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Deciphering the role of the histone H3 variant CENP-A in the adult *Drosophila* intestine

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Deciphering the role of the histone H3 variant CENP-A in the adult *Drosophila* intestine

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I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or material than those explicitly indicated.

The work was carried out at Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH) in the group „Chromatin & Centromere Biology“ of Prof. Dr. Sylvia Erhardt.

…….

To my grandfather

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Summary

The adult *Drosophila* midgut is an excellent model, in which to address how stem cell identity is defined and maintained, due to its simple structure, powerful genetics and similarity to the mammalian intestine. The epithelium turnover is carried out by multipotent intestinal stem cells (ISCs), which proliferate throughout life, renewing and generating transient committed cells called enteroblasts (EBs), which differentiate either into enterocytes (ECs) or enteroendocrine cells (EEs). The regulation for the progression from ISC to a terminally differentiated cell includes epigenetic mechanisms, however little is known about it in this specific stem cell system.

In this study, I analyzed the distribution pattern of the histone H3 variant CENP-A in the adult *Drosophila* midgut. CENP-A is the epigenetic mark of centromeres, which identify the specific chromatin regions that mediates spindle attachment during chromosome segregation. Even though centromeres orchestrate chromosome inheritance, their positions on chromosomes are primarily specified epigenetically rather than by a specific DNA sequence in multicellular organisms.

Employing different strategies, I found that CENP-A is asymmetrically inherited in cells of the midgut epithelium, where previously synthesized ('old') CENP-A is retained specifically in ISCs. Remarkably, long-term experiments revealed that CENP-A can persist in ISCs for more than 20 days. The stability and persistency of CENP-A supports the idea that CENP-A could act as an epigenetic mark responsible for regulating stem cell properties. Analyzing the distribution of this histone variant in somatic cells provided evidence that the asymmetric distribution of CENP-A is a mechanism specific of stem cells. In contrast to CENP-A, the histone variant H3.3 does not exhibit asymmetry during ISC division.

CENP-A and its loading factor CAL1 have always been studied in the context of cell division. However, data from this study suggest that CENP-A may play a role in non-dividing cells as well. I could show that the depletion of inner kinetochore proteins in the non-dividing committed progenitor cells leads to the loss of these cell types, indicating that CENP-A and CAL1 are important for EBs maintenance and differentiation. ECs also seem to be affected by the depletion of kinetochore proteins, ECs undergo endocycles, that are also characteristic for salivary glands, follicle cells and ovarian nurse cells. Cells of salivary glands lacking CAL1 failed to undergo endoreduplication and correct S-phase progression.

Taken all together, I propose a novel role of the histone H3 variant CENP-A in stemness, by which it contributes to the maintenance of intestinal stem cell identity. Furthermore, CENP-A and other inner kinetochore proteins are also important in non-dividing differentiated cells. Specifically, I identified CAL1 as a possible regulator of endocycle progression.

Zusammenfassung

Durch die Ähnlichkeit zum Säuger-Darm und durch die einfache Struktur und Genetik ist der adulte Mitteldarm von *Drosophila* ein exzellentes Modell dafür, wie die Identität von Stammzellen definiert und aufrecht erhalten wird. Die Integrität des Epitheliums wird durch multipotente intestinale Stammzellen (ISCs) bewerkstelligt, welche ein Leben lang proliferieren. Dadurch erneuern sie sich selbst und generieren transient determinierte Enteroblasten (EBs), welche in Enterozyten (ECs) oder enteroendokrine Zellen (EEs) differenzieren. Die Regulierung der Entwicklung von ISCs zu endgültig differenzierten Zellen schließt epigenetische Mechanismen ein, wenngleich Details in diesem Stammzellsystem weitestgehend unbekannt sind.

In dieser Studie habe ich die Verteilungsmuster der Histon H3-Variante CENP-A im adulten Mitteldarm von *Drosophila* untersucht. CENP-A ist der epigenetische Faktor, welcher Centromere als die spezifische Chromatin-Region markiert, welche die Verbindung der Chromosomen mit der Spindel während der Chromosom-Segregation bewerkstelligt. Obwohl Centromere die Vererbung von Chromosomen bewerkstelligen, wird ihre Position auf Chromosomen in multi-zellulären Organismen nicht durch die DNA-Sequenz, sondern primär epigenetisch bestimmt.

Unter Verwendung verschiedener Strategien habe ich herausgefunden, dass CENP-A asymmetrisch in Epithelzellen des Mitteldarms vererbt wird, wobei zuvor synthetisiertes (‚altes') CENP-A spezifisch in ISCs beibehalten wird. Bemerkenswerterweise zeigten Langzeit-Experimente dass CENP-A für mehr als 20 Tage in ISCs bestehen kann. Die Stabilität und Beständigkeit von CENP-A unterstützt die Idee, dass CENP-A als epigenetischer Faktor für die Regulation von Stammzell-Eigenschaften verantwortlich ist. Die Untersuchung von somatischen Zellen hat gezeigt, dass die asymmetrische Verteilung von CENP-A ein Stammzell-spezifischer Mechanismus ist. Im Gegensatz zu CENP-A zeigt die Histon Variante H3.3 keine Asymmetrie während der Zellteilung von ISCs.

CENP-A und der spezifische Beladungsfaktor CAL1 wurden stets im Kontext von Zellteilung untersucht. Daten dieser Studie schlagen hingegen eine zusätzliche Funktion in sich nicht-teilenden Zellen vor. Ich konnte zeigen, dass die Depletion von Proteinen des inneren Kinetochors in nicht-teilenden Vorläufer-Zellen zum Verlust dieses Zelltyps führt, was darauf hinweist, dass CENP-A und CAL1 wichtig für die Erhaltung und

Differenzierung von EBs sind. Auch ECs sind scheinbar betroffen von der Depletion von Kinetochor-Proteinen. ECs unterlaufen Endo-Zyklen, die auch charakteristisch für Speicheldrüsen, Follikel-Zellen und Nurse-Zellen der Eierstöcke sind. Speicheldrüsen-Zellen, die kein CAL1 enthalten, konnten keine Endoreduplikation durchführen und somit nicht durch die S-Phase fortschreiten.

Zusammengefasst schlage ich eine bisher unbekannte Rolle der Histon H3-Variante CENP-A für Stammzelleigenschaften vor, wobei es zum Erhalt der Identität intestinaler Stammzellen beiträgt. Außerdem sind CENP-A und andere Proteine des inneren Kinetochors wichtig für nicht-teilende, differenzierte Zellen. Dabei habe ich CAL1 als möglichen Regulierungsfaktor für endozyklischen Fortschritt identifiziert.

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List of Abbreviations

5mC	5-methylcytosine
β-Gal	beta-galactosidase
µg	microgram
µl	microliter
µm	micrometer
ACD	asymmetric cell division
APC	Anaphase-Promoting Complex
arm	armadillo
bp	base pairs
BTP	bromothenylpteridine
CAL1	Chromosome alignment defect 1
CATD	CENP-A targeting domain
CCAN	Constitutive Centromere Associated Network
Cdk2	Cyclin-dependent kinase 2
CENP-A	Centromere Protein-A
CENP-C	Centromere Protein-C
CID	Centromere identifier
CO-FISH	Chromosome Orientation Fluorescence In Situ Hybridization
CPC	Chromosomal Passenger Complex
CycE	Cyclin E
d	days
DAPI	4',6-Diamidin-2-phenylindol

ddH_2O	double distilled water
°C	degree Celsius
Dl	Delta
DNA	deoxyribonucleic acid
EB	enteroblast
EC	enterocyte
Ecc15	*Erwinia carotovora*
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EE	enteroendocrine cell
Esg	escargot
EtBr	ethidium bromide
F/O	Flip out
FLP	flippase
FRT	FLP Recombination target
g	gram(s)
Gem	geminin
GFP	Green Fluorescent Protein
h	hour(s)
HAC	Human Artificial Chromosome
HS	Heat shock
hsFLP	Heat-shock FLPase
HJURP	Holliday Junction Recognition Protein
ISC	Intestinal Stem Cell

IF	immunofluorescence
kb	kilobase pairs
kDa	kiloDalton
m	milli-
M	molar
min	minute(s)
mRNA	messenger RNA
MT	microtubule
MTOC	microtubule-organizing center
o/e	overexpression
o/n	overnight
ORF	open reading frame
PAGE	poly-acrylamide gel electrophoreses
PBS	Phosphate Buffer Saline
PCM	pericentriolar material
PCR	polymerase chain reaction
P.e	*Pseudomonas entomophila*
PFA	paraformaldehyde
PH3	phospho histone H3
Pros	prospero
PTM	post-translational modification
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference

rpkm	reads per kilobase million
rpm	revolutions per minute
ROI	region of interest
RT	room temperature
SCs	stem cells
SDS	sodium dodecyl-sulfate
smFPs	“spaghetti monster” fluorescent proteins
SSIS	Strand-specific imprinting and selective chromatid segregation
Su(H)	Suppressor of Hairless
TF	transcription factor
Tris	tris(hydroxymethyl)aminomethne hydochloride
UAS	Upstream Activating Sequence
UV	ultraviolet
VM	Visceral muscle
WB	western blot
WFE	whole fly extracts
W	white gene
wt	wildtype

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1 Introduction

1.1 The fruit fly as model for stem cell studies

1.1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster is one of many animal models widely used in genetic, molecular and biochemical studies. It is commonly known as the fruit fly and has been used as a model organism for genetic studies since the discovery of the *white* mutation by T. H. Morgan (Morgan, 1910). The advantages of *Drosophila* as experimental organism are numerous. It is small, economic, easy to handle and has a short generation time, only 10 days when maintained at 25°C (Dow and Romero, 2010). An additional advantage is the fact that flies have only four chromosomes (three autosomes and one sex chromosome), which have a high degree of genetic conservation. The genome of the fruit fly was sequenced already in 2000 and comparative genomic studies have shown that over 70% of human disease related genes are conserved in this small model organism (Reiter et al., 2001). Moreover, a huge amount of resources is available for researchers, including online databases and stock centers, such as Flybase, BDSC or VDRC.

A robust technique which revolutionized the generation of *Drosophila* transgenics was the P-element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). More than 30 years after its discovery, this technique was essential for this study. P-element mediated transposition permitted the first generation of enhancer trap lines which allowed the identification of cell and tissue-specific enhancers in the genome. An enhancer trap contains a minimal promoter region, insufficient itself to induce transcription. If, after transposition, it inserts in a region under the influence of a local genomic enhancer, the inserted sequence will be transcribed in a pattern reflecting the enhancer activity. The first used enhancer trap was P{lacZ} that used the *E.coli* gene lacZ which encodes β-galactosidase, the enhancer activity could be simply visualized by staining for β-Gal. A second generation of enhancer traps enabled the development of the GAL4/UAS system adapted from yeast (Brand and Perrimon, 1993; Duffy, 2002) which, instead of visualizing the reporter directly, expresses a yeast transcription factor functional in *Drosophila*, GAL4. GAL4 can be then used to drive cell/tissue-specific expression of

transgenic constructs placed downstream of the activation signal UAS, i.e. reporter fusions, overexpressors, RNAi.

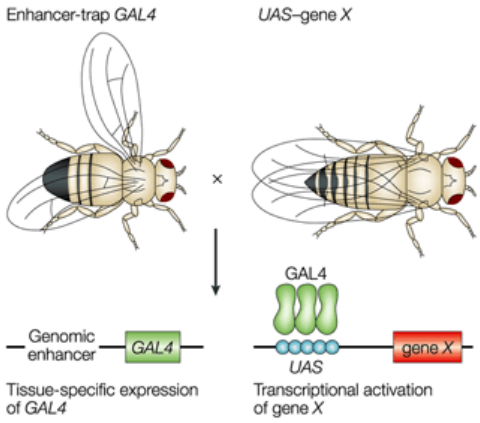

Figure 1.1 The GAL4/UAS system

The yeast transcription factor GAL4 can regulate gene expression by inserting the upstream activating sequence (UAS), to which GAL4 binds, next to a gene of interest (gene X). The expression of GAL4 is under the control of a nearby genomic enhancer. A lot of enhancer traps have been created, making possible to express GAL4 in a huge variety of cell- or tissue-specific patterns. By crossing the two lines containing the UAS and the GAL4 gene, the progeny will express GAL4, and this will stimulate expression of gene X in a pattern reflecting the genomic enhancer (adapted from (St Johnston, 2002)).

1.1.2 Lifecycle of *Drosophila melanogaster*

Drosophila melanogaster undergoes a four-stage life cycle, being each stage clearly identifiable: egg, larva, pupa and fly. The life cycle is temperature sensitive. At 25°C the life cycle is 10 days, whereas when kept at 18°C lasts about 20 days.

Once fertilized, the embryos develop in the egg chamber for around 24 hours before hatching as first instar larvae. The larval phase involves an exponential growth and it can be divided into three moult-separated instars with a total duration of five days, where the first and second instars last for one day, and the third lasts for two to three days. At the end of the third instar, the larvae stop feeding and pupate.

During the four days of pupation and metamorphosis, the imaginal discs give rise to adult structures and the clear majority of the remaining larval tissues undergoes histolysis, a degenerative process. Once the process is complete, adult flies emerge from the pupal case and will be sexually mature six to eight hours after eclosion (Ashburner et al., 2005).

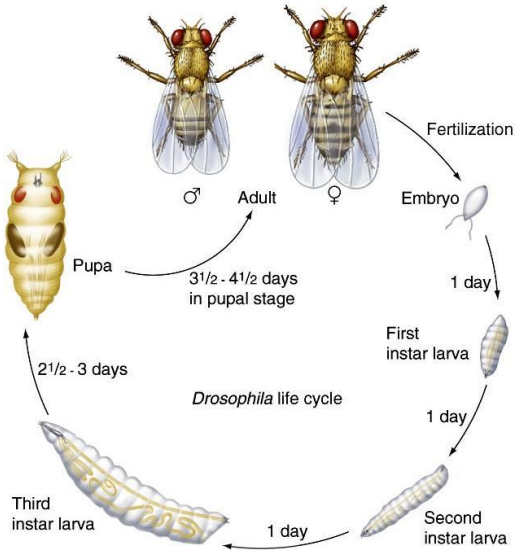

Figure 1.2 Lifecycle of *Drosophila melanogaster*

Embryogenesis last for one day before the egg hatches into a larva. The larval stages consist of three instars, where in total lasts for 4-5 days. During the pupal stage the animal undergoes metamorphosis and 5 days after the fly emerges. Image available on http://morphologicallydistbed.weebly.com/the-biology.html

1.1.3 Stem cells in *Drosophila melanogaster*

Stem cells (SCs) are undifferentiated cells that possess two essential characteristics. First, they safeguard their existence by their ability to self-renew. Secondly, they give rise to all distinct differentiating cell lineages of their respective tissue by having a defined developmental capacity (Till and Mc, 1961; Weissman, 2000).

One of the most popular invertebrate models for stem cell research is the fruit fly. *Drosophila melanogaster* retains several populations of stem cells during adulthood as well as transient populations of stem cells during development (Fig. 1.3, Pearson et al., 2009). These stem cell population include germline, stromal, hematopoietic, intestinal and neural stem cells. Additionally, it has been discovered, using a lineage tracing strategy, a population of small multipotent stem cells in the proximal segment, termed renal and nephric stem cells (RNSCs) (Singh et al., 2007).

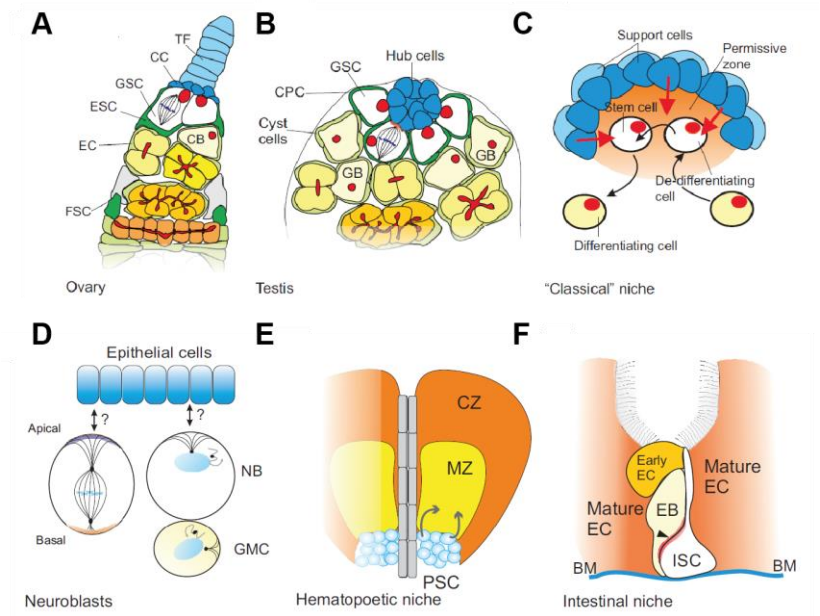

Figure 1.3 *Drosophila* stem cell models

(A) Schematic diagram of the *Drosophila* female GSC niche. (B) The testicular stem cell niche. (C) Stromal model of a stem cell niche. (D) *Drosophila* neural stem cells, the neuroblasts. (E) Larval hematopoietic niche. (F) Scheme of the *Drosophila* intestinal stem cell niche. Adapted from (Pearson et al., 2009).

