INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

EPIGENETIC REGULATION OF INTESTINAL STEM CELL AGING IN *DROSOPHILA MELANOGASTER*

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Mestrado em Tecnologias Clínico-Laboratoriais

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Abstract

Aging is characterized by a decline in the organism's physiological integrity. Adult stem cells are major regulators of organismal homeostasis and age-associated changes in stem cell populations have been proposed to be a major cause of the progressive deterioration of tissue health with aging.

Studies indicate that aging-associated stem cell functional decline can be reversed by manipulating epigenetic factors that become dysregulated during aging. The goal of this project is to identity new epigenetic regulators of stem cell aging.

We used *Drosophila melanogaster* as a model organism to screen a set of new candidate epigenetic factors for a role in intestinal stem cell (ISC) aging. As a screening method we used survival assays after multiple injuries. Positive hits were explored through the analysis of ISC behavior and morphology, including ISC proliferative capacity and differentiation potential in response to regenerative pressure, under conditions of gain or loss of candidate gene function.

Three genes were found to affect fly recovery and survival after gut injury: Su(var)2-10, Velo and Polo. Su(var)2-10 knockdown resulted in a phenotype of exacerbated proliferation of ISCs. Overexpression of Velo caused a deactivation defect and an irregular shape, size, and low number of cells. In Polo overexpression we found that the stem cells differentiate at an early stage and do not proliferate. This work identified three genes altered in aged ISCs that affect stem cell function at distinct levels and regulate regenerative capacity.

Keywords:

Aging, Stem Cells, Epigenetic regulators

Resumo

O envelhecimento é caracterizado por um declínio da integridade fisiológica do organismo. As células estaminais adultas são as principais reguladoras da homeostasia do organismo e as alterações associadas à idade nas populações destas células foram propostas como uma das principais causas da deterioração progressiva da saúde dos tecidos com o envelhecimento.

Estudos indicam que o declínio funcional das células estaminais associado ao envelhecimento pode ser revertido pela manipulação de fatores epigenéticos que se tornam desregulados durante o envelhecimento. O objetivo deste projeto é identificar novos reguladores epigenéticos de células estaminais no envelhecimento.

Usámos a *Drosophila melanogaster* como organismo modelo para rastrear um conjunto de novos fatores epigenéticos candidatos e perceber de que forma estão envolvidos no envelhecimento de células estaminais intestinais. Como método de triagem fizemos ensaios de sobrevivência após múltiplas lesões. Genes selecionados nesta triagem foram explorados através da análise do comportamento e morfologia das células estaminais intestinais, incluindo a capacidade proliferativa das células e a resposta do potencial de diferenciação à pressão regenerativa, sob condições de ganho ou perda da função do gene candidato.

Três genes foram selecionados por afetarem a recuperação e sobrevivência da mosca após lesão intestinal: Su(var)2-10, Velo e Polo.

Su(var)2-10 *knockdown* resultou num fenótipo de proliferação exacerbada de células estaminais intestinais. A subexpressão de Velo causou um defeito de desativação, e células com formato irregular, pequenas e em baixo número. Na subexpressão de Polo, descobrimos que as células estaminais se diferenciam num estágio inicial e não proliferam.

Este trabalho identificou três genes alterados em células estaminais intestinais em envelhecimento que afetam a função das células estaminais em níveis distintos e regulam a capacidade regenerativa das mesmas.

Palavras-Chave:

Envelhecimento, Células estaminais, Reguladores epigenéticos

Contents

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List of Abbreviations

Dl	Delta
DNA	Deoxiribonucleic acid
EB	Enteroblast
EC	Enterocyte
EE	Enteroendocrine cell
EGFR	Epidermal Growth Factor Receptor
ISC	Intestinal Stem Cell
JAK/STAT	Janus kinase / signal transducer and activator of transcription
JNK	Drosophila Jun N-terminal Kinase
PBS	Phosphate-buffered saline
pH3	Phospho-Histone H3 antibody
PQ	Paraquat
RNAi	Ribonucleic acid interference
SC	Stem Cell
SUC	Sucrose
SUMO	Small ubiquitin-related modifier
UAS	Upstream Activation Sequence
UbL	Ubiquitin-Like protein

1. Introduction

1.1. Stem Cells

Stem cells (SCs) are a population of undifferentiated cells characterized by their multi-potency and capability to self-renew, giving rise to progeny that differentiates to repair tissues and to daughter cells that retain SCs properties to ensure the extended maintenance of the SCs pool.[1,2]

Some of the progenitor cells that have contributed to organ formation do not terminally differentiate but are retained as tissue stem SC. These somatic SC populations differ according to regenerative necessities of the host tissue; in many tissues, they remain mostly quiescent, but in some tissues, for example, the intestine, most of the SCs population is active throughout life, proliferating to supplement cells during turnover or injury and repair.[1,3,4].

1.1.1. Stem cells in regenerative medicine

A central goal of regenerative medicine is to rejuvenate or restore the normal function of a tissue that has been damaged either by chemical exposure, a physical defect, or a genetic or infectious disease.[4,5] Thus, regenerative medicine is a major focus of research not only to find therapies but also to understand basic biology and the pathogenesis of disease. Although a number of ethical issues have arisen in stem cell research, due to the lack of organ donors, stem cells are now considered to be potential alternative sources for the replacement of organs and tissues.[1,5]

1.1.2. Stem cells in aging and regeneration

Adult stem cells show a progressive decline in functionality during aging.[6] Aging can be defined as a complex, time-dependent process that affects multiple tissues and organs leading to a progressive reduction in physiological integrity and the degeneration of tissue, organ, and organismal function. Stem cells are extremely vulnerable and their homeostasis can be challenged by many factors directly leading to decreased proliferation, which is one of the major features of stem cell aging, distorted differentiation, and dysregulation of self-renewal resulting in either increases or decreases in the stem cell number (Figure 1.1).[6,7] These factors are derived either from the systemic environment or the local environment. Both the local and systemic environment, as SCs themselves, change with age, and these changes limit the success of regenerative processes. [4,8]

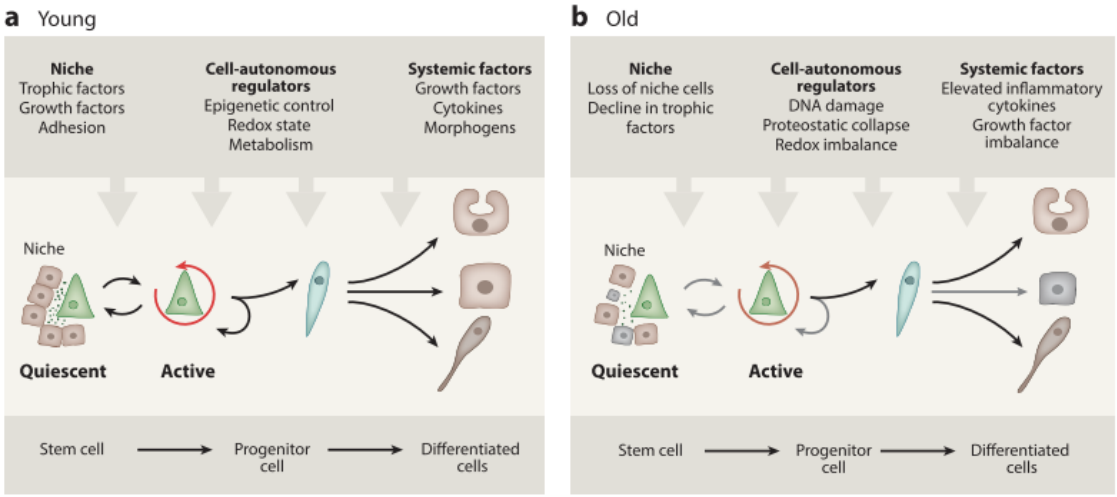

Figure 1.1 – Regulation and aging of stem cells. (a) Intrinsic (cell-autonomous), local (niche), and systemic factors regulate stem cell maintenance, quiescence, activation, lineage specification, and differentiation. (b) In the aging animal, many of these factors decline or become deregulated, resulting in the loss of stem cells, deregulated stem cell activity, distorted differentiation potential, and mis differentiation of daughter cells. (Adapted from [8])

1.2. *Drosophila melanogaster* gut as a study model

Drosophila melanogaster has served as a genetic model system for a century.[9] Is one of the most valuable organisms in biological research, particularly in the areas of genetics and developmental biology.[10] The simple architecture of intestinal epithelium, the availability of tools for genetic manipulation and the short lifespan makes *Drosophila* an ideal model for understanding the basic mechanisms of stem cell aging. Additionally, the *Drosophila* gut it's similar to the human gut, providing an asset for studies for the purpose of therapeutic applications in humans.[10,11]

The *Drosophila* gut is a self-renewing tissue in which a stem cell population generates new differentiated cell types to replace the damaged and lost ones during normal life.[12] In *Drosophila*, the regenerative response to intestinal injury induced by infection or stress depends on enterocytes which are large polyploid cells that differ markedly in morphology along the gut and secrete digestive enzymes and absorb nutrients.[11,13] If an injury occurs, it will kill enterocytes (causing stress), which will lead to the activation of intestinal stem cells to produce new enterocytes and heal the intestinal damaged.[12]

1.2.1. *Drosophila* Intestinal Stem Cells (ISCs)

