of e(z) in ISCs-driven intestinal homeostasis. In particular, we plan to identify putative targets of e(z) associated with ISCs and involved in developmental and differentiation pathways, dissecting the molecular mechanisms by which e(z) controls cell fate determination. Moreover, it is essential to be tested whether the observed reduction in ISCs division of e(z)-KD flies occurs due to precocious differentiation or cell cycle arrest. In the context of tissue repair, a combination of available genetic and immunofluorescence tools in DSS fed flies will allow us to investigate the role of e(z) in ISCs function upon tissue damage. Finally, it would be interesting to examine whether feeding flies with EZH2 inhibitors recapitulate our MARCM results and whether these inhibitors can rescue a tumorgenic phenotype in flies, paving the way for the establishment of Drosophila as an in vivo screening platform for evaluation of agents targeting the epigenetic machinery.

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