1.1.3.1 The stem cell niche

Stem cells reside in specific tissue microenvironments, known as 'niches' (Schofield, 1978). Niches have been identified in numerous tissues. These highly specialized zones regulate the formation of daughter cells with different developmental potentials providing the molecular signals and conditions required to both maintain stem cell fate and ensure proper regulation of the differentiation process, reviewed in (Morrison and Spradling, 2008). The signaling of the stem cell niche does not only play a role in homeostasis, but also in disease or tumor formation. It was recently published, how niche signals that are commonly used to activate intestinal stem cell proliferation in the *Drosophila* midgut after epithelial damage can also stimulate tumor growth creating so a special niche microenvironment that facilitates tumor progression (Patel et al., 2015).

1.1.3.2 Stem cell division

The main characteristic of stem cells is their ability to form more stem cells (self-renewal) and to produce differentiated cells. Stem cells can accomplish these important tasks by asymmetric cell division (ACD). However, stem cells can also undergo symmetric cell division (Morrison and Kimble, 2006). Symmetric cell divisions produce two daughter cells with the same fate, either two stem cells or two progenitor cells, whereas ACD generate one stem cell and one daughter cell committed to differentiate (Fig. 1.4). It is thought that ACD is the mechanism mostly employed to maintain tissue homeostasis and therefore the balance between self-renewal and differentiation, since it does not imply an increase in the stem cell population (Inaba and Yamashita, 2012).

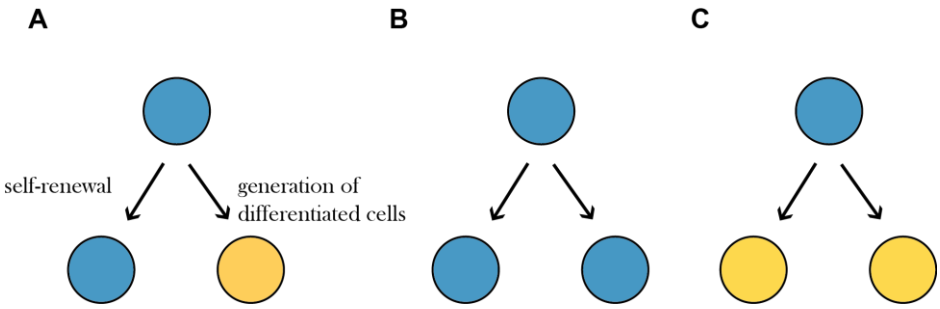

Figure 1.4 Stem cell division

(A) Stem cell dividing asymmetrically to generate a stem cell (blue) and a differentiated cell (yellow). (B-C) Possible outcomes of a symmetric stem cell division, formation of two stem cells (B) or the generation of two differentiated cell (C).

The equilibrium between these two modes of division is established by developmental and environmental signals to produce the appropriate number of stem cells and differentiated daughters that is required at any given moment. In the case of intestinal cells, which will be the matter of this study, they follow stochastic behavioral patterns, in which the choice between differentiation and stemness is balanced at the population level and not at the lineage level, leading so to the neutral competition of the ISC lineages (de Navascues et al., 2012). Misregulation of stem cell maintenance, proliferation or differentiation has been associated with a variety of disorders, including age-related diseases (Boyette and Tuan, 2014), rare genetic disorders (Scaffidi and Misteli, 2008) and many types of cancer (Reya et al., 2001). Thus, it is essential to characterize the regulating factors as well as the signaling pathways that control stem cell-related processes.

1.1.3.3 Centrosomes and ACD

The centrosome is the main microtubule-organizing center (MTOC) and consists of a pair of centrioles surrounded by pericentriolar material (PCM). The centrosome influences a wide variety of MT-related processes, including cell shape, cell motility, intracellular trafficking, organelle positioning and cell division. Centrioles are duplicated in a semiconservative manner and thus, centrosomes can be distinguished based on the age of their centrioles) (Nigg and Stearns, 2011). Differences in the age of the centrioles translate into differences in molecular composition, structure and function (Azimzadeh and Marshall, 2010).

Asymmetric centrosome segregation has been reported in *Drosophila* male germline stem cells (Yamashita et al., 2007), neuroblasts (Rebollo et al., 2007) and mouse neural progenitor cells (Wang et al., 2009). In these cases, either the mother or daughter centrosome is specifically segregated into the stem cells upon division. In male GSCs and mouse progenitor cells, the mother centrosome is retained by the stem cells, but in neuroblasts is the daughter centrosome the one inherited by the stem cell (Januschke et al., 2011). It is thought that the non-random centrosome segregation may contribute to the differential cell fate determination cell after division.

1.2 The adult *Drosophila* midgut

The adult *Drosophila* midgut is an excellent model, in which to address how stem cell identity is defined and maintained, due to its simple structure, powerful genetics and similarity to the mammalian intestine. The fruit fly intestine structurally consists of a monolayer cell epithelium enveloped by two layers of visceral muscle (VM) (Jiang and Edgar, 2009). Similar to the mammalian intestine, the epithelium turnover is carried out by multipotent intestinal stem cells (ISCs) (Micchelli & Perrimon, 2006). ISCs reside basally, show a wedge-like morphology and undergo cell division to form other stem cells (self-renew) and give rise to transient committed progenitor cells, termed enteroblasts (EBs). The progenitors however, do not further divide, but move apically to differentiate into either of two distinct cell types: absorptive enterocytes (ECs) or secretory enteroendocrine cells (EEs) (Ohlstein & Spradling, 2006). Interestingly, ISCs are the only-known cells in the midgut that proliferate, which makes them particularly suited to study stem cell proliferation and differentiation *in vivo*.

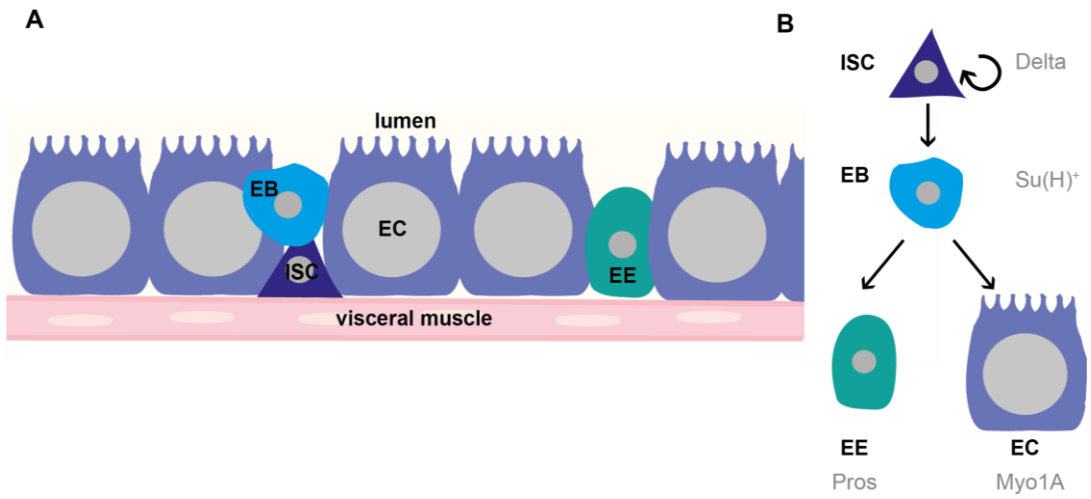

Figure 1.5 Organization of the adult *Drosophila* midgut

(A) Diagram of the adult midgut epithelium. (B) ISC division, ISCs self-renew themselves by division, and give rise to enteroblasts (EBs). EBs differentiate into either EC or EE. Some of the specific markers that can be used to specifically distinguish between the different cell types are shown in grey.

1.2.1 Intestinal stem cells of the *Drosophila* midgut

The basally located ISCs can give rise to both absorptive ECs and small secretory EE cells. Division of an ISC is morphologically symmetrical at first, producing two daughter cells that are initially similar. However, soon after division one cell remains an ISC while the other becomes an EB that differentiates to form either an EC or an EE. This process was shown to depend on the Delta/Notch signaling pathway – a pathway also conserved in higher vertebrates. Shortly after mitosis, one of the two daughter cells retains high levels of the Notch ligand Delta (Dl) at the plasma membrane as well as in cytoplasmic vesicles and remains as an ISC, while the other cell loses Dl and undergoes differentiation (Ohlstein and Spradling, 2006, 2007). High levels of Dl downregulate Notch receptor signaling in the ISC, suppressing differentiation, whereas Notch signaling is switched on in the EB, thereby triggering a transcriptional program that drives differentiation (Bardin et al., 2010; Ohlstein and Spradling, 2007; Perdigoto et al., 2011). Further studies have identified additional cell fate determinants and regulating factors in context of this stem cell niche. Escargot (esg), a zinc finger transcription factor of the Snail/Slug family (Fuse et al., 1994), expressed in both Delta-positive ISCs and EBs (Micchelli and Perrimon, 2006), was recently shown to be required for maintenance of ISC identity by acting as a transcriptional repressor of a diverse

set of differentiation genes, including transcription factors specific to ECs and EEs like Pdm1 and Prospero (Korzelius et al., 2014). Besides the identification of cell-autonomously acting ISC fate determinants such as Dl and Esg, nonautonomous regulation pathways of this niche have also been intensively characterized, among which the Wnt signaling pathway (Lin et al., 2008) and the insulin signaling pathway (Foronda et al., 2014).

1.2.2 Cell division mode of ISCs

The midgut epithelium undergoes constant development and renewal every week, when damaged or aged cells are lost from the epithelium, ISCs are responsible to respond and maintain the tissues homeostasis (Amcheslavsky et al., 2009; Buchon et al., 2009b).

Previously I have described how stem cells can follow two modes of division. In the *Drosophila* midgut, most ISC divisions have been proposed to be asymmetric, with ISC division resulting in the formation of a daughter ISC and an EB (Ohlstein and Spradling, 2006, 2007). When long-term clone studies were performed, clones with two or more ISCs or even lacking ISCs were observed, suggesting that ISCs can also divide symmetrically or even could be lost by neutral competition (de Navascues et al., 2012; Simons and Clevers, 2011). Moreover, the rate of ISC symmetric divisions in the epithelium can be increased by addition of insulin to the fly diet or in response to food abundance (McLeod et al., 2010). In contrast, starvation has been proofed to decrease the rate of symmetric division of stem cells (O'Brien et al., 2011). This indicates that a switch from a predominantly asymmetric division outcome to symmetric divisions could occur in response to environmental challenges or intestinal stress.

The maintenance of the stem cell pool in the adult midgut has been demonstrated not to only be kept by the balance of the asymmetric/symmetric ISC division rate, other mechanisms can also be used specially in situations of high damage where more than one response is needed. A recently discovered unexpected mechanism termed “amitosis” showed that in some situations where many stem cells are lost, ISCs can be replaced through a process of polyploidy reduction of differentiated ECs. These new generated diploid cells can replace the lost ISCs, however amitosis of polyploid cell can also induce deleterious mutations that cause tumor formation (Lucchetta and Ohlstein, 2017).

1.2.3 Intestinal response to pathogenic infection

Intestinal stem cells sense local cellular requirements and produce appropriate daughter cells in response, thus ISCs play a critical role in the physiology, longevity and pathology of the intestine. The adult *Drosophila* midgut has served as model for studying host-pathogen interactions. *Pseudomonas entomophila* (*P.e*) is highly pathogenic to both larvae and adult fly, and has the capacity to induce the systemic expression of antimicrobial peptide genes after ingestion (Buchon et al., 2009b). When enterocytes (ECs) are subjected to apoptosis, enteric infection, or JNK-mediated stress signaling, they produce cytokines (*Upd*, *Upd2* and *Upd3*) that activate Jak/Stat signaling in ISCs to promote their rapid (Jiang et al., 2009)

Oral infection of *P.e* induces a global translational blockage that impairs immune and repair programs in the fly midgut (Chakrabarti et al., 2012). This blockage is induced by the bacterial pore forming toxins and reactive oxygen species produced in response by the host. Analyzing changes in gene expression upon bacterial infection has shown that infection triggers a combination of immune, stress and developmental signaling pathways, providing so a link between infection and epithelial renewal (Buchon et al., 2009b; Cronin et al., 2009). Many genes affected by midgut infection are regulated by the IMD pathway as expected, but surprisingly, developmental pathways including Notch, Jak/Stat, and EGFR were also activated, indicating that the gut response to infection involves diverse aspects of gut physiology.

Bacterial infection has a dramatic impact on the gut physiology, causing a strong stress response that consequently stimulates stem cell proliferation and induces epithelial renewal. Regulatory mechanisms must ensure intestinal homeostasis, this is achieved partly by the integration of a complex set of stress responses that eliminate pathogens and tolerate indigenous microbiota (Buchon et al., 2013). The basally microbiota present in the intestine is important for the stimulation of intestinal turnover, studies with axenic flies have revealed that the proliferation rate in these flies is lower (Buchon et al., 2009a).

1.2.4 Endoreplication of ECs

Enterocytes are the most abundant cell type within the intestinal epithelium, approximately 90% of the enteroblasts become enterocytes (Ohlstein and Spradling, 2007). One of their main characteristic is their polyploidy that is achieved by a process termed

endoreplication. Endoreplication cycles (also referred as endocycles) occur by successive S phases taking place without occurrence of cytokinesis, consequently increasing the cellular DNA content (polyploidy). The strategy of endoreplication is believed to be an efficient way of increasing cellular mass and is often found in differentiated cells that are large or highly metabolically active (Edgar and Orr-Weaver, 2001). Acquisition of polyploid genomes has also been considered as a resistant mechanism to environmental stress. In contrast to diploid cells, polyploid cells tolerate genome alterations, and in some situations polyploidization occurs in response to stress, creating a genomic heterogeneity that facilitates the selection for stress-resistant phenotypes (Schoenfelder and Fox, 2015).

Enterocytes are the only cells within the *Drosophila* midgut epithelium which undergo endoreplication. They duplicate their genome 2-3 times to reach polyploidy levels of 16-32C (Jiang and Edgar, 2011). The Delta-Notch signaling pathway is responsible for promoting the endocycle onset, although the mechanism is not well understood. Endoreplication of ECs has been proposed as a mechanism to compensate cell loss and respond to epithelial damage, since larger ECs can be observed in midguts where the overall number of ECs is reduced (Edgar et al., 2014; Jiang et al., 2009).

Little is known about how endoreplication is regulated in ECs. It has been suggested to respond to external stimuli as nutrition, since low protein diets and reduced insulin signaling contributes to an increase in the number of lower ploidy enterocytes in the epithelium (Choi et al., 2011b). At molecular level, endocycles employ the same machinery as mitotic cycles to regulate the consecutive rounds of DNA replication, including Gap (G) phases between each S phase. In *Drosophila*, most of the knowledge of how endocycles are regulated comes from studies using salivary glands as model of study. The two major regulators of this process are Cyclin E (CycE) and its kinase partner cyclin-dependent kinase 2 (Cdk2) (Zielke et al., 2013), their specific removal from salivary glands causes elimination of endocycles (Zielke et al., 2011). E2F1 is also required for endocycles since it promotes CycE transcription, but its levels must be suppressed during S phase to achieve continuous endocycles. Moreover, E2f1 degradation promotes high $APC^{Fzr/Cdh1}$ activity suppressing so geminin accumulation (Fig 1.6, (Zielke et al., 2011)).

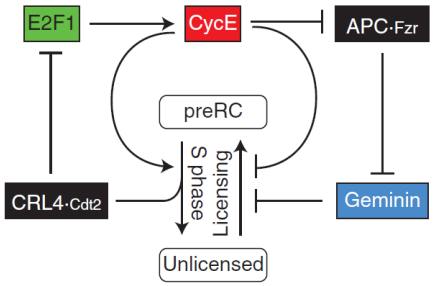

Figure 1.6 Regulatory network controlling salivary gland endocycles

E2F1 and CRL4·Cdt2 ensure that CycE activity peaks in late G phase. This allows the DNA replication and inhibits APC. Inhibition of APC results in the accumulation of geminin (Gem), preventing so relicensing of replication origins during S phase. Diagram of the regulatory network of salivary glands endocycles (Zielke et al., 2013).

1.2.5 Genetic tools in the *Drosophila* midgut

Considerable numbers of powerful genetic tools have been developed in the *Drosophila* midgut. The Gal4-UAS system (Brand and Perrimon, 1993) allows cell type specific expression of transgenes, and the temperature sensitive Gal80 protein ($Gal80^{ts}$) enables temporal control of Gal4 activity (McGuire et al., 2003). Therefore, cell type specific temperature-sensitive GAL4-UAS system (also termed as TARGET system, Temporal and Regional Gene Expression Targeting) enables spatial and temporal control of gene expression. Moreover, it has been established a lineage-tracing system called esg FlipOut (esg F/O) (Jiang et al., 2009), which allows the specific evaluation of ISCs and their progeny, since it can be used to permanently mark the progenitor cells and their progeny by expressing a heritable marker (i.e. GFP) forming the so called "clone".

In addition, T-TRACE lineage-tracing system was recently established, a method that combines TARGET system and Cre/loxP system (Zeng and Hou, 2015). The T-TRACE system induces the transgenes more meticulously, since the design only permits the induction of transgenes at 29°C along with presence of estrogen. Estrogen will induce Cre that consequently causes the expression of Ubi-p63<STOP<GFP by removing a loxP-flanked transcriptional termination cassette.