Drosophila ISCs have emerged as an excellent model for studying epithelial stem cells and homeostasis in the adult midgut of *Drosophila*, evenly distributed along the basal side of the epithelium.[12] The pseudostratified posterior midgut epithelium consists of four cell types: differentiating enteroblast progenitors; absorptive enterocytes; secretor enteroendocrine cells; and proliferating intestinal stem cells which are the ones we will focus on this project.[11]

Under normal conditions, ISCs are located in the basal region of the intestine and undergo regulated division to maintain tissue homeostasis.[14] The ISCs are the only mitotic cell type in the *Drosophila* adult midgut and are therefore critical for intestinal homeostasis. Coordination of stem cell proliferation and differentiation is required to maintain intestinal homeostasis, as is the incorporation and morphogenesis of new cells and the expulsion of damaged enterocytes.[15,16]

In *Drosophila*, intestinal regeneration (Figure 1.2) involves a well-studied process that relies on specific molecular mechanisms.[17,18] Intestinal tissue damage or stress triggers the activation of pre-existing ISCs. Multiple signaling pathways, including JAK/STAT and JNK pathways, are involved in ISC activation.[19,20] Activated ISCs undergo asymmetric division, generating new ISCs and enteroblast (EB) progenitor cells. This process is regulated by various factors, such as the transcription factors Escargot and Delta-Notch signaling.[21,22] The EB progenitor cells receive signals from neighboring cells through the Notch signaling pathway. Notch activation leads to the differentiation of the EB into enterocytes (EC) or enteroendocrine cells (EE).[18,21] Newly formed ECs and EEs undergo maturation and acquire their specific functions within the intestinal tissue. Several signaling pathways, including the EGFR and Hippo pathways, regulate the differentiation and maturation of these cells.[22,23] The newly formed ISCs replenish the pool of stem cells, maintaining the balance between stem cell self-renewal and differentiation to ensure intestinal tissue homeostasis.[19,21]

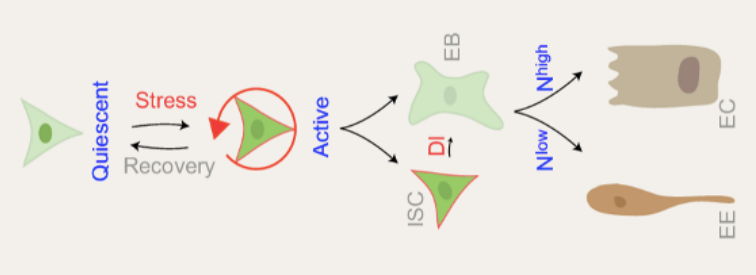

Figure 1.2 - The intestinal stem cell lineage in Drosophila. In response to stress, ISCs divide asymmetrically to give rise to a new ISC and an EB, which differentiates into either an EC or an EE. Notch (N) signaling, initiated by a Delta (Dl) signal from the ISC, drives EB differentiation. Depending on the levels of N signaling, EBs differentiate into either EEs or ECs. (Adapted from [24])

1.2.2. Intestinal Stem Cells in aging

During the aging process or after an infection, intestinal compartmentalization undergoes some changes, which are noted by a substantial alteration in gene expression patterns.[15,24]

In young animals, the epithelium consists of a monolayer of ECs with interspersed EEs and basally located ISCs.[25] In aging flies, ISCs become hyperproliferative, accumulating undifferentiated cells that co-express stem and progenitor cell markers and differentiation markers (Figure 1.3).[26,27]

Stem cell dysfunction is closely linked to tissue and body aging, leading to age-related diseases.[16] The effects that occur in the face of inflammation-induced changes in gut homeostasis can even lead to death as the animal ages. These age-related inflammatory changes are also strongly correlated with systemic metabolic dysfunction.[28]

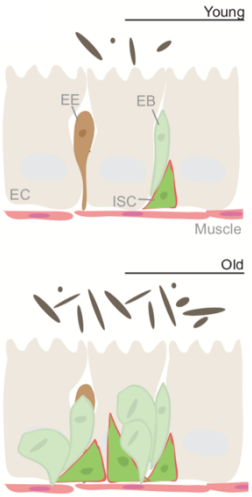

Figure 1.3 - Age-related changes in homeostasis of the intestinal epithelium. In aging flies, ISCs over proliferate, resulting in the accumulation of mis differentiated EB-like cells that disrupt structure and function of the intestinal epithelium. (Adapted from [24])

1.2.3. Epigenetic Regulation in *Drosophila melanogaster* ISCs

Epigenetic modifications play a critical role in gene expression regulation and cellular identity.[29] DNA methylation, which involves the addition of methyl groups to DNA, can impact gene transcription and stability. Histone modifications, such as methylation, acetylation, and phosphorylation, modulate chromatin structure and accessibility. Chromatin remodeling complexes further regulate gene expression by altering the positioning and packaging of DNA (Figure 1.4).[7,30]

Studies have demonstrated significant changes in epigenetic marks during ISC aging. For instance, alterations in DNA methylation patterns have been observed, including global changes and site-specific alterations, which may affect gene expression and

cellular function.[29] Histone modifications also show age-related changes, such as altered methylation and acetylation patterns, potentially influencing chromatin accessibility and gene expression.[30]

Several epigenetic regulators have been implicated in ISC aging. DNA methyltransferases, enzymes responsible for DNA methylation, and histone-modifying enzymes, such as histone methyltransferases and histone deacetylases, are involved in establishing and maintaining epigenetic patterns. Chromatin remodelers also contribute to epigenetic regulation during aging.[7,29]

Epigenetic modifications directly impact the expression of key transcription factors and signaling pathways involved in ISC maintenance and regeneration.[31] For example, altered histone modifications can affect the activity of transcription factors that regulate ISC self-renewal and differentiation. Dysregulation of epigenetic marks may contribute to age-related transcriptional changes observed in ISCs.[7,27]

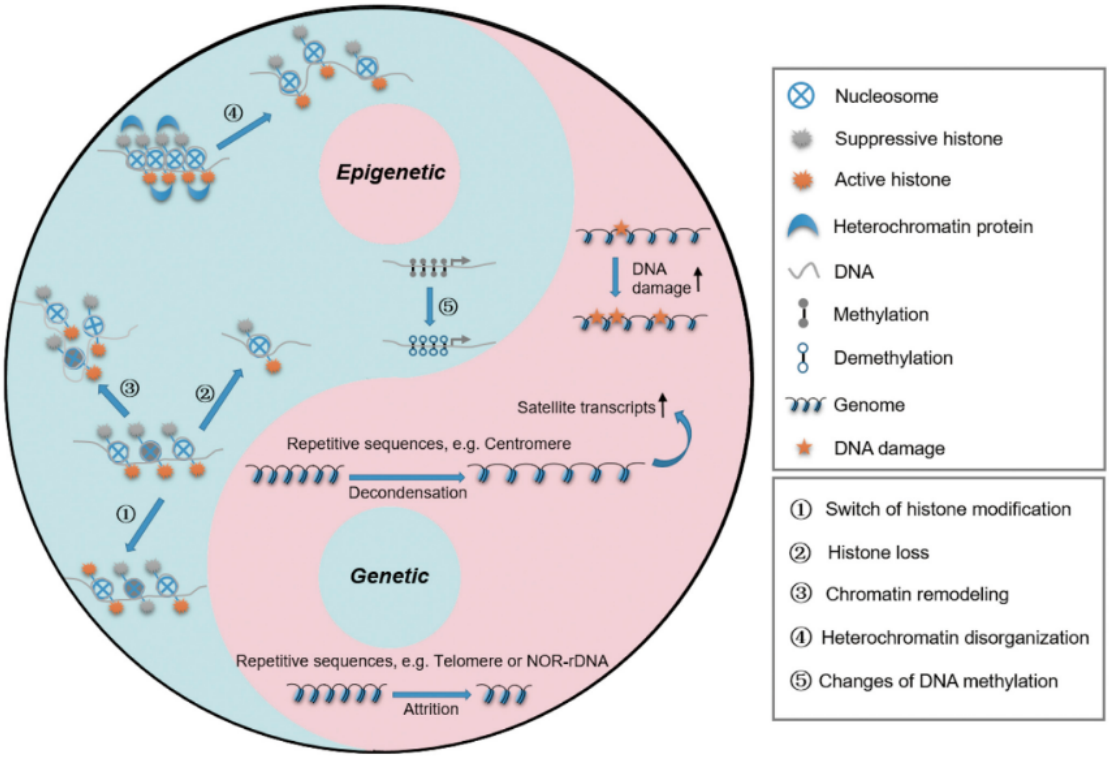

Figure 1.4 - Interplay between epigenetic and genetic instabilities in stem cell aging. During aging, major epigenetic changes are observed, including loss of core histones together with changes in histone modifications and chromatin remodeling enzymes. These changes lead to a loss of repressive heterochromatin, chromatin decondensation in centrometers and telomere attrition, and DNA damage. (Adapted from [29])

1.2.4. SUMOylation

Small ubiquitin-related modifier (SUMO) is a Ubiquitin-Like protein (UbL) with sequence and structural similarity to ubiquitin. SUMO has emerged as a crucial post-translational modification involved in diverse cellular processes. Increasing evidence suggests a strong correlation between SUMOylation and epigenetic regulation, where have been demonstrated that SUMOylation can directly modulate the activity and stability of key epigenetic regulators.[32,33]

SUMOylation has also been implicated in the regulation of chromatin remodeling complexes, which control the accessibility of DNA to transcription factors and other chromatin-modifying enzymes.[34]

These findings highlight the interplay between SUMOylation and epigenetic regulators.