ISC-specific driver Dl-Gal4 and EB-specific driver Su(H)-Gal4, which are expressed in *Drosophila* midgut ISCs and EB cells separately (Zeng et al., 2010), have been incorporated with both TARGET and T-TRACE system, so that they can be used for different cell type specific studies opening up a wide variety of techniques that can be

employed. For instance, the specific expression of esg^{RNAi} in EB cells using Su(H)-$Gal4^{ts}$ triggered EBs' differentiation into ECs, uncovering so the role of escargot in the maintenance of the transient EB state (Korzelius et al., 2014).

To summarize, the adult *Drosophila* midgut provides a versatile model to study stem cell biology *in vivo* both under physiological conditions, but also in the context of stress situations like infection, nutrient-deprivation or injury, which might contribute with new insights into the mechanisms that underlie both stem cell self-renewal and cell differentiation.

1.3 Epigenetic regulation to maintain stem cell identity

Epigenetics is generally defined as heritable changes in gene activity and expression that occur without altering the underlying DNA sequence (Bird, 2007; Goldberg et al., 2007). Epigenetic regulation is a requisite for specialization of cells with the same genetic information and thus indispensable for a functioning multicellular organism. Several lines of evidence are pointing to the fact that events on every possible epigenetic mechanism can be involved in control of stem cell fate regulation - reviewed in (Avgustinova and Benitah, 2016; Buszczak and Spradling, 2006; Lunyak and Rosenfeld, 2008; Tarayrah and Chen, 2013).

1.3.1 DNA methylation

DNA methylation consists in the addition of a methyl group to the 5 position of cytosine by DNA methyltransferases (DNMTs) and is probably the best understood mechanism in context of epigenetic inheritance (Holliday and Ho, 2002), specifically at CpG islands, which are maintained in a semi-conservative manner by the activity of the DNMT1 (Jones and Liang, 2009).

DNA methylation is essential for normal development and is associated with a variety of biological processes including genomic imprinting, X-chromosomes inactivation, regulation of stemness and tumor formation. Changes in the DNA methylation have been associated with development of most types of cancer (Jaenisch and Bird, 2003).

It has also been shown that DNA methylation mediated by Dnmt-1 is essential for self-renewal of hematopoietic stem cells (HSCs) in mice (Broske et al., 2009) and that

lineage potential of HSCs is influenced by site-specific alterations in DNA methylation patterns upon aging (Beerman et al., 2013).

The presence of 5-methylcytosine (5mC) in the *Drosophila* genome has been subject of debate, however, it has been proofed that DNA methylation does not occur in *Drosophila melanogaster* as evidenced by Raddatz et al. using whole genome bisulfite sequencing techniques (Raddatz et al., 2013), and thus it is not considered an epigenetic mechanism employed by stem cells of the fruit fly.

1.3.2 Histone modifications

Chromatin is organized in the dynamic structuring of nucleosomes, which represent the basic repeating unit of the chromatin fiber. Each nucleosome is formed by 146-147 bp of chromosomal DNA tightly wrapped around an octamer of proteins comprising two subunits each of the canonical histones H3, H4, H2A and H2B, or variants of these histones (Davey et al., 2002; Luger et al., 1997).

Histones are small basic proteins consisting of a globular domain, called the histone fold domain (HFD), and a more flexible and charged NH_2-terminus (histone tail). These flexible N-terminal tails of the four core histones undergo a range of post-translational modifications (PTMs), including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ribosylation, and many others (Hatakeyama et al., 2016). These covalent modifications reveal a "histone code" that is involved in generating epigenetic information, (Jenuwein and Allis, 2001; Kouzarides, 2007). Thus, histones function as the core proteins for chromatin packaging and play essential roles in gene regulation.

There are many reports about certain histone modifications playing important roles during epigenetic inheritance and 'cellular memory'. For example, the role of polycomb group (PcG) proteins in epigenetic regulation of gene expression has been characterized in depth (Ringrose and Paro, 2004) and the integrity of the PcG complexes also appears to be critical for stem cell maintenance (Richly et al., 2011; Sauvageau and Sauvageau, 2010). PcGs are required for the maintenance of both embryonic and adult stem cells through chromatin modification, which results in gene repression and delayed differentiation (Pietersen and van Lohuizen, 2008). For instance, the polycomb family transcriptional repressor *Bmi-1* is required for the self-renewal of adult neural stem cells in mice (Molofsky et al., 2003).

Similarly, other histone-modifying enzymes have been implicated in regulating stemness. In *Drosophila* ovary, the self-renewal of GSCs and SSCs require the ATP-dependent chromatin remodeling factors ISWI and DOM respectively (Xi and Xie, 2005). But these histone-modifying enzymes can be also involved in regulating differentiation, as the histone lysine methyltransferase dSETDB1 and Su(var)3-9 that function sequentially as GSCs differentiate (Yoon et al., 2008). Atac2, which encodes a histone acetyltransferase (HAT), has been proposed to act as a novel regulator of *Drosophila* intestinal stem cells, with Atac2 depletion increasing ISC proliferation and Atac2 overexpression promoting ISC differentiation (Ma et al., 2013).

1.3.3 Differential histone distribution

In addition to histone modifications, differential histone or histone variant distribution can influence epigenetic inheritance (Henikoff et al., 2004), also in a stem cell context.

Cardiac progenitor cells (CPCs), a population of resident cardiac stem cells known to account for physiological turnover of cardiac myocytes and vascular endothelial cells in mice, have been shown to depend on high levels of the histone H3 variant CENP-A in order to sustain proliferation and ensure survival after differentiation (McGregor et al., 2014). Isoforms of the histone variant macroH2A were shown to act as an epigenetic barrier in reprogramming of primary mouse fibroblasts to induced pluripotent stem cells *in vitro* by being deposited as differentiation 'lock' at loci of pluripotency genes (Gaspar-Maia et al., 2013).

Finally, during male germline stem cell (GSC) asymmetric divisions in *Drosophila*, preexisting canonical histone H3 is preferentially retained in the GSC, while newly synthesized H3 is enriched in the other daughter cell termed gonialblast (GB) committed for differentiation (Tran et al., 2012). A following up study on the asymmetric distribution of H3 during GSC division unraveled that the histone mark H3T3P is the key player responsible for distinguishing pre-existing versus newly synthesized H3. Loss of function of H3T3P by expressing H3T3A, which cannot be phosphorylated, leads to a symmetric H3 segregation pattern (Xie et al., 2015). This asymmetric inheritance of H3 could be a mechanism for the GSC to maintain its gene expression profile, as well as enabling GB to arrange its chromatin structure for differentiation.

1.3.4 Epigenetic regulation in intestinal stem cells

Several histone-modifying enzymes have been implicated in maintaining ISCs. One example is Scrawny (Scny), that deubiquitinates mono-ubiquitinates H2B and functions in gene silencing. Adult flies mutant for *scny* rapidly lose ISCs due to inappropriate activation of the Notch pathway, which leads to ISC differentiation. Cells mutant for *scny* have elevated ub-H2B and H3K4me3 signals, which probably leads to more open chromatin and active transcription of Notch target genes (Buszczak et al., 2009).

As mentioned before in section 1.3.2, a histone acetyltransferase (HAT) encoded by the *Atac2* gene has been shown to regulate the activity of ISCs (Ma et al., 2013). HATs transfer acetyl groups to specific lysine residues on histone tails, a modification that is mostly associated with active transcription. Loss of Atac2 leads to increased ISCs, whereas overexpression of Atac2 promotes ISC differentiation. The molecular mechanism by which Atac2 regulates ISC differentiation remains unknown, but one possibility is that Atac2 activates Notch target genes by generating the H4K16ac mark at their promoter regions (Ma et al., 2013). Furthermore, recently by performing transcriptome analysis of young versus old ISCs, it has been reported that another subunit of HAT complexes, Nipped-A, is important for ISC integrity and for regulating proliferation in aged midguts. When Nipped-A is depleted, the proliferative capacity of ISCs decreases (Tauc et al., 2017).

In addition to histone-modifying enzymes, dynamic regulation of ISC activities is achieved by DNA modifications. DNA methylation at cytosines is usually associated with repressive gene expression (reviewed in (Cedar and Bergman, 2009)). Unlike mammals, methylation on DNA is not present in *Drosophila* (Raddatz et al., 2013). Interestingly, expression of human MeCP2 (hMeCP2, methyl-CpG-binding protein 2) in *Drosophila* ECs in midgut alters the cytological distribution of heterochromatin protein-1 (HP-1), as determined by immunofluorescence, and stimulates ISC proliferation. These observations suggest that hMeCP2 misregulates the expression of genes important for ISC maintenance (Lee et al., 2011).

Other epigenetic programming such chromatin remodeling has also been identified as mechanism for ISC fate regulation. SWI/SNF is a well characterized ATP-dependent chromatin-remodeling complex that has been identified by RNAi screen to be important for ISC commitment to differentiation. Osa (a SWI/SNF component) seems to regulate

Delta expression in ISCs and thus maintaining Notch signal in EBs to promote their differentiation into ECs. When Osa is depleted, there is an increase in ISC self-renewal and a block of ISC differentiation that results in tumor formation (Zeng et al., 2013).

The development of single-cell transcriptome analysis techniques has facilitated the understanding of how ISCs define their unique properties by differences in gene expression (Dutta et al., 2015b; Kim et al., 2016). For instance, cell type specific profiling revealed cluster of differentiation and stemness genes, being a big fraction of all differentially expressed genes ISC specific. This indicates that stem cell express a unique gene repertoire with an autocrine regulation by TFs. Having access to such a resource facilitates the understanding of how stem cells maintain their unique properties.

1.4 Histone H3 variant CENP-A – the epigenetic mark for centromere identity

The proper segregation of genetic information during cell division is crucial to maintain genomic integrity. Errors in segregation can lead to abnormal chromosome number -known as aneuploidy- which is linked to human disease (Kops et al., 2005). Centromeres, which are defined as chromatin regions that serve as the primary constriction for kinetochore assembly, play an important role in maintaining genomic stability. The kinetochore is a multiprotein complex that attaches the mitotic microtubule spindle to chromosomes (Przewloka and Glover, 2009). Considering the critical role that centromeres play in kinetochore formation and thus in faithful transmission of chromosomes to dividing cells, the presence of single centromeric regions is crucial for genomic integrity. The loss or gain of additional centromeres must be avoided, and the centromere number on a specific chromosome must be tighly regulated (Runge et al., 1991).

Despite their important function during cell cycle, centromeres are not defined by their underlying DNA sequence, but by the presence of epigenetic marks (Malik and Henikoff, 2009). For most eukaryotes (Cse4 in *S.cerevisae*, Cnp1 in *S.pombe*, HTR12 in *Arabidopsis*, CID in *Drosophila*), centromeric chromatin is characterized by the presence of the special histone H3 variant CENP-A, that replaces canonical histone H3 in a subset of centromeric nucleosomes (Allshire and Karpen, 2008; Blower et al., 2002; Sullivan and Karpen, 2004; Zinkowski et al., 1991).

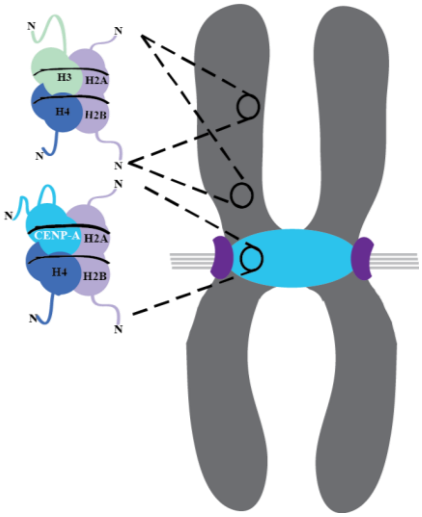

Figure 1.7 Centromeres are epigenetically defined by the presence of CENP-A

Canonical nucleosomes that are found along the chromosome arm are composed of an octamer containing two of each of the histones H2A, H2B (light purple), H3 (green) and H4 (dark blue). In centromeric chromosomes, histone H3 is replaced by the histone variant CENP-A (light blue). The centromere serves as foundation for the kinetochore (purple) where the spindle microtubules (grey) attached.

CENP-A is structurally similar to the canonical histone H3. The C terminus contains a globular HFD that shares 62% sequence homology with the HFD of canonical H3 (Sullivan et al., 1994). The HFD of CENP-A, like all histone proteins, consists of three α-helices linked by two loops (Arents et al., 1991). In addition to mediating the interaction with histone H4, CENP-A's HFD contains the critical structural features that are needed to deposit CENP-A to centromeres, i.e. loop1 (L1) and α-helix 2, which build up the CENP-A targeting domain (CATD), a region that is necessary and sufficient to promote centromeric targeting (Black et al., 2004). In contrast to the HFD, the N-terminal tail of CENP-A is very diverse and varies in length between different species as discussed later (Smith, 2002). X-ray crystallography has revealed that CENP-A and canonical nucleosomes are structurally very similar, and both types of nucleosomes wrap their DNA in a left-handed manner (Tachiwana et al., 2011). The precise composition of centromeric nucleosomes has been a subject of controversy over the past years, however, most evidence points to an octamer as the predominant centromeric structure (Dunleavy et al., 2013).

1.4.1 Centromeric DNA

The underlying DNA sequence of centromeres is fast evolving and not conserved between species (Murphy et al., 2005). With the exception of some yeast species such as *S. cerevisiae* and *K. lactis*, the centromeric DNA sequence alone seems insufficient to confer centromeric identity, and it is, therefore, widely accepted that centromeres are regulated epigenetically (Karpen and Allshire, 1997). Nevertheless, recent reports have shown preferences for specific DNA sequences that strongly indicate that they can contribute to centromere function.

One well-studied exception to the rule that the underlying DNA sequence is not enough for centromere specification is *Saccharomyces cerevisiae*. The point centromere present in budding yeast is genetically determined. The 125 bp conserved DNA sequence is called CEN DNA and can be found in all the chromosomes (Clarke and Carbon, 1985). This region contains CDEI, CDEII and CDEIII elements, being CDEII and III essential for mitosis (Fig. 1.8b). CDEIII recruits the CBF3 protein complex which leads to the incorporation of a single CENP-A-containing nucleosome to the CDEII element (De Wulf et al., 2003; Westermann et al., 2007).

In contrast to point centromeres, regional centromeres are usually composed of repetitive DNA sequences that may contribute, but are not sufficient for, centromere formation (Fig. 1.8). Centromeric DNA is generally highly repetitive, gene poor and AT-rich (Jiang et al., 2003; Schueler et al., 2001). In *Drosophila*, the model organism used in this study, there is also no common DNA sequence that can be found in all four chromosomes (Lamb and Birchler, 2003). All chromosomes except X contain simple and short repeats. The X chromosome has a complex AT-rich 359 bp long repeat called Satellite III (Sat III) (Lohe et al., 1993). This region is transcribed and the Sat III RNA localizes to centromeric and pericentromeric region of all the four chromosomes of the fruit fly. Furthermore, Sat III interacts with the kinetochore protein CENP-C affecting the loading and maintenance of different centromeric and kinetochore proteins. Consequently, depletion of Sat II in S2 cells leads to mitotic defects (Rosic et al., 2014).

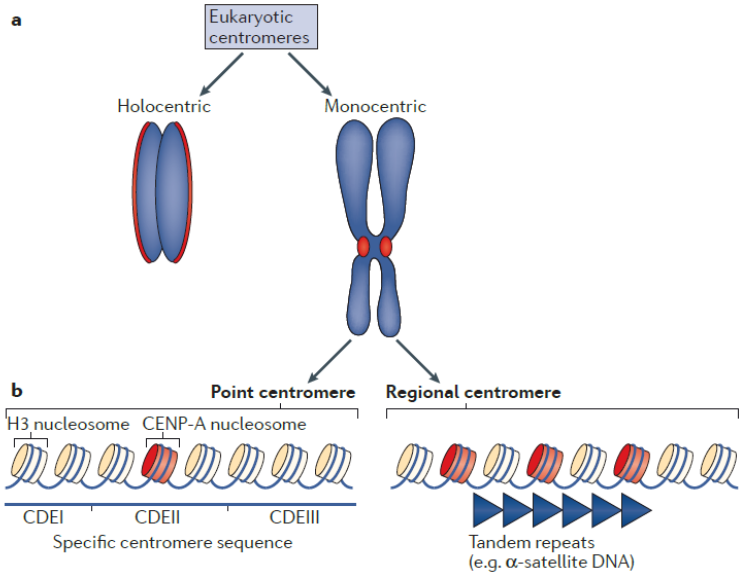

Figure 1.8 Centromeric DNA sequences

(A) Types of centromeres: holo or monocentric. Monocentric centromeres can be further sorted out in two categories: point or regional centromeres. (B) Point centromeres contain a DNA sequence that is sufficient for centromere function. Regional centromeres contain large regions of repetitive DNA and assemble numerous CENP-A nucleosomes. Modified from (McKinley and Cheeseman, 2016).

Apart from satellite III in *Drosophila*, transcripts originated from centromeric DNA have been described in a huge variety of organisms (Chen et al., 2015; Choi et al., 2011a; Li et al., 2008; Quenet and Dalal, 2014; Topp et al., 2004). RNA polymerase II (RNAPII) is the enzyme responsible for transcription and has been reported to be required for CENP-A deposition at ectopic sites in *Drosophila*. Using LacO-LacI system, it has been discovered a crosstalk between the CENP-A loading factor CAL1 and FACT (Facilitates Chromatin Transcription) that facilitates the recruitment of RNAPII, which transcribes the centromeric region leading to the removal of H3 containing nucleosomes and to the deposition of CENP-A (Chen et al., 2015) Therefore, transcription itself together with specific and non-specific transcripts are critical for centromere function.

1.4.2 Centromeric chromatin

Despite the essential role of CENP-A for most centromeres, the chromatin environment created by the presence of specific post translational modifications (PTMs) on all histone species at centromeres is just as important. In most species, centromeres are organized with a central region that is defined by the presence of CENP-A-containing

nucleosomes, surrounded on both sides by flanking heterochromatin (pericentric chromatin) (Blower and Karpen, 2001; Blower et al., 2002; Partridge et al., 2000). The PTMs on histones present at human centromeres indicate that centromeric chromatin is neither heterochromatic nor euchromatic. This unique mixture of repressive and permissive histone marks has been termed "centrochromatin" (Sullivan and Karpen, 2004).

Similar to centromeres, the surrounding pericentromeric heterochromatin is characterized by hypoacetylated canonical histones. However, in contrast to centromeres, pericentric chromatin is characterized by H3K9me2 (flies and fission yeast) or H3K9me3 (in mammalian cells) (Noma et al., 2001). Another repressive marks present are H3K27me3 (Lam et al., 2006) and H4K20me3 at DNA repetitive regions. The presence of heterochromatin in pericentromeric regions is also required to ensure recruitment of cohesin protein complex, which holds sister chromatids together until anaphase onset (Sakuno et al., 2009; Yamagishi et al., 2008). It is also important to note that there is a correlation between heterochromatin and neocentromere establishment at least in some species, stable hotspots of overexpressed CENP-A in *Drosophila* cells are preferentially established at euchromatin/heterochromatin boundaries (Olszak et al., 2011).

In contrast to the repressive marks at pericentromeres, canonical histone H3 within centromeric chromatin contains some marks that are usually specific for open chromatin, e.g. K36me2 (Bergmann et al., 2011). This modification is normally associated with transcription elongation, supporting observations that centromeres are transcriptionally active. This study also found that H3K4me2 plays a role in CENP-A maintenance. H3K4me2 depletion at the alphoid^{tetO} centromere of the HAC by tethering the lysine-specific demethylase 1 (LSD1) causes a reduction of CENP-A incorporation as a result of the loss of the CENP-A chaperone HJURP at centromeres, suggesting that this modification is involved in the recruitment of HJURP to centromeres.

In addition to establishing a unique chromatin environment, some marks established only during specific processes such as mitosis are also important for centromere function. For instance, the mitotic kinase haspin is responsible for H3T3 phosphorylation and this mark is specifically enriched at H3 nucleosomes of the centromeric core of mitotic chromosomes (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010) and has been proposed to guarantee proper chromosome congression to the metaphase plate for faithful segregation of sister chromatids during anaphase (Dai and Higgins, 2005).

H4 associated with pre-nucleosomal CENP-A is acetylated in a manner that is essentially identical to H4 in complex with pre-nucleosomal H3 (Chang et al., 1997; Hole et al., 2011). Acetylation of H4 at K5 and K12 is found within in the pre-nucleosomal CENP-A-H4-HJURP complex and requires RbAp46/48 for its subsequent successful localization of CENP-A to centromeres (Shang et al., 2016). In contrast to chromatin-associated centromeric H4, pre-nucleosomal CENP-A associated histone H4 lacks K20me (Bailey et al., 2015). H4K20me1 has been reported to be enriched at centromeres and essential for correct kinetochore assembly (Hori et al., 2014).

Not only canonical histones bear modifications, CENP-A itself can also be subjected to modifications. Depending on the modification, the effect will influence CENP-A stability, structure, or positioning. The CENP-A N-terminus is phosphorylated on S16 and S18 already in prenucleosomal CENP-A, and these marks are important for reliable chromosome portioning during division (Bailey et al., 2013). CDK1 phosphorylates CENP-A at S68, which interferes with CENP-A binding to its loading factor HJURP and, therefore, with its deposition to centromeric chromatin prior to mitotic exit (Yu et al., 2015; Zhao et al., 2016). At the time of CENP-A loading onto centromeric chromatin this phosphorylation is removed by the phosphatase PP1α. However, in long-term cell survival assays, S68 phosphorylation seems dispensable for CENP-A function and cellular survival, challenging the finding that S68 phosphorylation is necessary for CENP-A recognition by HJURP and therefore faithful loading (Fachinetti et al., 2017). CENP-A is also phosphorylated by Aurora A and B at S7 and this modification is required for mitotic progression and proper kinetochore function (Goutte-Gattat et al., 2013).

CENP-A N-terminus not only bears phosphorylation sites, but is also α-trimethylated on Gly1 by the N-terminal RCC1 methyltransferase NRMT (Bailey et al., 2013). In *S. cerevisiae,* R37 of Cse4 (CENP-A ortholog) is methylated and this modification is proposed to positively regulate the recruitment of the complete kinetochore complex and consequently control proper chromosome segregation (Samel et al., 2012).

Human CENP-A has also been reported to be acetylated at K124 in G1/S-phase-derived cells, a residue located within the HFD closer to the C-terminus. It was proposed that this CENP-A K124ac functions in "priming" or "blocking" CENP-A K124 for ubiquitylation until the M phase. At the same residue CENP-A can be ubiquitylated (K124ub) by the CUL4A-RBX1-COPS8 complex *in vivo* and *in vitro*. Acetylation of CENP-A serves as a signal for its deposition at centromeres. The ubiquitylation at this

residue occurs in the M and G1 phases and is required for efficient interaction with HJURP to properly localize CENP-A at centromeres and is, therefore, essential for CENP-A loading onto chromatin (Niikura et al., 2015). This study has recently been contradicted by Fachinetti et al. who found no evidence for CENP-A-K124ub to be important for loading or maintenance of CENP-A (Fachinetti et al., 2017). CENP-A mono-ubiquitylation seems epigenetically inherited through dimerization between cell divisions and this inheritance is important for the control of CENP-A deposition and maintenance at centromeres (Niikura et al., 2016). Similar to the human K124ub, mono-ubiquitylation of *Drosophila* CENP-A by the E3 ligase CUL3/RDX has been reported (Bade et al., 2014). Mono-ubiquitylation stabilizes CENP-A that is bound to its loading factor CAL1.

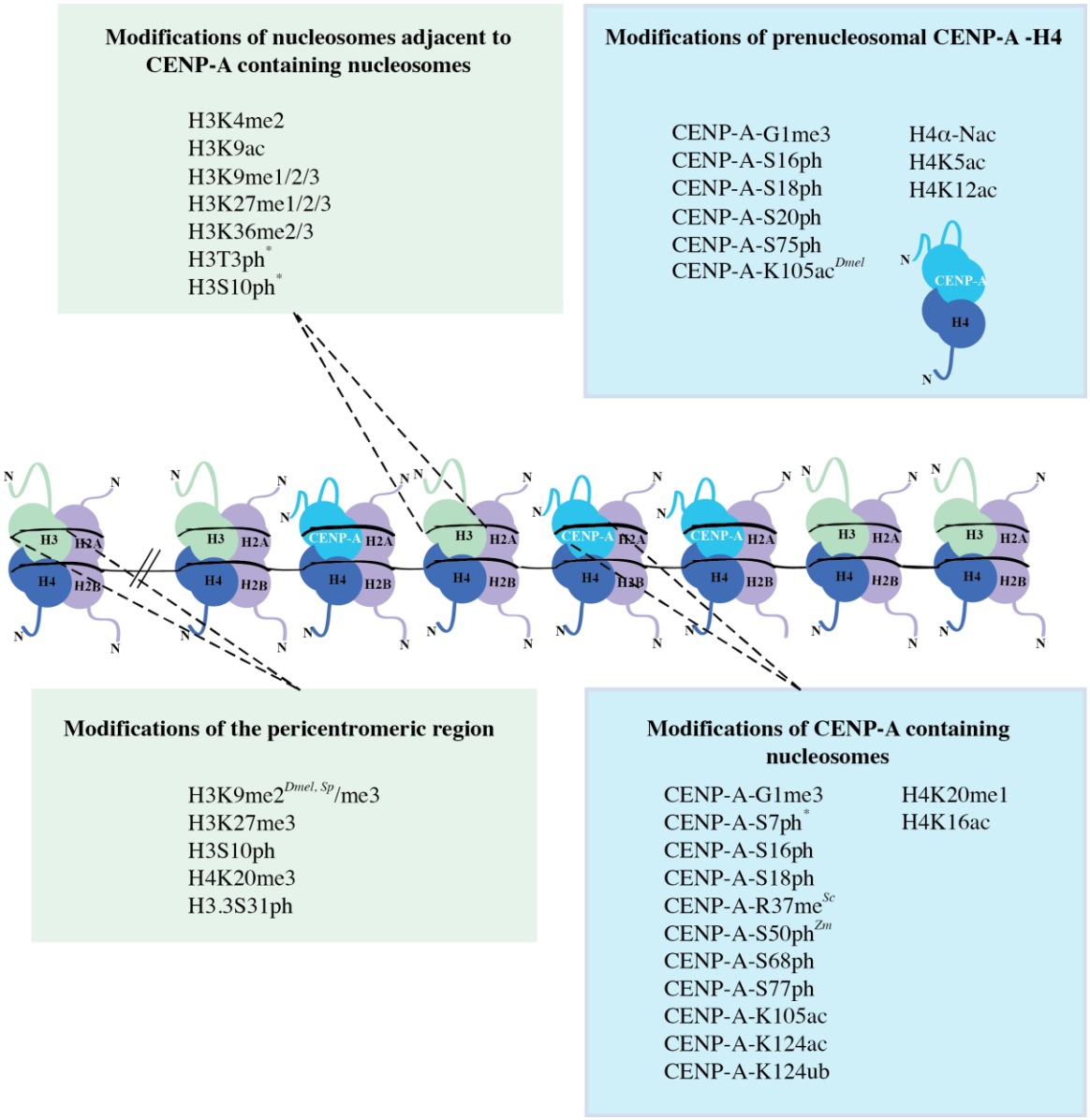

Figure 1.9 PTMs present on centromeric chromatin

Summary of the PTMs present at histones composing the core centromere and the pericentromeric region, and at CENP-A itself. Mitosis-specific PTMs are labelled with * and PTMs specific of certain species are also marked (*Dmel*=*Drosophila melanogaster*, *Sc*= *Saccharomyzes cerevisiae*, *Sp*= *Schizosaccharomyzes pombe*, *Zm*= *Zea mays*).

1.4.3 CENP-A loading in *Drosophila*

As already mentioned, the mechanisms in centromere and kinetochore regulation are conserved within eukaryotes. However, the fruit fly has a simplify version of the human interface that facilitates its study. The *Drosophila* kinetochore consists of only three KMN

proteins: CENP-A (termed also as CID), CENP-C and CAL1 (Przewloka et al., 2007), being CENP-C able to recruit all the proteins that formed the outer kinetochore (Przewloka et al., 2011). Remarkably, these three components of the *Drosophila* kinetochore are interdependent from each other for their localization and function (Erhardt et al., 2008).

Canonical histones are incorporated into chromatin during DNA replication (S phase) with the help of histone chaperones such as CAF1 and Asf1 in a replication-dependent manner (Allshire and Karpen, 2008; Mello and Almouzni, 2001). This is not the case for nucleosomes containing histone-variants.

In *Drosophila*, centromeric DNA is replicated in late S-phase (Sullivan and Karpen, 2001) but CENP-A is not produced until G2-phase and it will be loaded only during mitosis. The timing of CENP-A loadings is different in tissue culture cells and in embryos. In S2 cells, CENP-A is already incorporated during metaphase, but in fly embryos this event occurs at anaphase (Mellone et al., 2011; Schuh et al., 2007). Little is known about the loading mechanisms of CENP-A in stem cells, but the timing of CENP-A loading in neural stem cells of the fly larva is during late telophase/G1 (Dunleavy et al., 2012).

The loading mechanism of CENP-A into centromeric chromatin consists on the following steps: (i) CAL1, the loading chaperone of CENP-A, is binding to the centromeres during prophase, before newly synthesized CENP-A is incorporated (Mellone et al., 2011); then (ii), during metaphase in S2 cells, CAL1 mediates the loading of stabilized mono-ubiquitylated CENP-A (Bade et al., 2014). CENP-C also gets recruited at the same time together with CENP-A (Bade et al., 2014; Pauleau and Erhardt, 2011; Schuh et al., 2007).

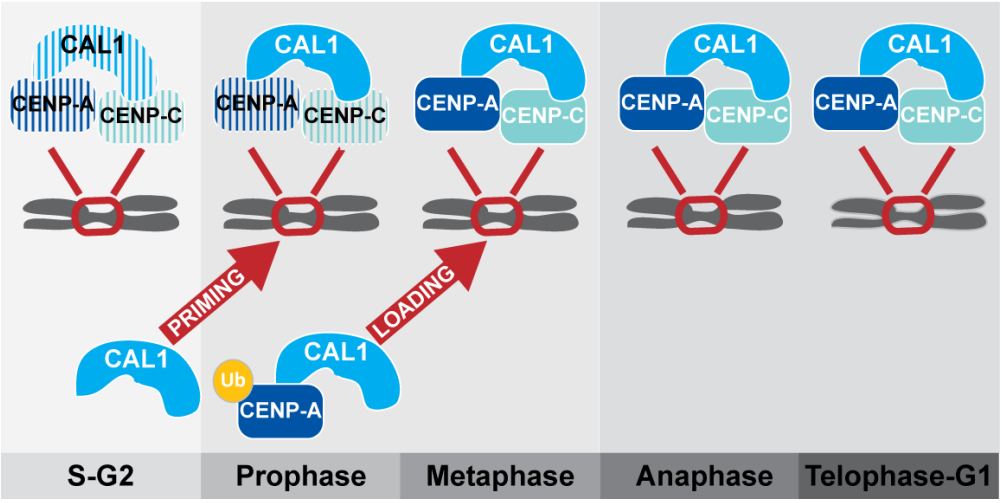

Figure 1.10 Model for centromere assembly in *Drosophila*

Striped boxes indicate that new proteins have not been loaded yet. CAL1, the loading chaperone of CENP-A, is binding to the centromeres during prophase, before newly synthesized CENP-A is incorporated. Then,

during metaphase in S2 cells, CAL1 mediates the loading of stabilized mono-ubiquitylated CENP-A and CENP-C also gets recruited at the same time. Adapted from (Pauleau and Erhardt, 2011) by Dr. Pauleau.

1.5 Other Histone H3 variants

In humans, the family of H3 histones contains eight isoforms including the canonical histone H3.1, H3.2 and the histone variants CENP-A, H3.3, H3.4 H3T), H3.5, H3.X and H3.Y (reviewed in (Filipescu et al., 2014)). These can be grouped in two different categories based on their incorporation into chromatin: (i) canonical – replication dependent (H3.1 and H3.2) and (ii) replication independent histone H3 variants (Gunjan et al., 2005). Two of these H3 variants, CENP-A and H3.3 are relevant for the work in this thesis and thus they will be introduced in more detail (see sections 1.4 and 1.5.1)

1.5.1 Histone variant H3.3

Histone H3.3 differs from canonical H3 in terms of primary sequence only by four amino acids (Franklin and Zweidler, 1977) and is extraordinarily conserved in mouse, humans and *Drosophila* (Akhmanova et al., 1995; Frank et al., 2003; Krimer et al., 1993).

In contrast to CENP-A, H3.3 loading is not cell cycle regulated, but can occur throughout the cell cycle (Wu et al., 1982). Deposition of H3.3 occurs in a transcription-coupled manner and requires the assistance of the Histone Regulator A (HIRA) complex (Tagami et al., 2004). In addition to HIRA, other studies have revealed that the ATRX-DAXX (α-thalassemia X linked mental retardation protein – death associated protein) complex and DEK are involved in the loading of H3.3 at telomeres, pericentromeres and centromeres (Goldberg et al., 2010).

A main property of H3.3 is its preferential association with transcriptionally active chromatin. Chromatin immunoprecipitation combined with high-resolution genome mapping in *Drosophila* and mammalian cells has revealed that H3.3 is particularly enriched in the gene body as well as at the promoter regions of transcribed genes (Chow et al., 2005; Goldberg et al., 2010; Wirbelauer et al., 2005). Moreover, a study points at the potential involvement of H3.3 in transmission of active chromatin states from one cell generation to the next and thus relate H3.3 deposition to cellular memory. Memory of MyoD target gene expression in *Xenopus* is maintained in the absence of transcription

during 24 mitotic divisions and this is dependent on H3.3K4me (Ng and Gurdon, 2008).

1.6 Aim of the study

Current studies suggest that differential histone H3 distribution between *Drosophila* germline stem cells and differentiating cells is important for the regulation of stem cell properties. A mechanism of retaining preexisting histones (and their modifications) throughout cell division might be a way of preserving information important to maintain stem cell properties in this particular niche. Understanding if these observations are specific to a specific stem cell niche or to a precise histone is crucial for unravelling the mechanisms behind stem cell regulation at this level. Therefore, the aim of this study is to investigate the distribution of histone H3 variant CENP-A in cells of the *Drosophila* midgut in order to understand whether this particular histone variant shows an asymmetric distribution pattern related to intestinal stem cell or non-stem cell identity.