1.3. Main objectives of the project

This project proposes to use the Drosophila ISCs as a model to discover and characterize new epigenetic regulators involved in the process of stem cell aging, starting from the question of how epigenetic regulators are involved in the aged-related decline in SC function.

Using fly strains with drivers that allow knockdown or overexpression of genes specifically in ISCs, we will collect our data from candidate genes through survival assays to study the ISC function for effective regeneration and survival. Positive hits on this screen will be further explored through the analysis of ISC behavior, including ISC proliferative capacity and differentiation potential response to regenerative pressure, under conditions of gain or loss of candidate gene function. Data regarding ISC proliferation will be evaluated by immunohistochemistry to detect phosphohistone-3 presence.

This work will contribute to a better understanding of how epigenetic regulators are involved in age-related decline in stem cell function.

2. Materials and Methods

2.1. Drosophila stocks and culture

Fly stocks were raised on standard cornmeal and molasses-based food produced at Champalimaud Foundation. Flies were maintained at 25ºC with a 12h light/dark cycle and 70% humidity. All flies used in the experiment reported were females. The Blomington Drosophila Stock Center provided fly lines and those were maintained at 18ºC and flipped to new food once a month.

2.2. Genetic manipulation of flies

We cross between virgin females and sexually mature young males to make flies with specific genetic manipulations. The virgin females carry a Driver Transgene (GAL4), expressing GAL4 in specific cell types. On the other hand, males carry an overexpression or knockdown transgene for the gene of interest, i.e., they express a sequence of the gene or an RNAi for the gene after a UAS sequence. When a GAL4 driver line is crossed to a line with the UAS-target gene, the progeny expresses the gene of interest only in the cells in which the GAL4 is present (Figure 2.1).[35] In this project we used the Esg G4 and Esg G4/G80 driver. Escargot (Esg) is a transcription factor that marks the ISCs and enteroblasts in *Drosophila*.[36]

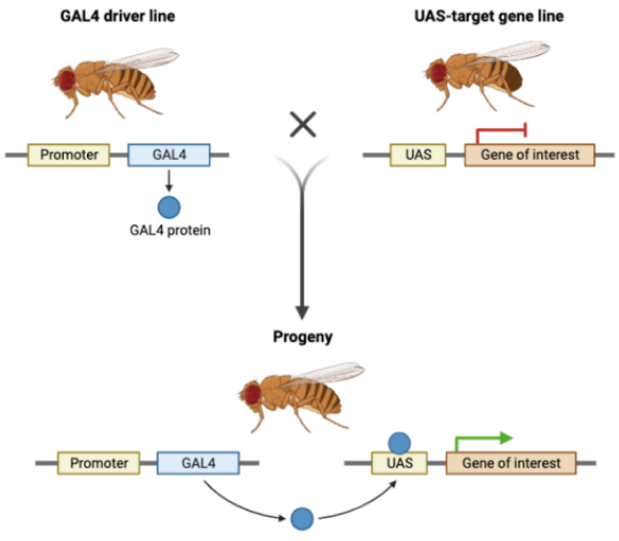

Figure 2.1 - The Basic GAL4 System. GAL4 driver and UAS-target gene fly lines are generated and maintained as separate stocks. In the absence of GAL4 there is no expression of the target gene. Crossing a fly expressing GAL4 to a fly carrying a UAS-target genes results in targeted gene expression in the progeny of the cross. (Figure created with Biorender.com based on [31])

2.2.1. GAL80 temperature-sensitive repressor

The GAL4/GAL80 system, was used when the genetic manipulation using the GAL4 system led to embryonic lethality, thus not allowing the study of the gene function in adult flies. GAL80 binds the transactivation domain of GAL4 and prevents GAL4 from activating transcription (Figure 2.2). This allows the flies to produce offspring (at 18ºC) without the knockdown or the overexpression of the gene in the embryonic stage. GAL80 repression of GAL4 is alleviated by a simple temperature shift (18ºC to 29ºC), enabling more precise control of the onset of target gene expression (Figure 2.2)[35,37]. This alternative benefits from being fully compatible with the array of established GAL4 lines.

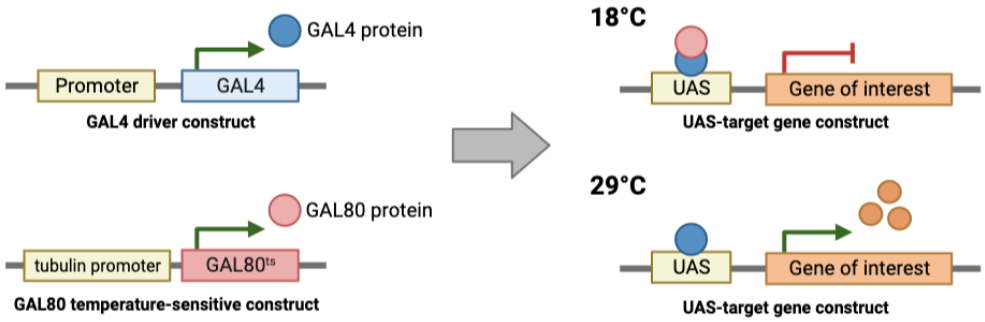

Figure 2.2 - Modification to the basic GAL4 system. The presence of a ubiquitously expressed temperature-sensitive allele of GAL80 will result in inhibition of GAL4 activity at the permissive temperature of 18ºC. At the restrictive temperature of 29ºC Gal80^{ts} cannot bind GAL4, therefore GAL4 activity is not inhibited, and GAL4-dependent transgenes will be expressed. (Figure created with Biorender.com based on [33])

2.3. Experimental design of inducing a gut injury

To study the epigenetic regulation of intestinal stem cells throughout this project, it was necessary to induce intestinal damage in the flies using paraquat (PQ), a toxic chemical (the range of PQ concentration used depends on the effect in control flies' proliferation and the batch). For this we separated female flies in two groups: control (SUC) and injury (PQ). Then the flies were moved to empty vials for starvation for 2 hours. While the flies were starving, empty vials with two paper filters were prepared and SUC and PQ solutions were added. After the 2 hours the flies were moved to the respective vials with the drug for 16 hours (Figure 2.3). After 16 hours the next step depends on what experiment it's going to be performed.

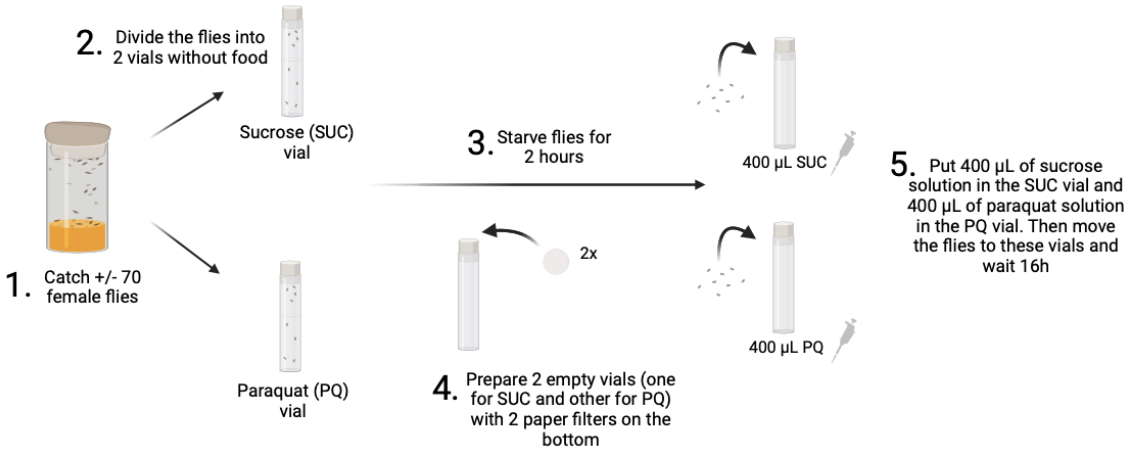

Figure 2.3 - Protocol of the experimental design for inducing an intestinal injury. Figure created with Biorender.com.

2.4. Survival tests

For the survival experiments, female flies previously crossed, of the indicated genotypes, were collected and sorted into vials with a density of 35 flies maximum and 14 flies minimum per vial. For each gene there were a vial for control (SUC) and one vial for the injury (PQ) in each survival test. Before each injury, the dead flies were counted, discarded and the alive ones were kept in empty vials for starving for 2 hours. While the flies were starving, empty vials with two paper filters were prepared and 400 µL of SUC and PQ solutions were added in the respective vials. After the 2 hours the flies were moved to the vials with the control and the drug for 16 hours, as mentioned above (Figure 2.3). After the 16h the flies were moved to normal food and the fly deaths were counted. This process was repeated for 4 injuries, in a total of 15 days of analysis, for flies expressing the gene under the control of Esg G4 driver, and 3 injuries, in a total of 12 days of analysis, for flies expressing the gene under the control of the temperature sensitive Esg G4/G80 driver. The number of dead flies were counted before and after each injury (Figure 2.4). For each gene screened were performed 2-3 survival tests.