If this is the case, I would like to understand the role in stem cell function and self-renewal and identify important players involved in establishment or maintenance of this asymmetry. Obtained results are supposed to clarify the function of CENP-A in intestinal stem cells to provide a basis for further experiments that aim to unravel the general role of epigenetic inheritance in stem cells.

2 Results

2.1 Asymmetric CENP-A inheritance in *Drosophila* intestinal stem cells

2.1.1 CENP-A and CENP-C are predominantly present in the progenitor cells of the *Drosophila* midgut

Stem cells' main characteristic is their potential to self-renew and differentiate. This is mainly achieved by asymmetric cell division (ACD) that gives rise to two daughter cells with different fate. SC and their differentiating progeny share the same genomic information and thus epigenetic mechanisms regulate stem cell fate determination. Among the different epigenetic mechanisms involved in the regulation of stem cell identity and ACD, the asymmetric inheritance of canonical histones was discovered recently.

CENP-A is the epigenetic mark of centromeres, which identify the specific chromatin regions that mediates spindle attachment during chromosome segregation. Due to its important role during sister chromatids segregation, we wanted to explore the possibility of CENP-A distribution also playing a role in the establishment of the asymmetry required in stem cell division.

Our starting hypothesis was that centromeres could follow an asymmetric segregation pattern during asymmetric cell division of intestinal stem cells. To determine whether this is the case, first I decided to look at the pattern of endogenous CENP-A in the different cell types of the midgut. As shown in Fig. 2.1A and C, CENP-A can be detected in small cells of the adult *Drosophila* midgut. The so called "small cells" include the following cell types: intestinal stem cells (ISCs), enteroblasts (EBs) and enteroendocrines (EEs). This distribution was observed not only in flies expressing CENP-A-GFP under the control of the endogenous promoter, but also in wildtype OregonR flies using CENP-A and CENP-C antibodies and IF against the endogenous proteins.

To further validate this, I compared the expression level of CENP-A in the different cell types of the midgut (http://flygutseq.buchonlab.com/), a transcriptomic resource, which provides cell- and region-specific RNAseq data as well as cell-specific regulation in response to bacterial infection. CENP-A expression level was remarkably higher in progenitor cells compared to differentiated ECs (data not shown). Intriguing for me was that transient EBs showed almost double the amount of CENP-A mRNA than ISCs (2.501 rpkm in EBs, 1.439 rpkm in ISCs), EBs are transient cells that without further division will directly different either into ECs or EEs, so this opens the question of why a non-mitotic cell will need to express a histone variant required during cell division.

Surprisingly, CENP-A and CENP-C were not always present within the same cell (see zoom images of panel 2.1C), where the bottom cell lacks CENP-C signal by IF analysis, but stains for CENP-A. This staining differences may indicate an asymmetric segregation pattern of centromeric factors. To further understand this, distinguishing which cell type shows both signal and which not must be accomplished.

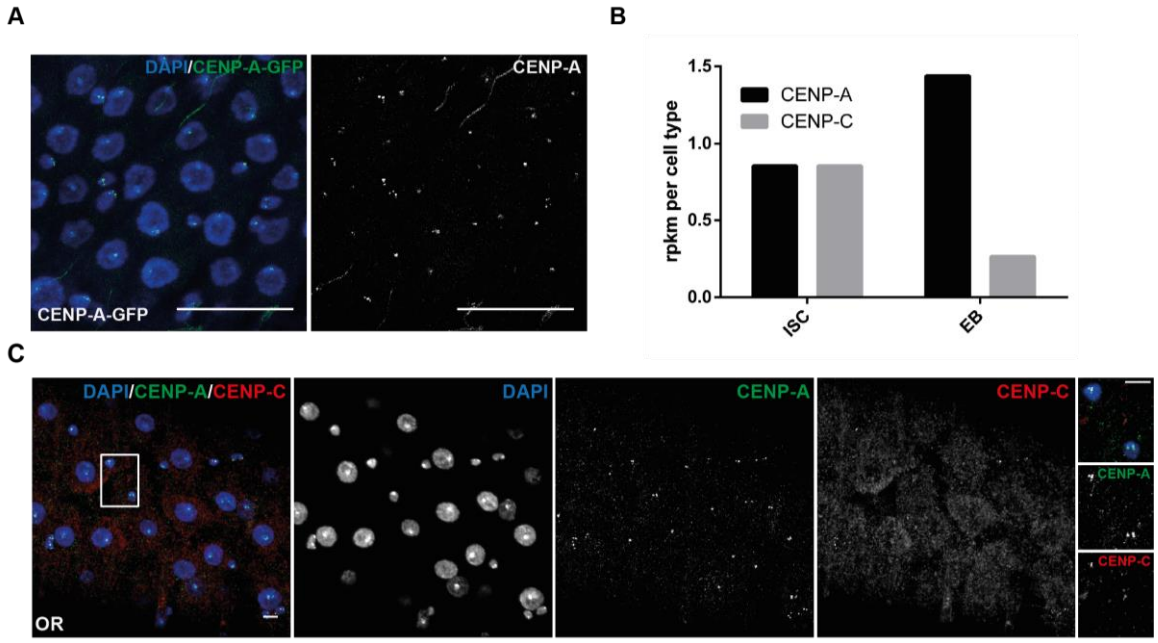

Figure 2.1 Endogenous CENP-A expression is restricted to small cells of the adult *Drosophila* midgut.

(A) CENP-A-GFP in midguts expressing CENP-A GFP transgene under the control of the endogenous promoter. (B) Rpkm data of CENP-A and CENP-C expression in ISCs and EBs (source: http://flygutseq.buchonlab.com/). (C) Midguts of Oregon-R flies (wt) stained for CENP-A (green) and CENP-C (red). The inlets on the right show zooms of the cells marked with a white box. Scale bar, 25 µm and 5µm for the zoom.

2.1.2 Old synthesized CENP-A is preferentially retained by ISCs

To investigate how CENP-A is inherited in the different midgut cells, it was important to develop a method that allowed us to distinguish between "old" versus "new" CENP-A pool, which means pre-existing and newly synthesized CENP-A protein. In 2012 Tran et. al published a switchable dual-color method to differentially label canonical histone H3 (Tran et al., 2012). We have obtained the constructs for this dual labeling and I cloned CENP-A into this system and made transgenic flies by germ line transformation (injections were kindly performed by Katrine Weischenfeldt from Prof. Dr. Aurelio Teleman laboratory, DKFZ, Heidelberg).

This method uses two different controls, spatially -by GAL4/UAS system-, and temporally -by heat shock induction-, to switch labeled CENP-A from green (GFP) to red (mKO) (see Fig. 2.2). The heat shock treatment induces an irreversible DNA recombination between the FRT sites of the transgene, that ends with the expression of GFP-labeled old CENP-A and induces the expression of mKO-labeled newly synthesized CENP-A. First, I used a ubiquitous Gal4 driver (ub. Ga4) to drive the expression of the transgene to all cell types and tissues of the fly.

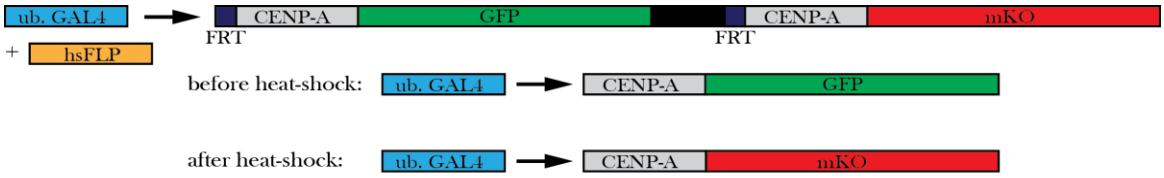

Figure 2.2 The UASp-FRT-CENP-A-GFP-PolyA-FRT-CENP-A-mKO-PolyA transgene.

UAS: upstream activating sequence; FRT: FLP (flippase) recombination target; CENP-A; GFP: green fluorescent protein; mKO, monomeric Kusabira-Orange fluorescent protein. ub-Gal4: ubiquitous GAL4 driver. hs-FLP: heat shock inducible FLP recombinase

The flies were kept at 25°C and young adult female flies at 2-7 days after eclosion were heat shocked in a 37°C water bath for 90 minutes (Fig. 2.3).

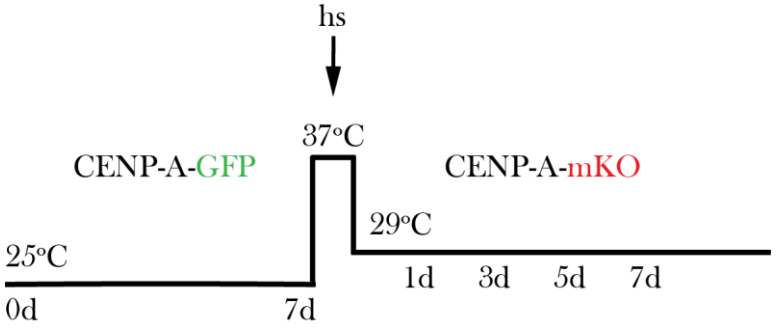

Figure 2.3. Heat shock regimen for analysis of CENP-A segregation.

Adult female flies at 7 days after eclosion were heat shock in a 37°C water bath for 90 minutes. Guts were analyzed at different timepoints after heat shock, during the recovery period flies were kept at 29°C.

Subsequently, the flies were kept at 29°C to assess the optimal expression of the transgene. To analyze the distribution of old vs. new synthesized CENP-A in ISCs and differentiated progeny, midguts were studied at different timepoints. Samples from heat shocked flies were collected 1, 3, 5 and 7 days after heatshock (HS), fixed and the numbers of GFP, mKO and GFP/mKO positive cells were evaluated (Fig. 2.3.). Qualitative analysis of the distribution of old and newly synthesized CENP-A in summarized in Fig. 2.4. To specifically address the question of how CENP-A is distributed in ISCs compared to their differentiating progeny, I included the Delta-lacZ construct in the genetic background of the flies to be able to easily distinguish ISCs from other small cells by staining against β-Galactosidase.

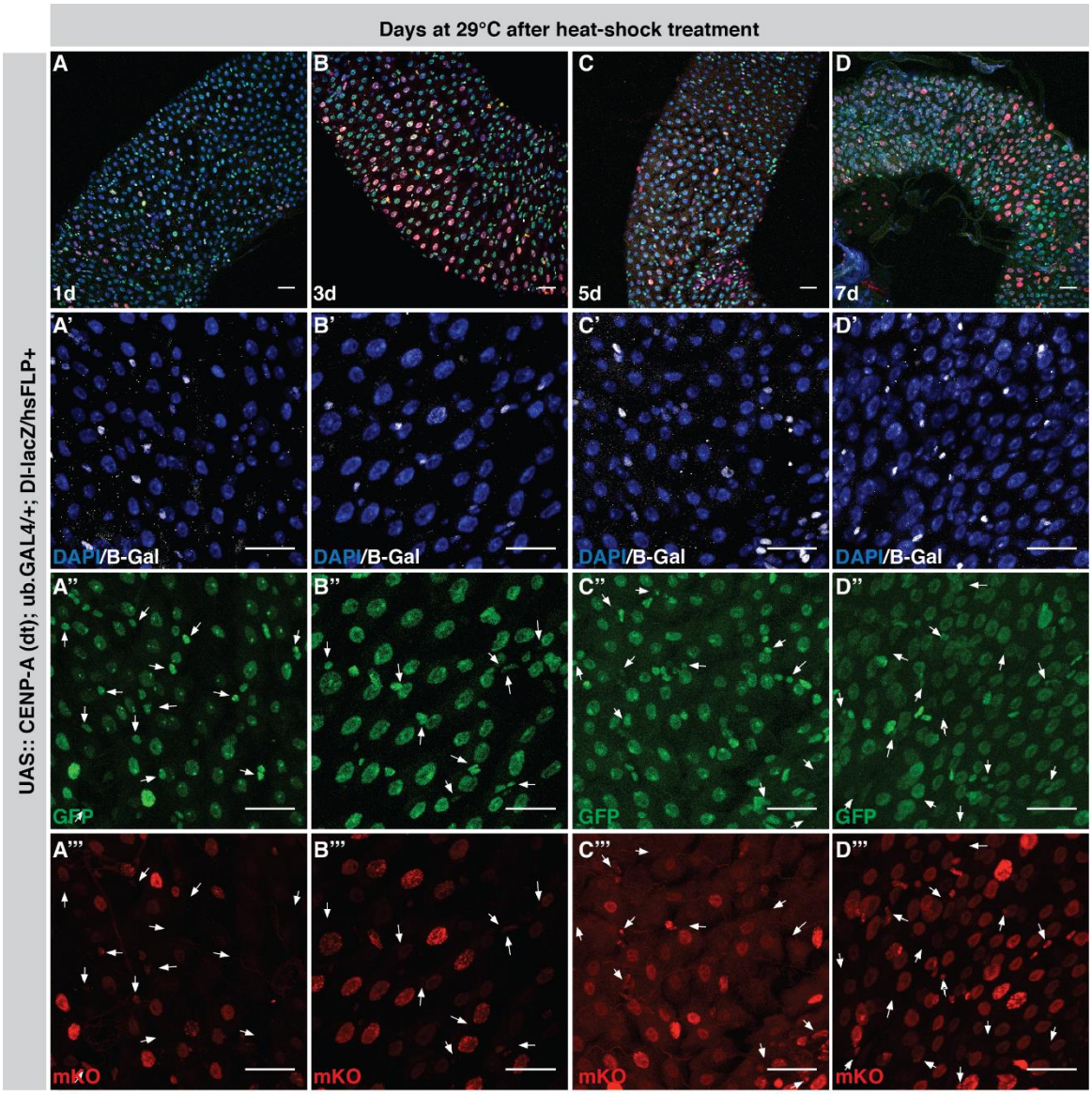

Figure 2.4. CENP-A is asymmetrically distributed in adult midgut cells.

Fixed and stained female fly guts show the distribution of old CENP-A (green) and newly synthesized CENP-A (red) at different recovery timepoints (A 1 day, B 3 days, C 5 days, D 7 days). No antibody was added to enhance GFP or mKO signal. Samples were stained with β-Galactosidase to detect intestinal stem cells marked by Delta-lacZ (white signal). The arrows mark the ISCs. (A'-D') Zoom in images of the same region showed in the upper panel, DAPI and β-Galactosidase. (A''-D'' and A'''-D''') Zoom in images of the same region but showing GFO and mKO signal, arrows point to β-Gal +ve cell. Scale bars, 25 μm.

One day after heat shock I found 73.5% of ISCs keeping old synthesized CENP-A-GFP, whereas only 4.5% showed signal for new CENP-A (labeled with mKO). Seven days after HS, 73% of the ISCs analyzed still retained CENP-A-GFP signal (GFP only 41%, both 32%), though no GFP expression was possible after the HS. Comparing the distribution of

both CENP-A signals between ISCs and ECs is when the difference is more remarkable. Only 51.5% of the ECs analyzed retained CENP-A-GFP (33% GFP only, 18.5% both), whereas 48.5% completely showed signal for newly synthesized CENP-A-mKO (Fig. 2.5). These data suggest that CENP-A is asymmetrically inherited in cells of the midgut epithelium, being the old synthesized CENP-A (CENP-A-GFP) mostly retained by ISCs, indicating a role of CENP-A in stemness.

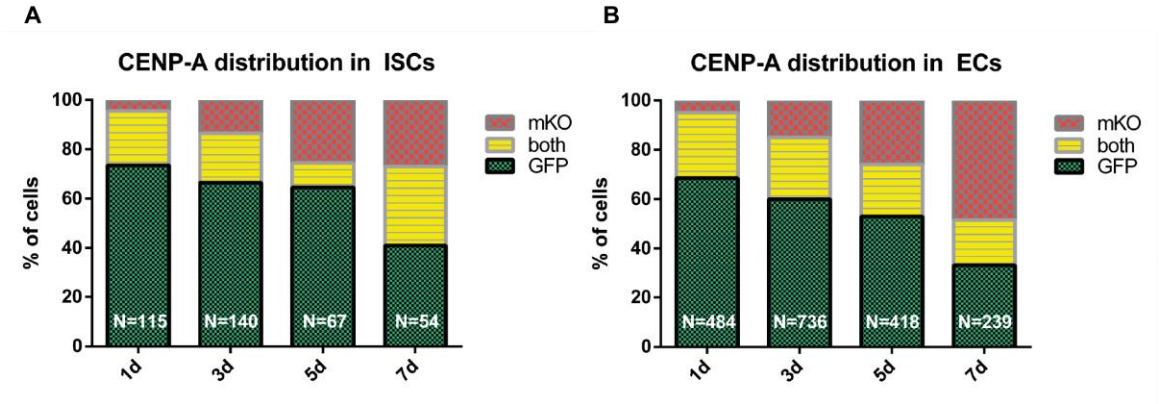

Figure 2.5 Quantification of CENP-A distribution in adult midgut cells show an asymmetric distribution of old synthesized CENP-A in ISCs.