At the end of the survival tests, the number of deaths count for each gene, for each survival test performed, was gathered and graphs with survival curves were created to study the survival rate. The survival curves are the result of the pool of the multiple survival tests performed for each gene.

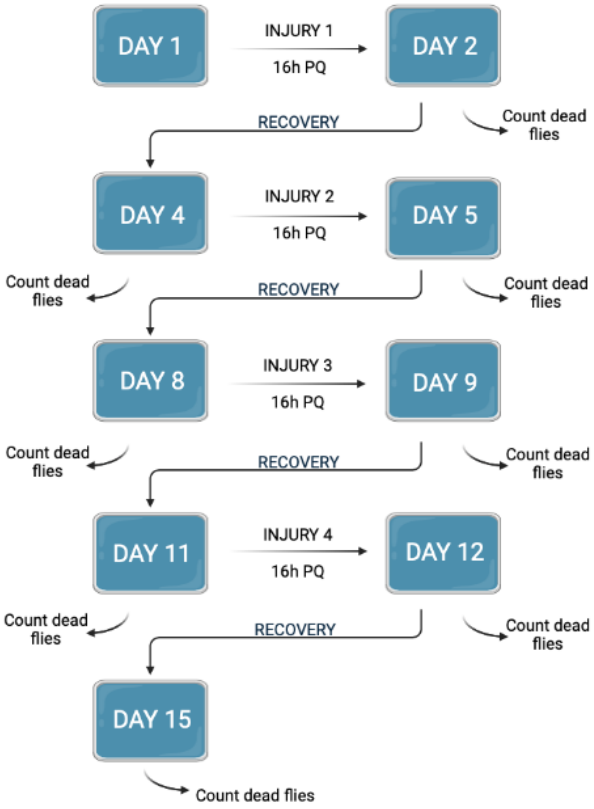

Figure 2.4 – Schematic representation of the survival test protocol. Figure created with Biorender.com.

2.5. Staining of *Drosophila* gut

Adult female *Drosophila* guts were dissected in PBS 1x, fixed for 45min at room temperature in fixative (100 mm glutamic acid, 25 mm KCL, 20 mm MgSO4, 4 mm sodium phosphate, 1 mm MgCl2 and 4% formaldehyde), washed for 10 min and then again for 45 min in wash buffer (PBS 1x, 0.5% bovine serum albumin, and 0.1% TRITON X-100), and then incubated in primary antibody (4ºC overnight). The next day a 45 min wash was done, and the secondary antibody was placed (2 h at room temperature) and followed by another wash. Finally, DAPI was added to stain the DNA incubating for 30 min. The slides were mounted with Mowiol mounting medium and microscope cover glass. For information on the antibodies used, see Table 2.1. The number of pH3-positive cells/midgut was counted by direct observation under the Motorized Wideflield Fluorescence Microscope Zeiss Axiovert 200M.

Table 2.1 – Antibodies used for immunofluorescence analysis.

Primary Antibody target	Species	Source	Dilution in IHC	Secondary antibody (target species/conjugated fluorophore)	Source	Dilution in IHC
pH3	Rabbit	Millipore (06-570)	1:1000	Anti-rabbit/Alexa Fluor® 555	Abcam (ab150066)	1:500

2.6. Image acquisition and quantification

All preparations were imaged using a Zeiss LSM 710 confocal laser scanning microscope. Images were analyzed using ZEN 2012 SP5 FP3 (black) software. Quantification of ISCs was performed using Fiji Software, and the scale was defined at 7.5758 pixels/μm. An outline was established for the cells and the midguts imaged. With these analysis three parameters were evaluated: cells size, total cell number and cell density.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). An unpaired two-tailed Student´s t-test was used to compare two groups to determine statistical significance. Two tests were used for the statistical analysis of survival tests: the Mantel-Cox test and the Wilcoxon test. Statistical significance was set at a value of $p < 0.05$.

3. Results

3.1. Selection of genes for screening

As it was already stated, we wanted to understand how epigenetic regulators are involved in age-related decline in stem cell function. For this, we used the *Drosophila* as a model to do a screening of genes that are known to be implicated in epigenetic processes. The analyses were performed using candidate genes. These genes had been previously selected for fulfilling four essential points for the study: being present in ISCs, regulated in aging in ISCs, expressed in mammalian SCs, and regulated in mammals with age. In total, 12 genes were screened (Table 3.1). The screening strategy was based on causing stress in the gut of flies with a knockdown or overexpression of a gene, and with a control to compare, we were able to identify the genes that show to have a relevant role in regenerative response.

Table 3.1 - List of screened genes. Gene symbol and name in *Drosophila melanogaster*; regulation in aging; cellular processes that the gene is involved in. (Information gathered at flybase.org)

Gene symbol	Gene name	Regulation in aging	Involved in
Kdm4A	Histone demethylase 4A	Down	Transcription; DNA repair and heterochromatin silencing
gpp	grappa	Down	Maintenance of expression of some protein groups; telomeric silencing
Aos1	Activator of SUMO 1	Down	Encodes one subunit of the heterodimeric SUMO activating enzyme
Velo	Veloren	Down	Encodes a SUMO protease
lwr	lesswright	Down	Encodes Ubc9 (SUMO conjugating enzyme); biological functions in innate immunity, meiosis, and anterior patterning of the embryo
Su(var)2-10	Suppressor of variegation 2-10	Down	Encodes a member of the PIAS protein family; involved in SUMOylation; JAK/STAT pathway regulator
rno	rhinoceros	Up	Cell differentiation and developmental patterning
Nph	Nucleophosmin	Down	Encodes histone binding protein involved in chromatin remodeling and sperm chromatin decondensation
mRe11	Meiotic recombination 11	Down	Encodes protein involved in the mitotic G2 DNA damage checkpoint and telomere capping
Polo	polo	Up	Regulation of nuclear and cytoplasmic aspects of the mitotic cycle
Iswi	Imitation SWI	Down	Energy-transducing component of chromatin-remodeling complexes NURF, ACF and CHRAC
MBD-like	Methyl-CpG binding domain protein-like	Up and down	Encodes protein involved in chromosome condensation and transcription repression

At an early stage of performing the genetic manipulations (mentioned in 2.2.) we noticed that, for some of these genes, we were not having enough flies to do the experiments.

This led us to understand that those genes are essential for *Drosophila melanogaster* embryonic development. For this reason, we performed the genetic manipulations for those genes with a temperature sensitive driver, Esg G4/G80 (Table 3.2). This driver allows the normal embryonic development of the fly without the knockdown or overexpression of the gene (explained in detail in 2.2.1.).

Table 3.2 - Genes divided by driver. The genes that were crossed with Esg G4 are not essential for embryonic development and the ones that were crossed with the temperature sensitive Esg G4/G80 are essential for embryonic development. Number (N) of survival tests and number of flies used per survival test for each gene.

Gene symbol	Target	Driver	N of Survival Tests	N of flies/survival test
Kdm4A	Knockdown	Esg G4	2	32, 35
Gpp	Knockdown	Esg G4	2	32, 35
Iwr	Knockdown	Esg G4	2	35, 35
Su(var)2-10	Knockdown	Esg G4	2	24,35
Nph	Knockdown	Esg G4	2	32, 35
Iswi	Knockdown	Esg G4	2	25, 30
Aos1	Knockdown	Esg G4/G80	2	35, 22
Velo	Knockdown	Esg G4/G80	3	24, 28, 30
	Overexpression	Esg G4/G80	2	35, 18
rno	Knockdown	Esg G4/G80	3	35, 15, 19
mRe11	Knockdown	Esg G4/G80	2	35, 35
Polo	Knockdown	Esg G4/G80	3	21, 20, 30
	Overexpression	Esg G4/G80	3	24, 23, 26
MBD-like	Knockdown	Esg G4 and Esg G4/G80	2	24, 14

3.2. Screen of essential genes in ISCs involved in regenerative response

To select the genes that are essential in regenerative response to intestinal injury, we performed survival tests. For the survival tests, we recorded the deaths of the flies in the face of consecutive intestinal damage through ingestion of PQ. As we injure the fly with PQ the differentiated cells in the fly gut die, and the SCs start to replace the cells that are lost. If the SCs cannot replace those cells over the time the fly dies. If the SCs are functional and manage to repair the damage to the gut, the fly survives.

The results are graphs with survival curves. Increased death means that the specific gene has a direct and robust contribution to gut regeneration. Thus, survival curves

approaching 0% survival represent the essential genes for regeneration (Figure 3.1 and Table 3.3).

This can be seen because, in the graphs, control flies that are expressing Cherry-RNAi were compared with flies in which there was a knockdown or overexpression of the gene. The Cherry-RNAi flies serve as a control do to targeting the mCherry protein, a fluorescent protein that is not present in *Drosophil*a. It does not do knockdown, but in any case, the RNAi machinery is activated.

To analyze the graphs, we have two conditions, SUC and PQ, control solution and injury solution, respectively. For death to be related to regeneration, death must happen when we inhibit a gene only in the PQ condition and not in the SUC condition. If the flies die in SUC condition, it means that the gene has an essential function for the animal's survival independently of regeneration.

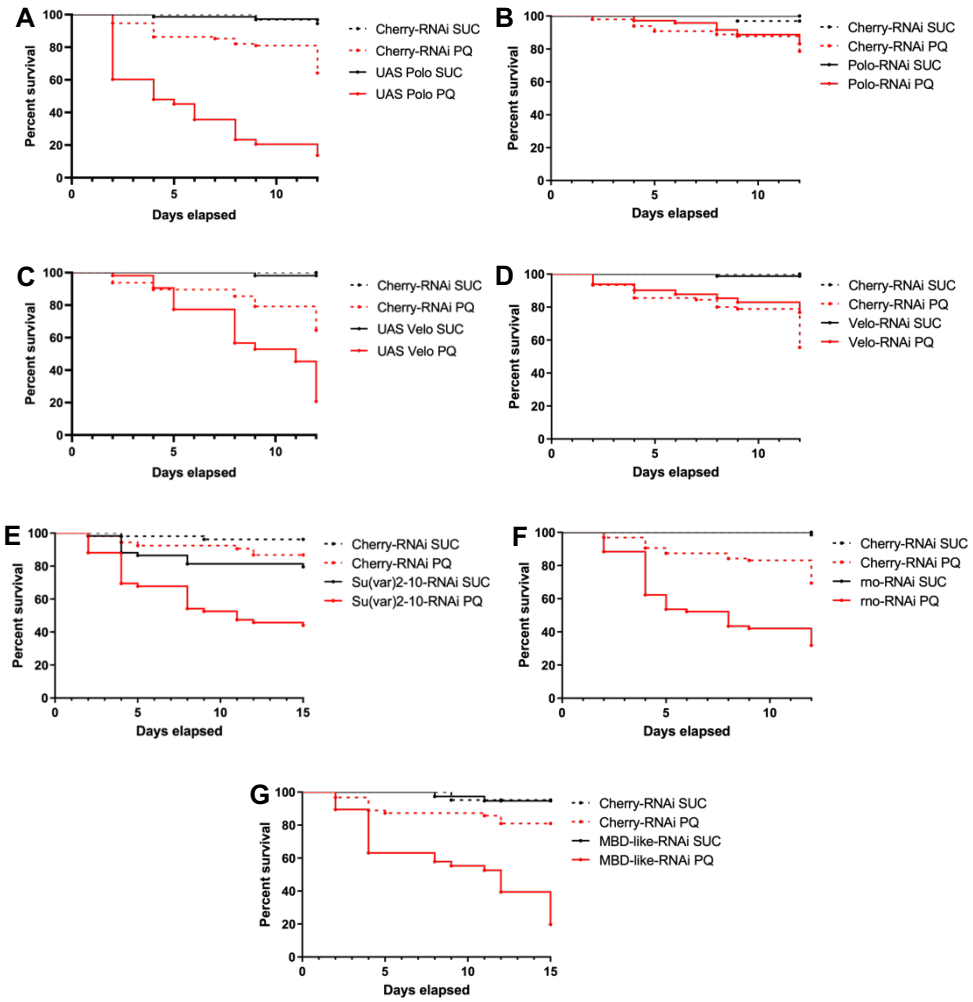

Figure 3.1 – Demography of flies expressing Polo transgene (**A**), Polo-RNAi (**B**), Velo transgene (**C**), Velo-RNAi (**D**), Su(var)2-10-RNAi (**E**), rno-RNAi (**F**) and MBD-like-RNAi (**G**) under the control of a stem cell-specific driver (Esg G4 and/or Esg G4/G80). All flies are female, and the number of flies (N) for each genotype/condition and for a total of 2-3 survival tests performed varies between 35-14 flies.

Table 3.3 - Positive hits in survival tests statistical analysis. Comparation between the PQ curves and between the SUC curves.

Genotype	Condition	Chi-square		p-value	
		Mantel-Cox	Wilcoxon	Mantel-Cox	Wilcoxon
Esg G4/G80 x UAS Polo	PQ	65.27	63.21	<0.0001	<0.0001
Esg G4/G80 x Polo-RNAi	PQ	0.5594	0.5926	0.4586	0.4414
Esg G4/G80 x UAS Velo	PQ	18.77	16.14	<0.0001	<0.0001
Esg G4/G80 x Velo-RNAi	PQ	6.997	6.288	0.0082	0.0122
Esg G4 x Su(var)2-10-RNAi	PQ	22.65	22.39	<0.0001	<0.0001
	SUC	6.993	7.055	0.0082	0.0079
Esg G4/G80 x rno-RNAi	PQ	29.23	30.23	<0.0001	<0.0001
Esg G4/G80 x MBD-like-RNAi	PQ	27.36	21.32	<0.0001	<0.0001

These data indicates that the overexpression of Polo gene significantly reduces fly survival (Figure 3.1 (A)), suggesting that this gene has a preponderant role during intestinal regeneration, affecting the stem cell's function. In contrast, knockdown of Polo appears to have an opposite effect, increasing fly survival (Figure 3.1 (B)). We cannot, with this analysis, conclude about the increase in survival, as this experiment was not designed for that purpose. However, these results may be interesting to evaluate further if there is an actual increase in survival.

For the Velo gene, interestingly, the results indicate that the overexpression of Velo gene reduces fly survival (Figure 3.1 (C)) and that the knockdown of the gene does not show an effect (Figure 3.1 (D)), which goes against what was expected since this gene is down regulated in aging.

We can also observe that the absence of the Su(var)2-10, rno and MBD-like genes reduces fly survival (Figure 3.1 (E, F and G)), and that for Su(var)2-10 there is also a decrease of the gene survival in SUC but, even though the value is significant (Table 3.3), the magnitude of death in PQ is bigger which indicates that this gene has a preponderant role during intestinal regeneration, affecting the stem cell's function.

From these five genes, only the Su(var)2-10, Velo and Polo genes were selected for the next step of the study.

The graphs for the genes we could not discern differences in the survival of flies with knockdown and/or overexpression, as only a small mortality was observed, are shown in appendices, in Figure A3.1 (Esg G4) and Figure A3.2 (Esg G4/G80), with the statistical analysis for both at Table A3.1.

3.3. Stem cell function

Since we identified the genes (Su(var)2-10; Velo and Polo) as having an effect on fly death during repeated injuries, we evaluated the proliferative capacity and other properties of the fly ISCs where these genes were manipulated, in response to regenerative pressure, by again exposing the flies to PQ and performing an immunohistochemical analysis. Thus, we asked, how do these genes affect the properties of the ISC?

3.3.1. Proliferation

The regulation of gene expression through epigenetic mechanisms is essential for governing the proliferation of SCs and controlling their ability to self-renew and differentiate. [38,39]

Epigenetic modifications, such as DNA methylation, histone modifications, and noncoding RNA-mediated processes, contribute to the precise control of gene activity in SCs. Research has shown that these epigenetic modifications can impact the expression of specific genes that play a vital role in maintaining the undifferentiated state of SCs and facilitating their proliferation.[40–42]

Disruptions in epigenetic regulation in these processes have been associated with various diseases and disorders, making it central to understand the role of epigenetic regulation in the proliferation of SCs.[43]

As mentioned above, we wanted to understand what is wrong with the ISCs that do not have the gene or have an overexpression of the gene. For this, we only used flies with the genetic manipulations where we saw positive hits in survival tests.

The first thing we evaluated was the SC ability to activate and proliferate, which is the first step in the stem cell response to stress and happens during the first 16h after the injury. After proliferating, the SC must give rise to new enterocytes and return to quiescence (to the deactivated state), recovering from the injury. To evaluate stem cell activation and proliferation, we performed immunohistochemical analysis to detect phosphohistone-3 (pH3), an immunomarker specific for cells undergoing mitoses, employed to track the proliferative SCs.

We studied at 16h post injury because that is when there is maximum proliferation, and at 24h, when the quiescence is re-established. We also studied at 48h to give a safety margin for the re-establishment of quiescence. If pH3 is present, the SCs have not return to quiescence. The results for flies expressing Su(var)2-10-RNAi and Velo transgene are shown below in Figure 3.4.