(A) Quantification of GFP and/or mKO β-GAL+ve cells, and therefore ISCs from 3 or more independent experiments, the number of cells analyzed is indicated. For the three first timepoints more than 65% of the ISCs retained old synthesized CENP-A (GFP labelled). It is at timepoint 7 days when the percentage of ISCs retaining old synthesized CENP-A decreases to 41%, but 32% of the ISCs analyzed were keeping old synthesized CENP-A as well as newly. (B) Quantification of GFP and/or mKO distribution in ECs from 3 or more independent experiments. In total, 484 ECs were analyzed for 1 day after heatshock, 736 for 3 days, 418 for 5 days and 239 for 7 days. In contrast with the stem cell behavior, the differentiated cell in the adult midgut do not retain old synthesized CENP-A as the graph shows with a decrease of 68.5% (day 1) to 33% (day 7) of ECs having GFP-CENP-A.

A hypothetical asymmetric distribution of CENP-A during stem cell division would implicate the establishment of two different asymmetries at cellular and molecular level: (i) an asymmetric cell division of ISCs and (ii) an asymmetric distribution of CENP-A within sister chromatids during its loading and in mitosis. However, nothing is known about CENP-A loading cycle in stem cells. Since the timing of CENP-A loading in flies differs among developmental stages and tissues, i.e. during metaphase for S2 cells and during anaphase for embryos (Mellone et al., 2011; Schuh et al., 2007), a prediction of the precise timing of CENP-A loading in stem cells is impossible.

To validate the asymmetric CENP-A distribution, I decided to analyze the distribution pattern during ISC division, for that I tried to analyze mitotic ISCs undergoing anaphase. I followed the same timeline as depicted in Fig. 2.3, but instead of staining midguts against β-galactosidase for detecting ISCs, I stained them with pH3, a mitotic marker (data not shown). Unfortunately, mitotic ISCs show a very bright pH3 signal due to chromatin compaction at the metaphase plate. This bright signal caused bleed-through into the mKO channel making so impossible to distinguish real signal from not. To overcome this problem, I tried to use other mitotic markers such as H3T3P (data not shown), a phosphorylation mediated by haspin that is specifically enriched at H3 nucleosomes of the centromeric core of mitotic chromosomes (Kelly et al., 2010; Wang et al., 2010; Yamagishi et al., 2010). This mark again allowed to specifically distinguished mitotic ISCs but also produced a staining signal that caused crosstalk between the mKO detection channel and the far red.

2.1.2.1 CENP-A distribution in ISC-EB pairs follows an asymmetric distribution pattern

Since I could not validate the asymmetric CENP-A inheritance analyzing anaphase mitotic ISCs, I decided to quantify the distribution of CENP-A within the ISC-EB pair. In the midgut epithelium, ISCs are basally localized and are usually observed to form small nests of most often a single ISC and one or two undifferentiated ISC daughters, the EBs (Edgar, 2012).

The ISC-EB pairs analyzed showed six different distributions of CENP-A that I sorted out in different models to facilitate the quantification (Fig. 2.6A). In model 1, each cell of the pair retains CENP-A differentially labeled; in model 2, there is a clear difference where each cell also retains CENP-A with different label, but one of the cells also shows a low signal for CENP-A labeled with the other fluorophore. In model 3 and model 5, both cells of the pair show signal for CENP-A labeled with the same fluorophore; and, in model 4, both cells show signal for CENP-A labeled with the two fluorophores. Finally, in model 6, one of the cell shows signal for CENP-A labeled but the other not. Model 1, 2 and 6 correspond to an asymmetric distribution model and the other 3 to a symmetric model.

One day after heat shock I found that 16.67% of the ISC-EB pairs analyzed followed model 1, 61.11% model 2, 0% model 3, 13.89% model 4, 0% model 5 and 8.33% model 6 (Fig. 2.6B-C). This means that 78% of the ISC-EB pairs showed an asymmetric distribution model for old vs. newly synthesized CENP-A (keeping out model 6, because

one of the cells of the pair lacks signal for CENP-A). It has been reported that in homeostasis, 80% of the ISCs divide asymmetrically, whereas the other 20% give rise to a symmetric fate (de Navascues et al., 2012), these numbers almost perfectly correlate with my data for CENP-A distribution in ISC-EB pairs 1 day after heat shock. Remarkably these percentages are not constant through the different time points analyzed, 3 days after HS, 63% of the ISC-EB pairs show a distribution of CENP-A that can be considered as asymmetric and 7 days after it drops to 40%. But keeping in mind that in homeostasis, 20% of the ISC divisions are symmetric and that once the HS is induced it is not possible to synthesize CENP-A-GFP is expected that the numbers for CENP-A distribution within the ISC-EB pair after certain time point do not show the asymmetric inheritance of this histone variant. This is because during symmetric ISC division, two ISCs having CENP-A-mKO can be formed, and those once they divide, even if they do asymmetrically, afterwards will give rise to an outcome like depicted in model 4 and 5, and these models can be categorized as a symmetric distribution model for CENP-A.

My analysis of CENP-A distribution within ISC-EB pairs validated the previous conclusion that CENP-A is asymmetrically distributed during ISC division. However, it has been assumed that old synthesized CENP-A is retained by the ISC, but no clear evidence was shown so far. Therefore, I checked ISC-EB pairs stained for Delta or β-Galactosidase (when the flies contained the Delta-lacZ reporter) to specifically distinguish which cell of the pair was the ISC and which the EB, and by so, validating whether ISCs retained old synthesized CENP-A or not. In Fig. 2.6D there are two panels showing either Delta staining (upper panel) or β-Gal (lower panel) in ISC-EB pairs, in both the cases, the cell with Delta and thus the ISCs retained CENP-A-GFP. These data show that indeed CENP-A is asymmetrically inherited in cells of the midgut epithelium, being the old synthesized CENP-A (CENP-A-GFP) retained specifically by ISCs, as possible mechanism to retain stem cell properties.

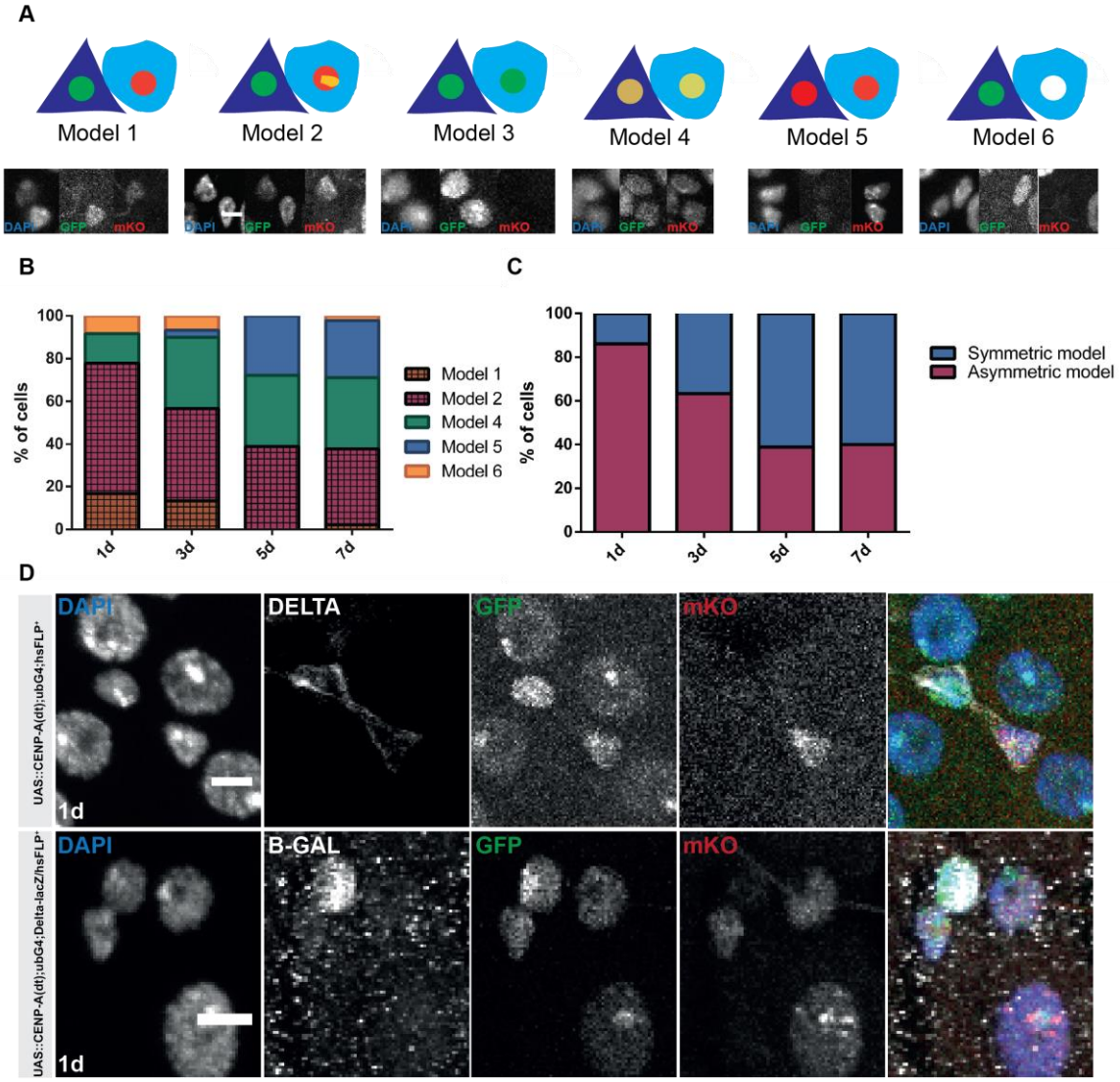

Figure 2.6 CENP-A is asymmetrically distributed towards the ISCs in ISC-EB pairs.

(A) Different models of possible outcomes in CENP-A distribution within the ISC-EB pair (all these models can be found in fixed tissue as showed in the panel underneath the model draw). (B) Qualitative measurement of CENP-A distribution in ISC-EB pairs within the different models from 3 or more independent experiments. (C) Distribution of the CENP-A behavior in ISC-EB pairs according to either an asymmetric model or symmetric (from the previous graph). For day 1, 79% of the ISC-EB pairs shows how CENP-A is distributed asymmetrically, matching perfectly with the known percentage of asymmetry vs symmetry fate division occurring in homeostasis in the gut (80% to 20% respectively, (de Navascues et al., 2012)). (D) Fixed and stained female fly guts show the distribution of old CENP-A (green) and newly synthesized CENP-A (red) at 1 day recovery timepoint. In the upper panel, the ISC can be distinguished by high accumulation of Delta in the cytoplasm. In the lower panel, the ISC can be detected with β-Galactosidase staining. In both panels, old synthesized CENP-A (GFP) is retained by the ISC. Scale bars, 5 μm.

2.1.2.2 Alternative systems supporting the asymmetric distribution of CENP-A in ISCs

The switch between CENP-A-GFP (pre-existing) and CENP-mKO (newly synthesized) in the previous experiments was always induced by a heat shock treatment. The HS applied to the adult flies aimed to induce an irreversible DNA recombination between the FRT sites of the transgene, ending so with the expression of GFP-labeled old CENP-A and starting from that point on with the expression of mKO-labeled newly synthesized CENP-A. However, to avoid possible concerns regarding the efficiency of the recombination mediated by the hs-flp, I decided to check the CENP-A distribution in another system where the flippase activity can be controlled.

The system I chose is the so called esg flip-out system (esg F/O), a lineage-tracing system, which allows the permanent labeling with GFP of ISCs and all their progeny and, importantly, includes an UAS-flp in the genetic background of the fly (Jiang et al., 2009). With this induction system *esg* positive cells and all their progeny will express Gal4, UAS-GFP, UAS-flp and CENP-A(dt) after a temperature shift. However, since the UAS-flp is active at the same time as the expression transgene, it will produce the recombination between the FRT sites of the dual labeling transgene for CENP-A and only newly synthesized CENP-A mKO will be detectable (Fig 2.7A). Therefore, to prove my previous observations, I should find ISCs lacking signal for this newly synthesized CENP-A-mKO.

As mentioned before, one of remarkable features of the esg F/O system is that allows the specific evaluation of ISCs and their progeny, since all will be permanently labeled with GFP forming the so called "clone". To distinguish ISCs within the clone, I performed Delta staining. In Fig 2.7B it is shown how CENP-mKO behaves once it is induced by the temperature shift. I evaluated clones at different timepoints after the temperature drift, and in all the cases I found ISCs within the clone marked by expression of Delta (arrows marked Delta+ve cells) that were lacking or expressing less signal compared to their differentiated progeny (all the cells labeled with GFP are originally from the same ISC). These data give more evidences to strengthen my hypothesis that ISCs retained old synthesized CENP-A, and therefore are negative or show less CENP-A-mKO compared to daughter cells.

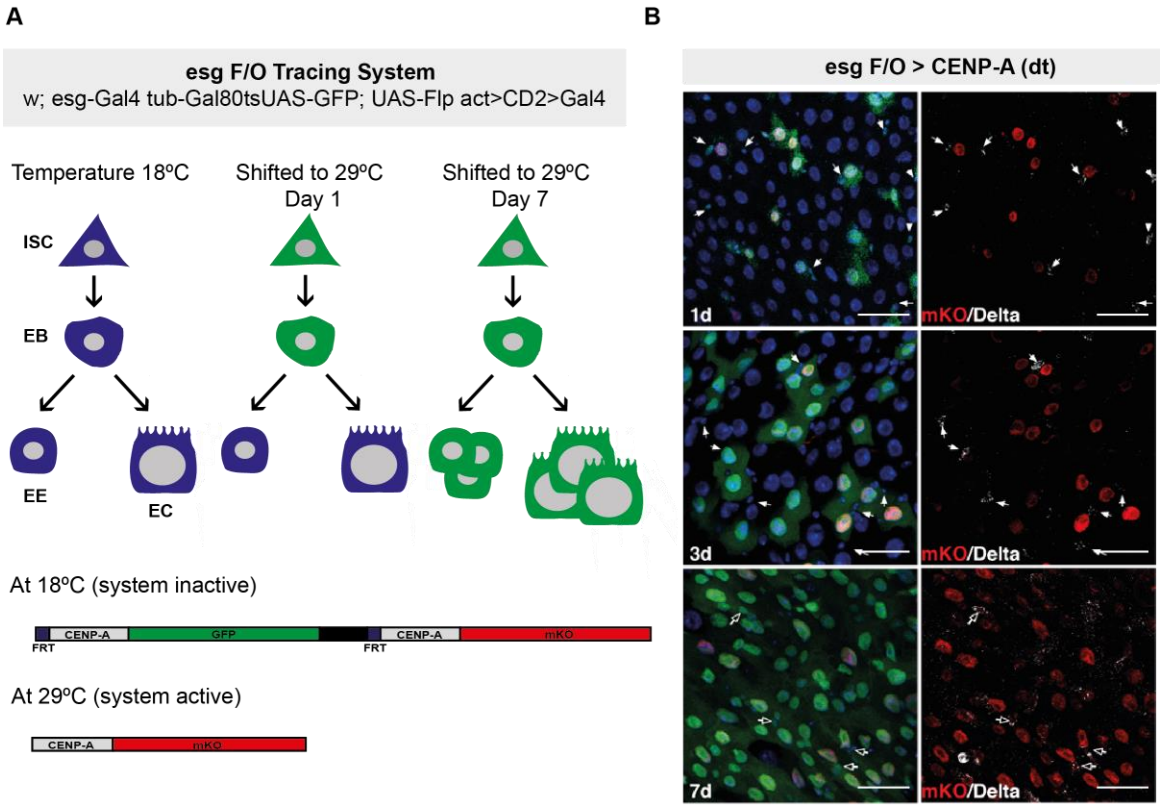

Figure 2.7 Clonal analysis assays using esg F/O system show that newly synthesized CENP-A is reduced from ISCs.

(A) Diagram outlining the esg F/O system (adapted from (Korzelius et al., 2014)). At 18°C the system is held inactive by the temperature-sensitive suppressor Gal80^{ts} (left). 1 day shift to 29°C inactivates the Gal80^{ts}, and esg-Gal4 will drive the expression of UAS-GFP and UAS-Flp, thereby activating the Act>STOP>Gal4 cassette and the FRT sites of my CENP-A(dt) transgene. After several days at 29°C, the esg F/O system will express UAS-GFP and UAS-CENP-A-mKO (newly synthesized) in both the progenitor cells and in the descendants from these progenitors due to the activated actin-Gal4 driver. (B) Distribution of CENP-A-mKO after clone induction for 1, 3 and 7 days. Samples were stained with Delta to selectively mark ISCs within the clone, marked with arrows in the right panel. ISCs show less CENP-A-mKO signal than their descendants. Scale bars, 25 μm.

2.1.2.3 Old synthesized CENP-A can persist in ISC for more than 20 days

It is known that it takes 12 days for flies fed on normal food to completely renew the posterior midgut epithelium (Jiang et al., 2009). Therefore, I decided to check CENP-A distribution at later time points. So far I have observed how ISCs retained old CENP-A (GFP labeled) seven days after heat shock induction, but if the epithelium takes more time for a complete renew, it is worth to check the distribution 10 and 20 days after HS.