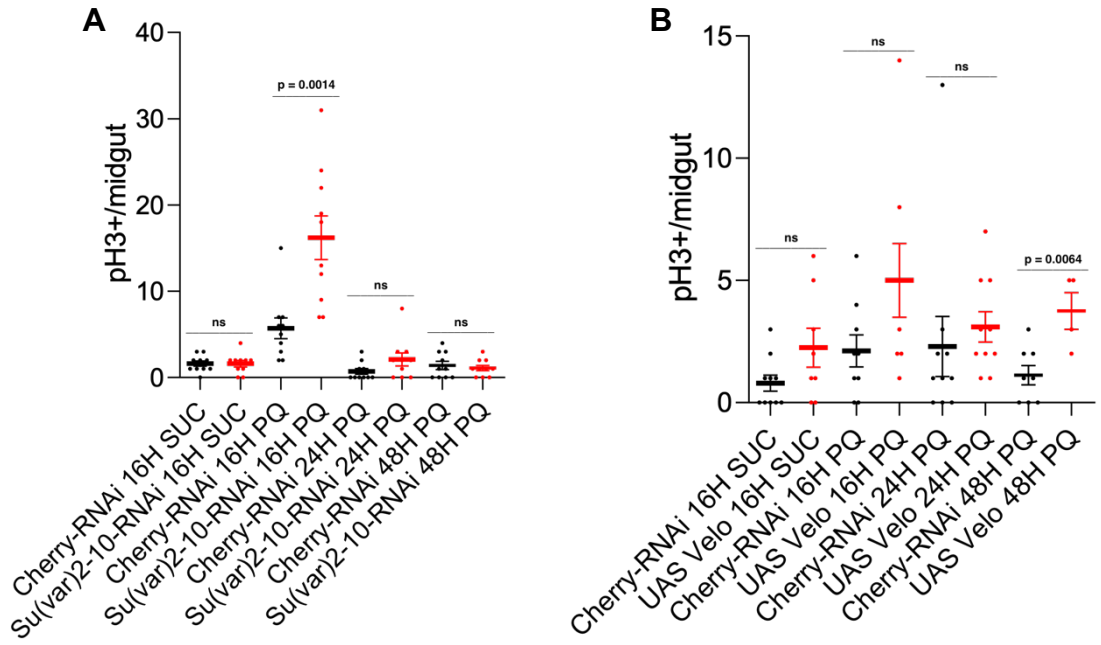

Figure 3.2 - Quantification of mitotic figures (pH3+ cells) from flies expressing Su(var)2-10-RNAi (A) and Velo transgene (B) under the control of a stem cell-specific driver (Esg G4 in A; Esg G4/G80 in B) for 16h in sucrose, and 16h, 24h and 48h after intestinal damage. An unpaired two-tailed Student´s t-test was used in the analysis (n=8-12 guts/condition).

The results obtained for Su(var)2-10-RNAi (Figure 3.2 (A)) suggest that the mitotic response of ISCs to intestinal acute oxidative stress damage (induced by PQ) is exacerbated when there is absence of Su(var)2-10. These results are demonstrated by the maximum proliferation of ISCs at 16h in the absence of Su(var)2-10 being higher than the maximum proliferation in the control (Cherry-RNAi). This over-proliferation can result in a disturbance in the normal cycle of the ISCs differentiation and incomplete replacement of the lost cells. There were no differences in proliferation between conditions at 16h in sucrose, nor in the re-establishment of quiescence at 24h and 48h after injury. At these time points, the number of pH3 positive ISCs was low in both conditions since the SCs are at the deactivated state. These results indicate that the absence of Su(var)2-10 in ISCs during a regenerative event does not cause defects in the ISC return to quiescence.

We observed in Velo overexpression (Figure 3.2 (B)) a trend for increase activation of stem cells at 16h post injury, and an over proliferation at 48h, suggesting a prolonged activation, meaning that the overexpression of Velo leads to a deactivation problem.

We could not take precise conclusions from the immunohistochemical analysis of the overexpression of Polo since the death for those flies, even with one cycle of PQ and despite the various attempts, was too high to obtain sufficient flies for analysis.

3.3.2. Morphology

The next step in our project was to observe and quantify the stem cells for the three genes that gave positive hits in the survival tests. The slides analyzed for effect of stem cell proliferation were also used for quantification using the confocal laser scanning microscope. In these sections ISCs were identified based on the expression of Green Fluorescent Protein (GFP) under the control of the Esg promotor. After imaging we then quantified the cells, using the Fiji Software, for the 3 genotypes and for all conditions in three aspects: cells size, total cell number and cell density.

In general, it is known that the SCs tend to grow as they differentiate.[6] These data (Figure 3.3 (A and B)) indicate that at a deactivated state (16h SUC) the ISCs are smaller in the absence of Su(var)2-10 and that in the peak of proliferation (16h PQ) they are bigger when compared with the control, meaning that there are cells differentiating at the same time point that there is an exacerbated proliferation of ISCs.

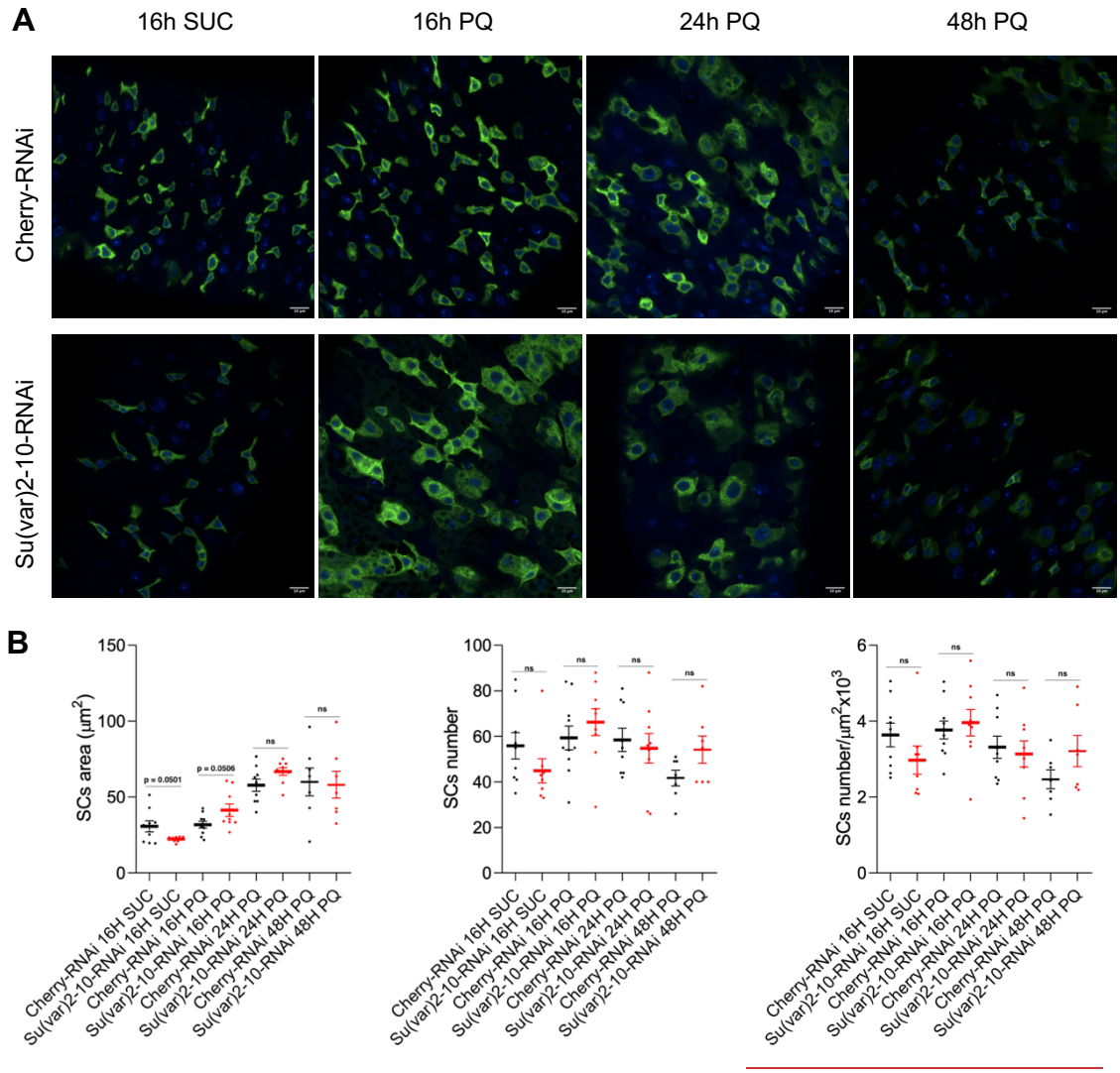

Figure 3.3 – (A) Representative micrographs of stem cells and nucleus, scale bar = 10 µm (Esg EGFP – green; DAPI - blue); **(B) Quantification of stem cells from flies expressing Su(var)2-10-RNAi** under the control of a stem cell-specific driver (Esg G4) for 16h in sucrose, and 16h, 24h and 48h after intestinal damage. An unpaired two-tailed Student´s t-test was used in the analysis (n=8-12 guts/condition).

These data (Figure 3.4 (A and B)) show that in general, and for all the conditions, the ISCs in Velo overexpression are smaller and fewer than in the control and have an irregular shape. As previously mentioned, Velo overexpression showed a trend for increase proliferation of ISCs at 16h post injury, and an over proliferation and continuous activation at 48h post injury, potentially related with deactivation and differentiation defects. These data and the fact that the stem cells are smaller, suggests that the overexpression of Velo keeps the cells in a stem state (non-differentiated stem) in which the cells proliferate more, in higher levels when activated, and keep proliferating since they cannot differentiate. We conclude this by their size because, as mentioned before, the stem cells tend to grow as they differentiate, suggesting they stay at a stem state.