Analysis of female midguts 20 days after heat shock uncovered that old synthesized CENP-A (GFP labeled) can be retained in midgut cells and be detectable 20 days after its synthesis (Fig. 2.8, arrows point to ISC-EB pairs). The stability and persistency of CENP-A supports the idea that CENP-A could act as an epigenetic factor responsible for regulating stem cell properties. However, it remains to be tested that the tag used in the transgene does not favor the stability of the protein (Appendix 6.1).

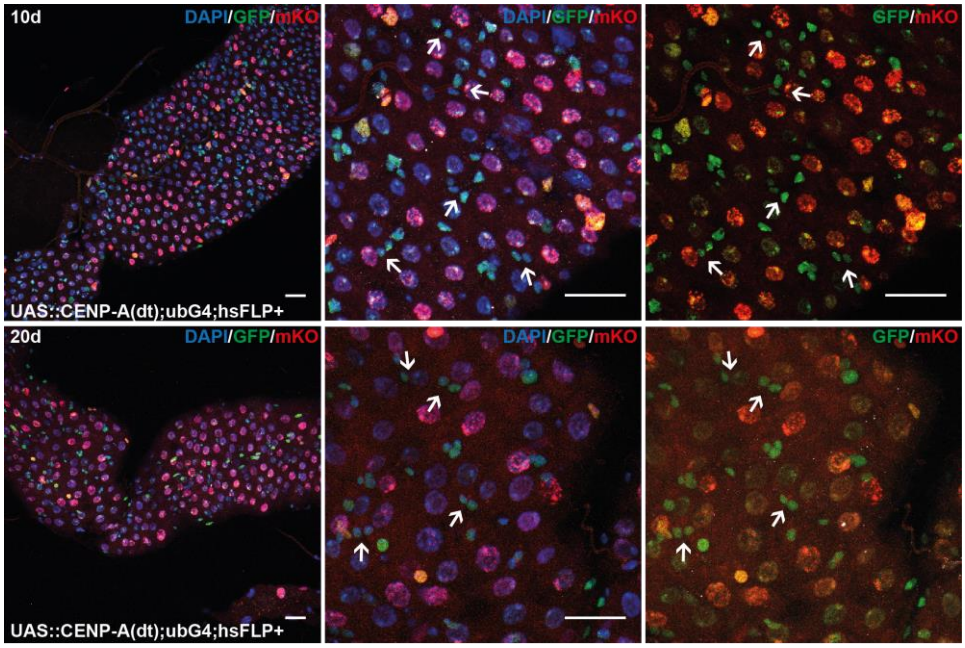

Figure 2.8 CENP-A persists at high levels in progenitor cells even after 20 days.

Distribution of old CENP-A (green) and newly synthesized CENP-A (red) in fixed female fly guts at 10 and 20 days recovery time point after heat shock. The arrows mark the ISC-EB pairs, where CENP-A-GFP can still be detectable. Scale bars, 25 μm.

2.1.2.4 CENP-A-GFP ISCs undergo cell division

The retention of a histone variant for long time could be because the cell of study has remained quiescent and thus loading of the newly synthesized has not occurred. To investigate the proliferation capacity of the ISCs after the heatshock event, I performed an EdU incorporation assay in cells of the midgut at the same time points after HS that I analyzed previously in section 2.1.2. When added to the dissecting medium, EdU is incorporated into the newly synthesized DNA during replication and in endoreplicating cells (Micchelli and Perrimon, 2006). EdU was diluted in Ringer's solution where the guts were incubated for 5 hours after their dissection.

EdU+ve cells can be detected in the different time points (Fig. 2.9). The fact that "small cells" show signal for EdU (ISCs are the only cell type of the epithelium that proliferates, but ECs undergo endocycles and thus also show EdU incorporation), clearly demonstrates that ISCs undergo mitosis and they have developed a specific mechanism to retain the old CENP-A, ruling out the possibility that the retention of CENP-A is an artifact caused by non-proliferating ISCs.

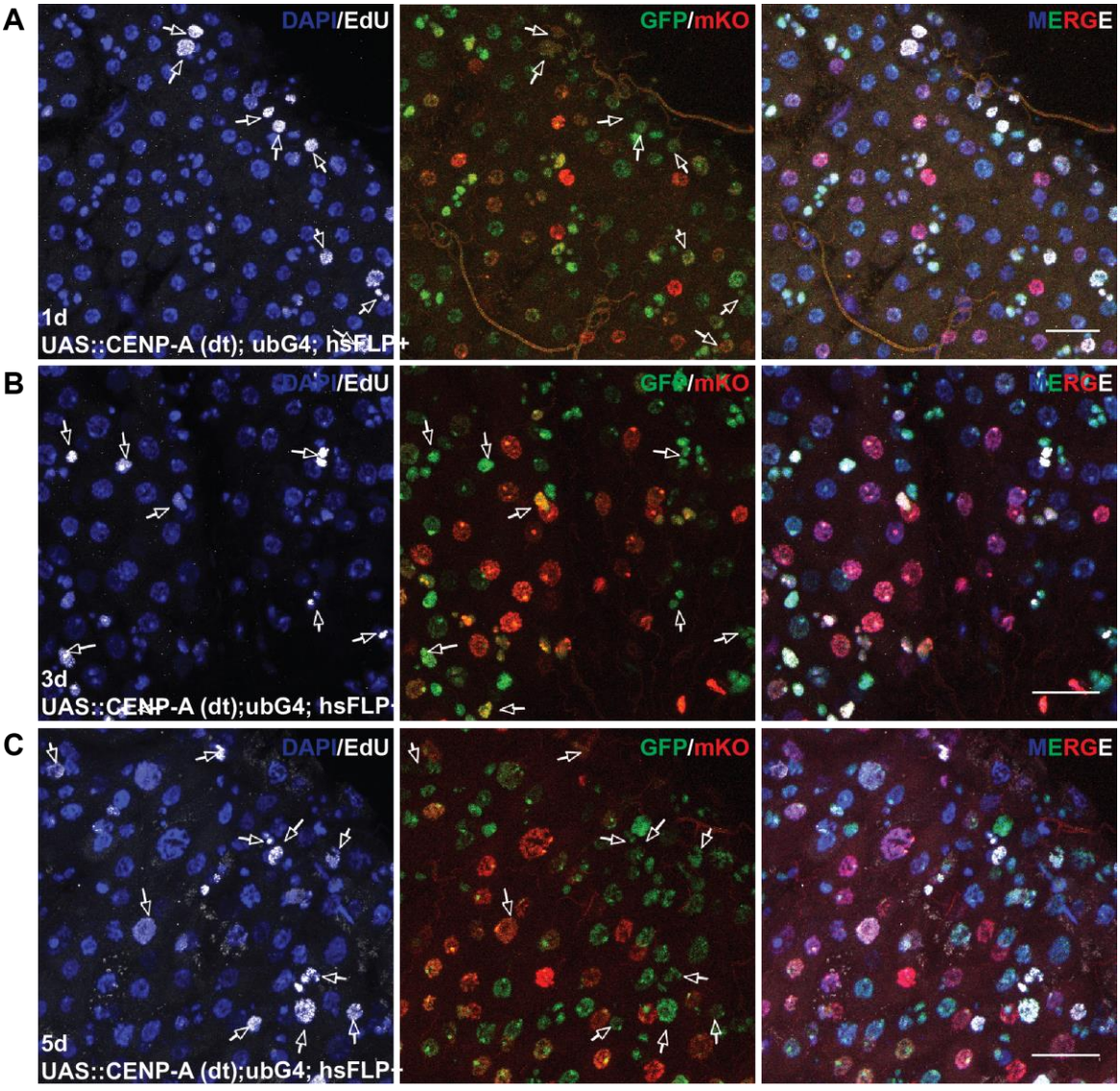

Figure 2.9 ISCs retaining old synthesized CENP-A GFP do proliferate

EdU incorporation assay in heat shocked female fly guts show that ISCs retaining CENP-A-GFP do proliferate. (A-C) Female fly guts were dissected at the specified time point after heat shock (A, 1 day; B, 3 days; C, 5 days) and incubated for 5 hours in 10μM EdU. Then, the samples were fixed and the Click-iT® Plus kit was used to detect cells which have incorporated EdU during the incubation time. Arrows point to the cells, which have undergone S phase and therefore have incorporated EdU. Scale bars, 25 μm.

2.1.2.5 Overexpression of CENP-A in the midgut epithelium does not affect the flies' lifespan

The histone H3 variant CENP-A is usually restricted to centromeric chromatin and is loaded into chromosome arms only upon its overexpression, leading so to the formation of ectopic kinetochores and consequently genomic instability (Heun et al., 2006). The existence of an asymmetric CENP-A distribution in ISCs discovered in this study is based on *in vivo* data where CENP-A was overexpressed and not only restricted to centromeres. To investigate if the overexpression of my transgene is detrimental for the flies, I carefully checked the mitotic rate and the lifespan of the overexpressing CENP-A(dt) flies in comparison with wt flies.

An increased number of mitotic ISCs in the midgut epithelium is an indication of stress or damage to the intestine. If the overexpression of CENP-A(dt) in ISCs caused genomic instability, I would expect that the number of mitotic ISCs will be increased in the given situation to replenish the ISC loss. However, the number of mitoses per midgut in o/e of CENP-A(dt) was not affected and similar number to wt situation were quantified (Fig. 2.10A). Furthermore, I analyzed the lifespan of the flies to investigate whether the o/e in all the cell types accomplished by the ub.Gal4 was detrimental for the animal. In this case, no significant difference in the median survival of control flies compared to o/e flies was detected. All these data suggest that the overexpression of CENP-A(dt) has not detectable effect in the adult midgut epithelium or in the viability of the animal (Fig. 2.10B).

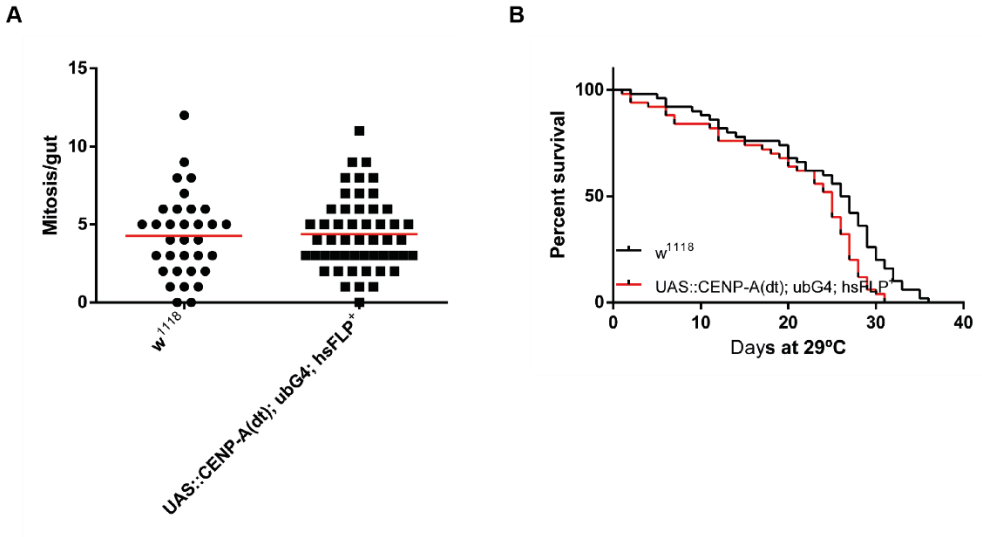

Figure 2.10 Overexpression of CENP-A(dt) does not affect ISC proliferation nor the flies' lifespan.

(A) Quantification of PH3+ cells in adult midguts of the indicated genotype. No difference in the number of dividing ISCs (PH3 positive cells) was observed in overexpressing CENP-A midguts compared to wildtype

flies w1118. (B) Kaplan-Meier survival curve of flies of the indicated genotype, n= 50. No change in the flies lifespan either was observed upon overexpression of CENP-A.

2.1.2.6 Somatic embryonic cells symmetrically distribute CENP-A during cell division

From previous observations in this study, I can conclude that CENP-A could have a role in the establishment of the asymmetry required during cell division of ISCs. To test if this is a mechanism employed specifically by stem cells, I studied the distribution of CENP-A in non-stem cells, which divide symmetrically giving rise to two equal cells indistinguishable from each other, with no asymmetric distribution of fate determinants. To do so, I used embryos ubiquitously expressing the dual labeling transgene. The cross was kept at 25°C and embryos were collected 6-8 hours after egg laying. These embryos were heat shocked for 45 minutes at 37°C, and one hour later they were dechorionated and mounted on a slide to analyze CENP-A distribution.

The differential distribution of old vs. new CENP-A was not detected for symmetrically dividing somatic cells of fly embryos (Fig. 2.11). This provides evidence that the asymmetric distribution of CENP-A is a mechanism specifically employed by stem cells.

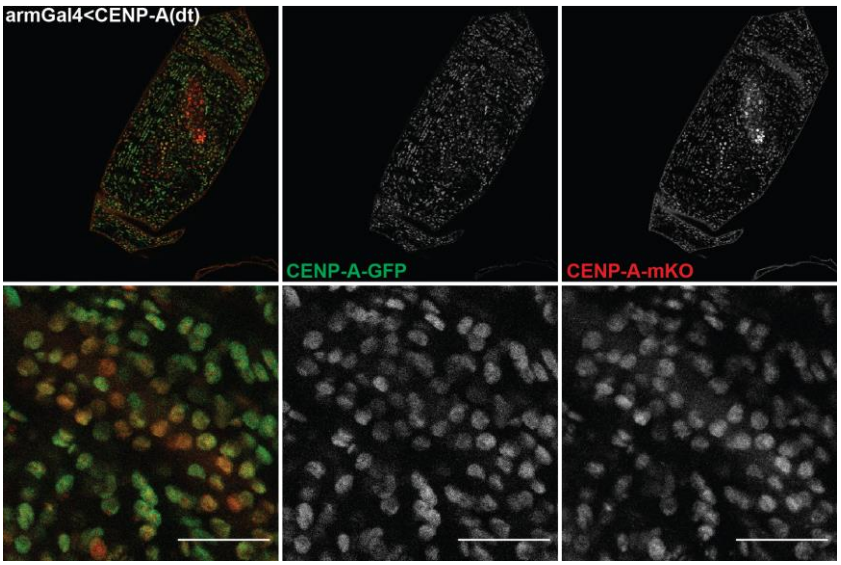

Figure 2.11 The asymmetric distribution of CENP-A is specific of stem cell like cells, embryonic somatic cells distribute equally old and newly synthesized CENP-A.

CENP-A distribution in embryonic somatic cells. The CENP-A(dt) transgene was driven by the arm-Gal4 driver. Embryos were collected overnight and then heat shocked for 45 minutes at 37°C in a water bath. All somatic cells show signal for both old (GFP) and newly (mKO) synthesized CENP-A. Scale bars, 25 μm.

2.1.3 H3.3 is not preferentially retained by ISCs

It seems that the asymmetric CENP-A distribution is a specific mechanism employed by ISCs, however it remains unclear whether other histone variants share the same behavior. To rule this out, I investigated the distribution of the histone H3 variant H3.3, which is often regarded as a mark for transcriptionally active chromatin. To do so, I used the same set-up but with flies ubiquitously expressing the dual labeling transgene for H3.3. The cross was kept at 25°C and newly eclosed flies (2-7 days) were heat shocked for 90 minutes at 37°C. Then, they were kept at 29°C and to analyze the distribution of old vs. new synthesized H3.3 in progenitor cells compared to differentiated progeny, midguts were studied at different timepoints. Samples from heat shocked flies were collected 1, 3, 5 and 7 days after HS, fixed and the number of GFP, mKO and GFP/mKO positive cells was evaluated. The expression of H3.3 was weaker compared to CENP-A and though the same driver was used to induce the expression, not all the cells of the midgut epithelium showed signal (Fig. 2.12).