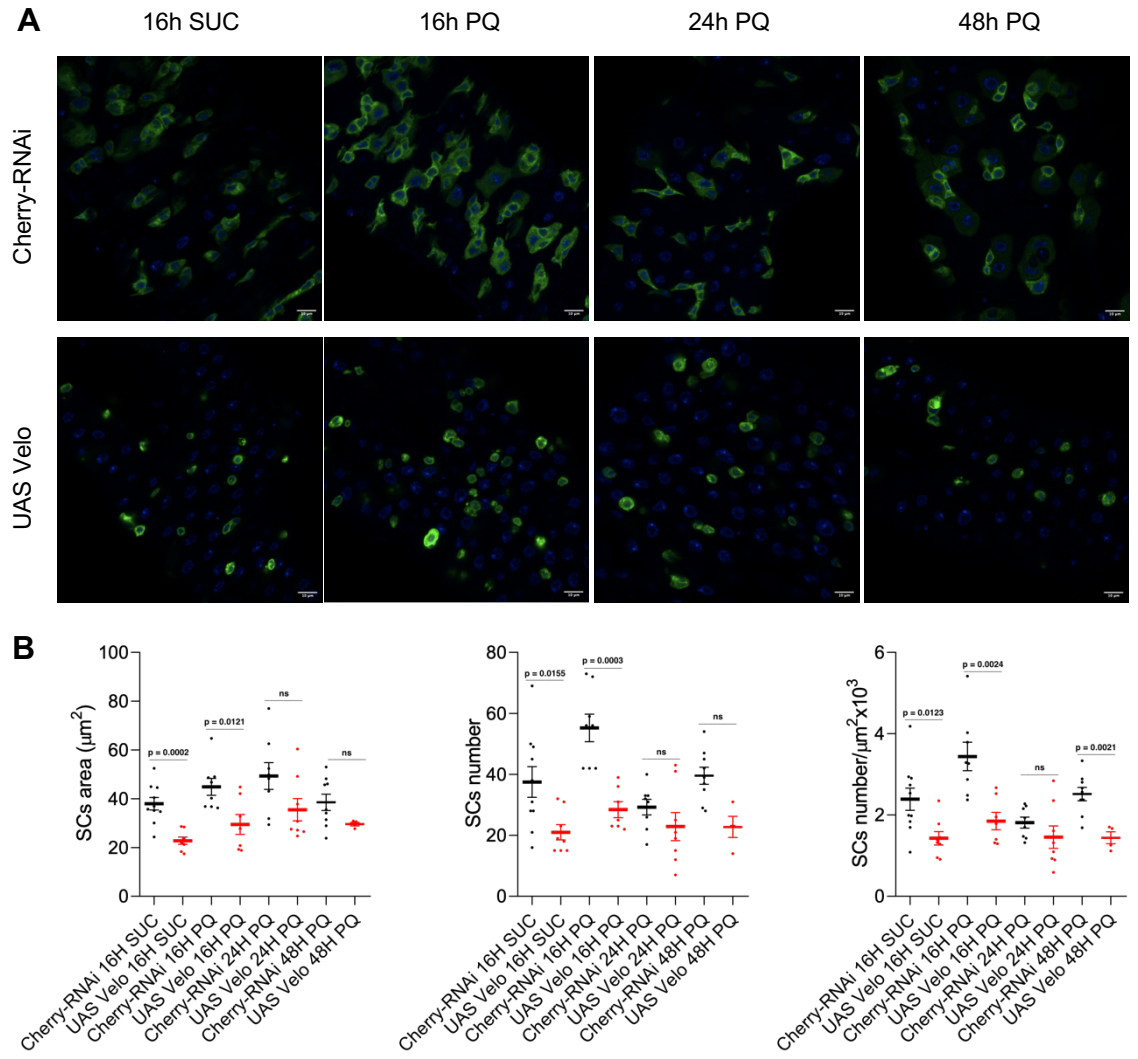

Figure 3.4 - (A) Representative micrographs of stem cells and nucleus, scale bar = 10 µm (Esg EGFP – green; DAPI - blue); **(B) Quantification of stem cells from flies expressing Velo transgene** under the control of a temperature sensitive stem cell-specific driver (Esg G4/G80) for 16h in sucrose, and 16h, 24h and 48h after intestinal damage. An unpaired two-tailed Student´s t-test was used in the analysis (n=8-12 guts/condition).

Even though we did not have enough guts to take conclusions about the proliferative aspect of the ISCs overexpressing Polo, we took the images for the conditions of 16h SUC, and 16h and 48h PQ to evaluate changes in cell morphology.

The data (Figure 3.5 (A and B)) showed that, for all the conditions, the ISCs overexpressing Polo were larger and in a much lower amount than the ISCs expressing Cherry-RNAi. This suggests that the overexpression of Polo may disturb the activation and normal cycle of the SCs, since at 16h post injury we observed gigantic cells, possibly meaning that they were differentiating at an early stage and not proliferating, justifying the little amount of ISCs. The ISCs overexpressing Polo were not functional, could not

give rise to all the cells that were lost, and the homeostasis of the gut was never recovered, which explains the high rate of death.

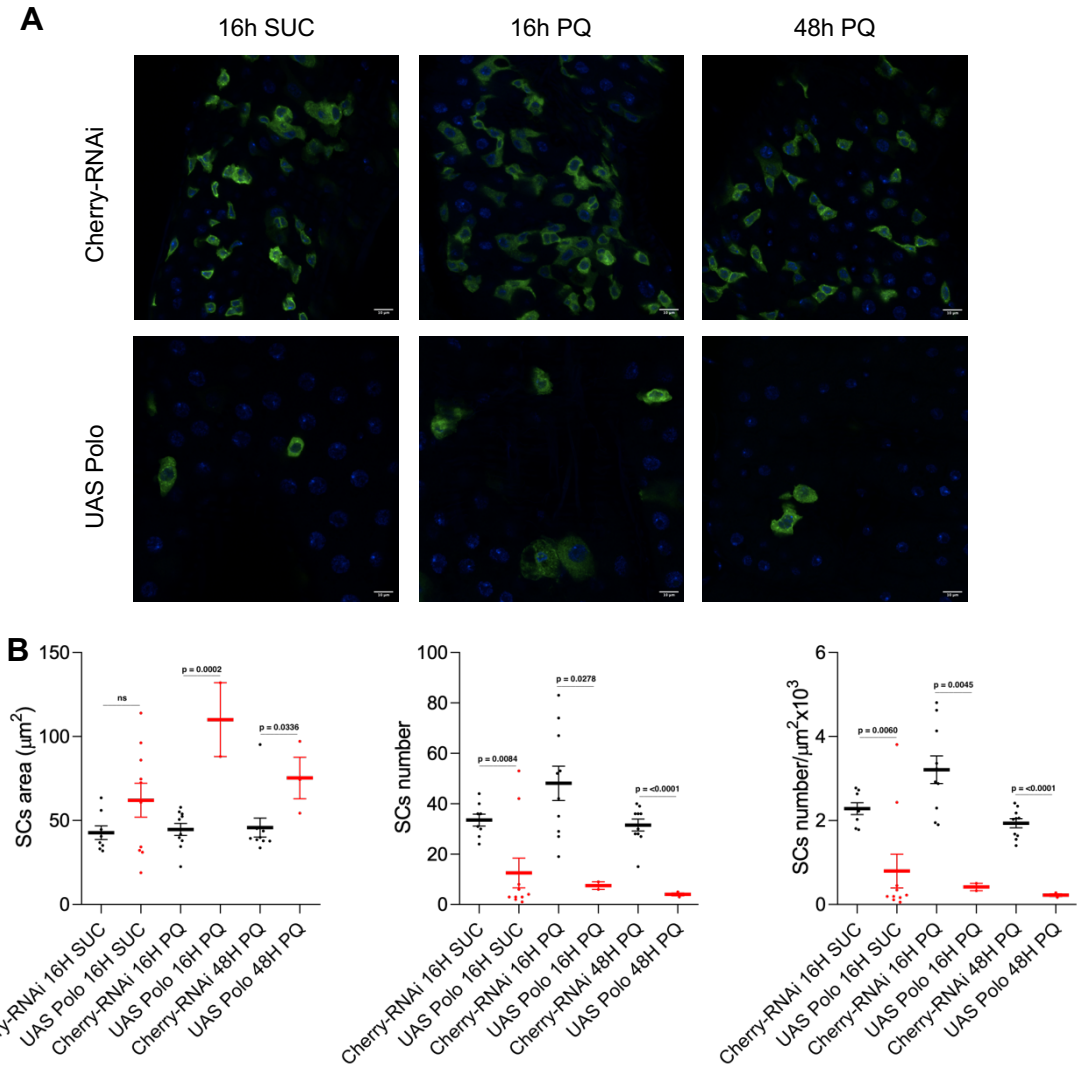

Figure 3.5 - (A) Representative micrographs of stem cells and nucleus, scale bar = 10 µm (Esg EGFP – green; DAPI - blue); **(B) Quantification of stem cells from flies expressing Polo transgene** under the control of a temperature sensitive stem cell-specific driver (Esg G4/G80) for 16h in sucrose, and 16h and 48h after intestinal damage. An unpaired two-tailed Student´s t-test was used in the analysis (n = 2-10 guts/condition).

4. Discussion and Conclusions

This project focused on further investigating and discovering new epigenetic regulators with a role in SC aging and consequent characterization of the role of these regulators in the functioning of the SC during regeneration. Epigenetic modifications directly impact the expression of key transcription factors and signaling pathways involved in ISC maintenance and regeneration. Therefore, this project relied on inducing intestinal damage to assess cell behavior during the cycle of regeneration in the face of damage. This was done by feeding the *Drosophila melanogaster* flies (our animal model) a diet composed by a toxic component that causes intestinal damage.

In this project, the analyses were performed using candidate genes that had been previously selected for being present in ISCs, regulated in aging ISCs, expressed in mammalian SCs, and regulated in mammals.

Our first approach was to identify the genes from *Drosophila melanogaster* that are essential in regenerative response to intestinal injury. For this, we performed fly survival tests, causing controlled intestinal damage on the flies that had a knockdown and/or overexpression of the genes. We identified three genes of interest from the results, Su(var)2-10, Velo and Polo.

Polo gene was our most exciting result, as fly survival when this gene is overexpressed decreased dramatically. This gene is up regulated in aged ISCs, meaning that as the fly gets older there will be a higher expression of the gene. By doing knockdown of Polo, we are confirming that nothing happens if we keep the levels of the gene low, and by doing overexpression of Polo we are mimicking what happens when the flies get older. Through the study of morphology in stem cell function we found that the overexpression of Polo appears to disturb the activation and normal cycle of the ISCs, which goes against the phenotype of aged ISCs, possibly meaning that they are differentiating at an early stage and not proliferating, justifying the little amount of ISCs and the high rate of death. This is corroborated by a recent finding (published during this work) that shows that enforced expression of Polo causes the loss of ISCs in *Drosophila midgut*. There is also evidence that indicates that just the ISC-specific activation of Polo restricts the proliferation of SCs, meaning that Polo function in regulating nuclear and cytoplasmic aspects of the mitotic cycle itself is a major cause for the loss of ISCs.[44,45]

Su(var)2-10 gene is down regulated in aging in ISCs, which means that as the fly gets older there will be a lower expression of the gene, meaning that we are mimicking that when we do the knockdown of Su(var)2-10 and that the low rate of survival in this case means that the gene is necessary for regeneration. Analyzing the proliferative capacity of the ISCs in the absence of Su(var)2-10 we observed that these cells exhibit an

exacerbated proliferation and bigger cells than the control, which goes accordingly with what happens in aged ISCs, where there is an increased proliferation and a decline in function.[46] This loss of function can possibly lead to a defective SC cycle and compromise the reestablishment of gut homeostasis since the cells must activate, proliferate, and give rise to new differentiated cells in a defined time. The exacerbated proliferation can also give rise to tumor cells.[47]

For Velo we know that is down regulated in aging, meaning a higher expression of the gene at a younger stage of the fly age. Interestingly, the results indicate that the overexpression of Velo gene reduces fly survival and that the knockdown of the gene may increase it, which goes against what was expected. As mentioned above, one of the reasons why these genes were selected for the screening was because they are regulated in aging, but there may be other physiological conditions that lead to stem cell dysregulation that cause expression of Velo to increase. It does not explain the aging phenotype, but it can explain the phenotype in another condition where there is upregulation of Velo. We performed the proliferative capacity analysis for the ISCs for overexpression of Velo and detected a trend for increase activation, suggesting a prolonged activation, meaning that the overexpression of Velo leads to a deactivation problem. The results for the ISCs morphology suggest that the overexpression of Velo keeps the cells in a stem state (non-differentiated stem) in which the cells proliferate more, in higher levels when activated, and keep proliferating since they cannot differentiate. We conclude this by their size because, as mentioned before, the stem cells tend to grow as they differentiate, suggesting they stay at a stem state.

There is not much information in the literature about the role of Velo gene in the SUMOylation pathway besides encoding a SUMO protease, meaning that the overexpression of Velo affects this enzyme in some way. For the Su(var)2-10 gene it is known to have a role in which SUMOylation is involved: normal heterochromatic functions, being involved in maintenance of chromosomal structure and chromosomal inheritance, being a negative regulator of the JAK/STAT pathway and being associated with telomeres and nuclear periphery during interphase.[48] If Su(var)2-10 is a negative regulator of the JAK/STAT pathway, it means that the protein that the gene encodes plays a role in downregulating the activity of the pathway.[22] Since the JAK/STAT pathway leads to activation and proliferation of SCs, by doing the knockdown of the Su(var)2-10 gene, the protein is absent and promote ISCs over proliferation.

With this work, as proposed, we were able to identify three genes altered in aged ISCs that affect stem cell function at distinct levels and regulate regenerative capacity.

5. Future perspectives

In the future, further investigation of these three genes and they exact role as epigenetic regulators would be necessary. A future experiment would be to evaluate the ISC function maintenance in aging maintaining the selected genes expression at youthful levels.

It would also be promising that, from the discovery of these new epigenetic regulators in *Drosophila*, these could be used in future studies in mammals.

Overall, the study's results provided insight into the impact and importance of epigenetic regulators in ISC aging.

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Appendixes

A1

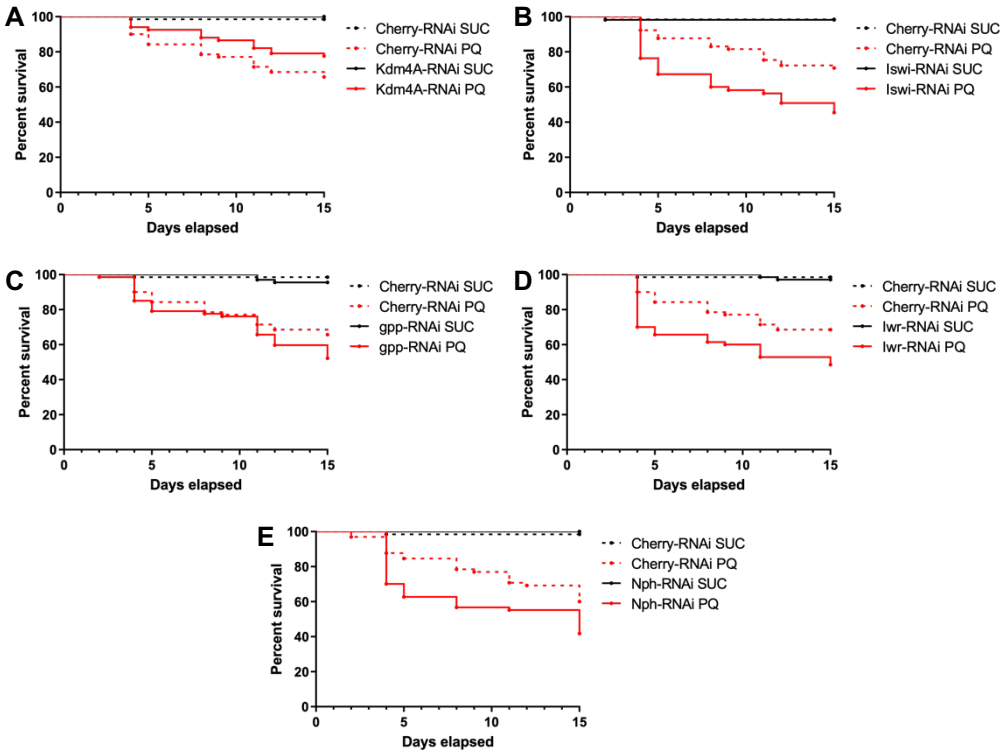

Figure A3.1 – Demography of flies expressing Kdm4A-RNAi (A), Iswi-RNAi (B), gpp-RNAi (C), Iwr-RNAi (D) and Nph-RNAi (E) under the control of of a stem cell-specific driver (Esg G4). All flies are female, and the number of flies (N) for each genotype/condition and for a total of 2-3 survival tests performed varies between 35-14 flies.

A2

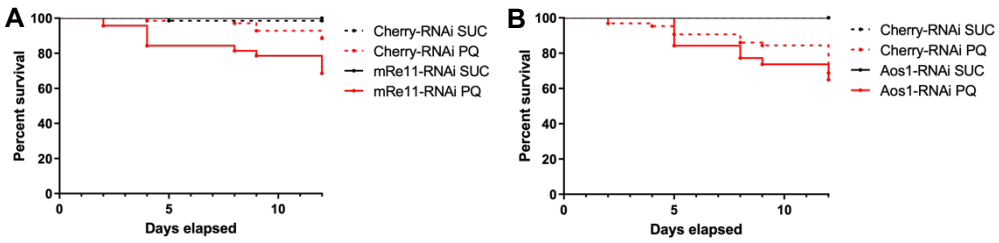

Figure A3.2 - Demography of flies expressing mRe11-RNAi (A) and Aos1-RNAi (B) under the control of a temperature sensitive stem cell-specific driver (Esg G4/G80). All flies are female, and the number of flies (N) for each genotype/condition and for a total of 2-3 survival tests performed varies between 35-14 flies.

A3

Table A 3.1 - Remaining genes survival tests statistical analysis. Comparation of the PQ curves for the genes graphed at Figure A3.1 and Figure A3.2.

Genotype	Chi-square		p-value	
	Mantel-Cox	Wilcoxon	Mantel-Cox	Wilcoxon
Esg G4 x Iswi-RNAi	8.410	8.807	0.0037	0.0030
Esg G4 x Kdm4A-RNAi	2.482	2.556	0.1152	0.1099
Esg G4 x gpp-RNAi	2.159	1.826	0.1418	0.1767
Esg G4 x Iwr-RNAi	6.279	7.302	0.0122	0.0069
Esg G4 x Nph-RNAi	4.780	5.728	0.0288	0.0167
Esg G4/G80 x mRe11-RNAi	8.703	9.110	0.0032	0.0025
Esg G4/G80 x Aos1-RNAi	0.1112	0.4320	0.7388	0.5110