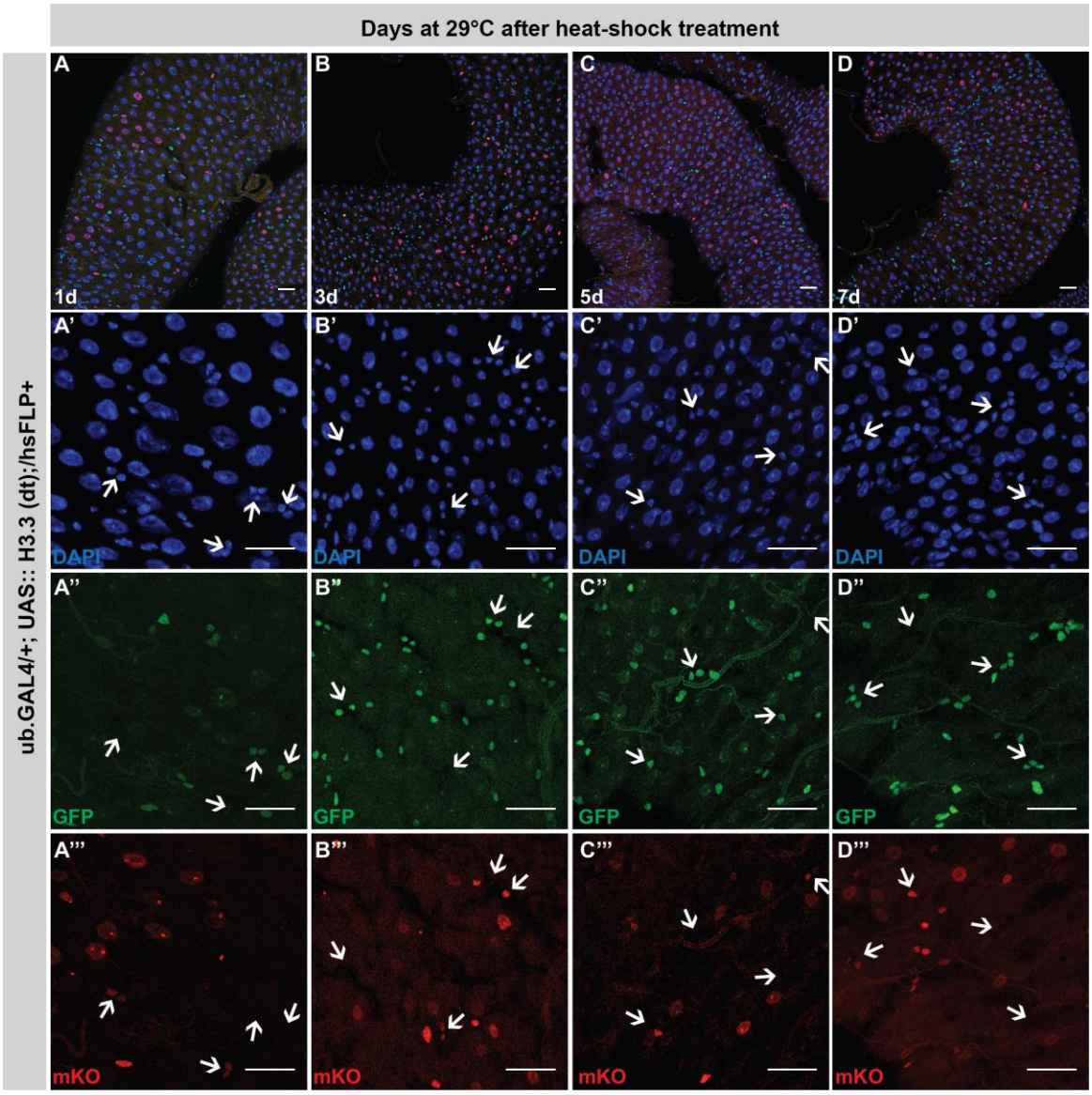

Figure 2.12 H3.3 histone variant has ambiguous distribution pattern in progenitor cells vs differentiating progeny.

Fixed and stained female fly guts show the distribution of old H3.3 (green) and newly synthesized H3.3 (red) at different recovery timepoints (A 1 day, B 3 days, C 5 days, D 7 days). No antibody was added to enhance GFP or mKO signal. (A'-D') Zoom in images of the same region showed in the upper panel, DAPI. (A''-D'' and A'''-D''') Zoom in images of the same region but showing GFO and mKO signal. Arrows point to the progenitor cells, 25 μm.

In this set of experiments, I could not include Delta-lacZ construct in the genetic background of the flies, so to specifically address the question of how H3.3 is distributed in ISCs compared to their differentiating progeny, I performed instead staining with Delta antibody. Unfortunately, Delta staining was very reliable between different midguts regions

and samples, not allowing to specifically identify ISCs. To be able to do quantifications, I decided to test the expression of the ubiquitous Gal4 driver in the different cell types by driving the expression of UAS::nls-GFP. Surprisingly, though this driver is supposed to be ubiquitously expressed and, therefore, driving UAS::nls-GFP to all the cell types within the midgut epithelium, it was not expressed in the differentiated enteroendocrine cells (EEs) (Fig.2.13). The advantage of this was that I could quantify H3.3 distribution in progenitor cells (ISCs+EBs) and in ECs, because they can easily be distinguished according to their size. Based on their nuclear size, the cells were sorted automatically into the two categories using Fiji.

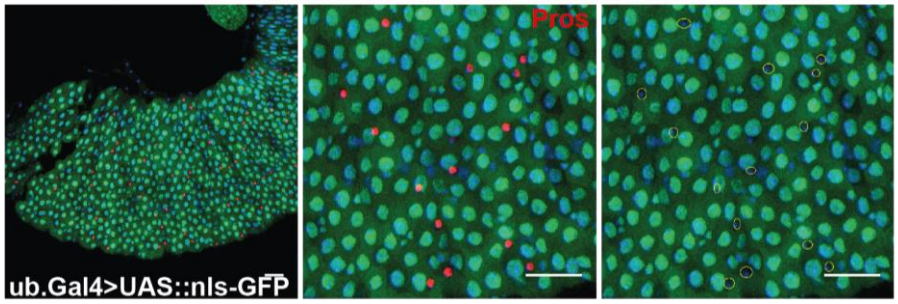

Figure 2.13 Ub.Gal4 is not expressed in enteroendocrine cells of the adult midgut

Fixed and stained female fly guts show nls-GFP drive by ub.Gal4. Samples were stained with Prospero (Pros), a specific marker for enteroendocrine cells (red signal). Pros+ve cells were not expressing nls-GFP. Circles mark the cells with signal for Pros. No antibody was added to enhance GFP signal. Scale bars, 25 μm.

One day after heat shock I found that only 68% of the progenitor cells analyzed showed signal for H3.3. 55% of the progenitor analyzed one day after heat shock retained H3.3-GFP whereas only 10% displayed H3.3-mKO signal. Seven days after heatshock, the percentage of progenitor cells with H3.3-GFP stayed almost constant (49% only GFP, 3% both), but the percentage of progenitor cells with newly synthesized H3.3 increased up to 20%. In the case of differentiated ECs, the transgene was virtually no expressed and therefore I could not draw clear conclusions out of the H3.3 distribution.

In contrast to CENP-A, the histone variant H3.3 does not exhibit asymmetry during ISC division. The ambiguous distribution of H3.3 suggest that the asymmetric inheritance mode is not a common feature of all histone variants and perhaps specific for CENP-A (Fig. 2.14).

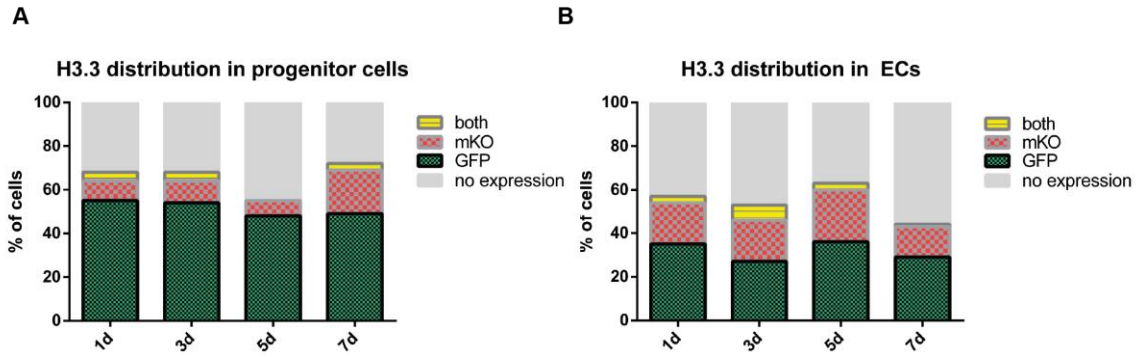

Figure 2.14 Quantification of histone variant H3.3 in adult *Drosophila* midgut.

(A) Quantification of GFP and/or mKO positive-progenitor cells, and therefore ISCs and EBs from 3 or more independent experiments. For the all the timepoints, half of the population of the progenitor cells retained old synthesized H3.3 (GFP labelled), whereas only 10% of the progenitor cells analyzed showed signal for newly synthesized H3.3. Remarkably, more than 30% of the progenitor cells do not show any expression of the transgene. (B) Quantification of GFP and/or mKO distribution in ECs from 3 or more independent experiments. Half the population of the differentiated cells in the adult midgut which express the transgene retain old synthesized H3.3, whereas the other half inherit the newly synthesized H3.3 pointing to a symmetric H3.3 distribution in differentiated ECs.

2.1.4 Canonical H3 is asymmetrically distributed in the midgut epithelium

Tran et al. reported that canonical histone H3 is asymmetrically partitioned in germline stem cells of *Drosophila* testis. It seems that the asymmetric distribution of the histone variant CENP-A is a specific mechanism employed by ISCs, however it remains unclear whether canonical H3 share the same behavior in other stem cell niches. To find out how H3 is inherited in progenitor cells compared to differentiated cells in the intestine, I investigated the distribution of canonical histone H3 in the adult midgut. To do so, I used the same set-up but with flies ubiquitously expressing the dual labeling transgene for H3. The cross was kept at 25°C and newly eclosed flies (2-7 days) were heat shocked for 90 minutes at 37°C. Then, they were kept at 29°C and to analyze the distribution of old vs. new synthesized H3 in progenitor cells compared to differentiated progeny, midguts were studied at different time points. Samples from heat shocked flies were collected 1, 3, 5 and 7 days after HS, fixed and the number of GFP, mKO and GFP/mKO positive cells was evaluated. The expression of H3 was not penetrant to all the progenitor cells of the epithelium compared to CENP-A though the same driver was used to induce the expression (Fig. 2.15-16).

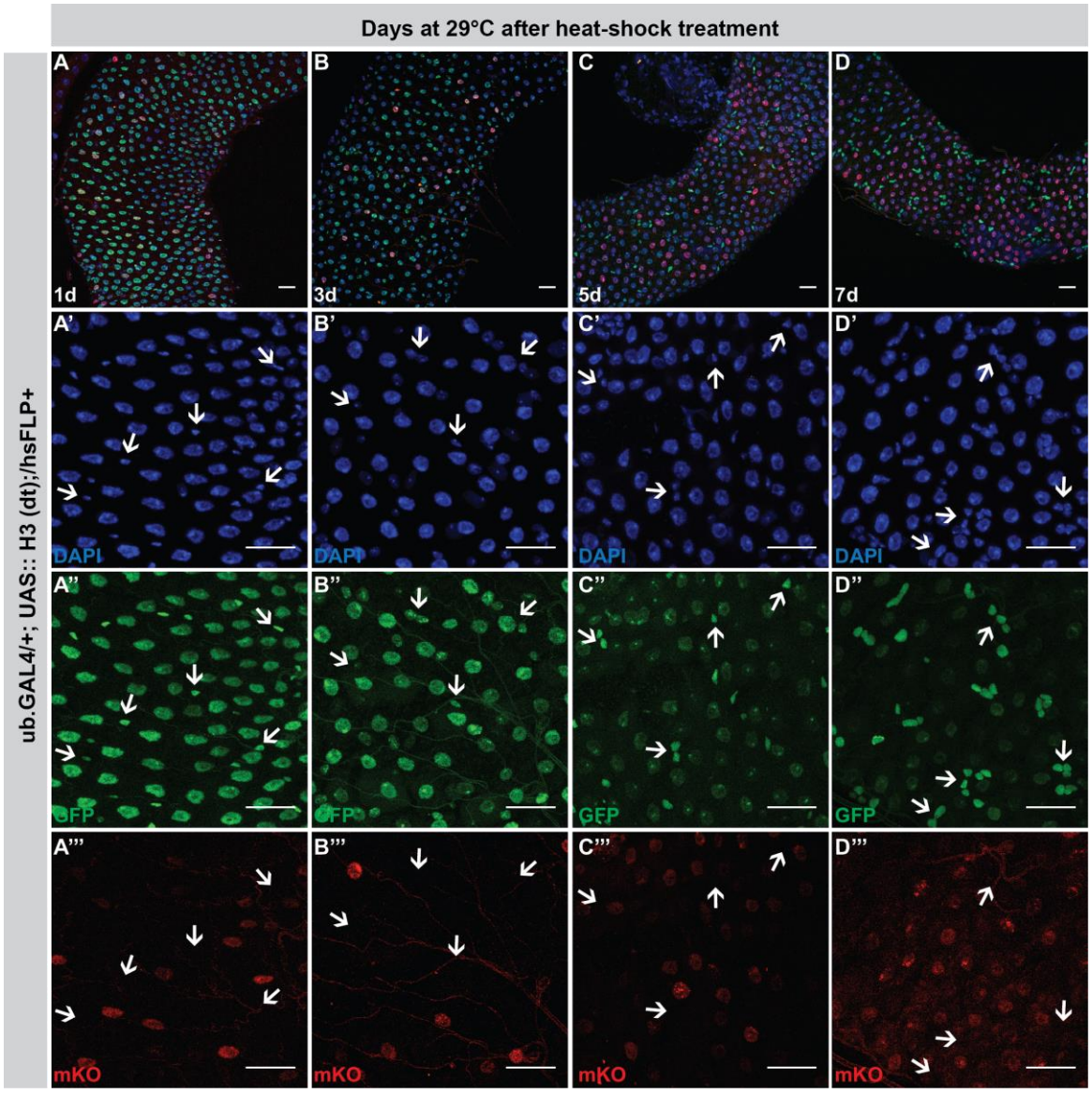

Figure 2.15 Canonical histone H3 also distributes asymmetrically in adult *Drosophila* midgut.

Fixed and stained female fly guts show the distribution of old H3 (green) and newly synthesized H3 (red) at different recovery timepoints (A 1 day, B 3 days, C 5 days, D 7 days). No antibody was added to enhance GFP or mKO signal. (A'-D') Zoom in images of the same region showed in the upper panel, DAPI. (A''-D'' and A'''-D''') Zoom in images of the same region but showing GFO and mKO signal. Scale bars, 25 μm.

One day after heat shock, 65% of the progenitor cells analyzed showed signal for H3-GFP, while only 1% of the progenitor cells were positive for newly synthesized H3-mKO. Seven days after heatshock, the percentage of progenitor cells with H3-GFP stayed almost constant (60%), but I could not find progenitor cells with newly synthesized H3-mKO. In the case of differentiated ECs, overexpressed H3 seems not to be so efficiently

loaded as CENP-A was, but still 24% of the ECs analyzed were positive for H3-mKO, and the percentage of differentiated cells expressing H3-GFP was significant reduced.

Similar to CENP-A, the canonical histone H3 exhibits asymmetry during ISC division. These data suggest that canonical H3 is also asymmetrically distributed during ISC division as it is during GSC, being so a mechanism most likely employed in all stem cell niches to maintain stem cell properties. CENP-A and H3 could act as important factors during stemness and further experiments would be needed to unravel the specific molecular mechanism behind. The analysis of the distribution of CENP-A in other stem cell niches, such as GSCs, would be one of the next steps to assess its function as a key stemness factor.

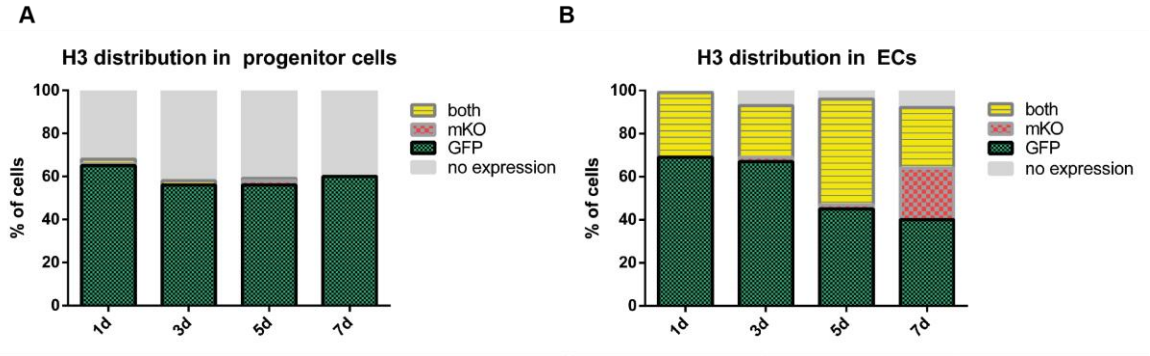

Figure 2.16 Quantification of canonical H3 distribution in adult midgut cells show a clear asymmetric distribution of old synthesized H3 in ISCs.

(A) Quantification of GFP and/or positive progenitor cells, and therefore ISCs and EBs from 3 or more independent experiments. The percentage of progenitor cells retaining old synthesized H3 (GFP) remains stable through the different time points and almost none progenitor cells with H3-mKO can be detected. (B) Quantification of GFP and/or mKO distribution in ECs from 3 or more independent experiments. In contrast with the stem cell behavior, the differentiated cells in the adult midgut do not retain old synthesized H3 as the graph shows with a gradually decrease of 64% (day 1) to 42% (day 7) of ECs having GFP-H3. Remarkably, ECs only having H3-mKO cannot be detected until 7 days after the heatshock.

2.2 CENP-A loading is required in proliferating ISC and non-mitotic cells of the adult midgut

2.2.1 Centromere and inner kinetochore proteins are required for ISC proliferation

Previously, I have shown how CENP-A is asymmetrically distributed during stem cell division and how ISCs retain old synthesized CENP-A, probably as a mechanism to maintain their stem cell properties. If CENP-A is important for stem cell regulation, I next ask what happens to the fruit fly midgut epithelium upon depletion of key centromere and inner kinetochore proteins. In *Drosophila*, the inner kinetochore contains only two more proteins: CENP-C and CAL1. These proteins together with CENP-A are mutually co-dependent for their centromeric localization and function (Erhardt et al., 2008).

To test how the depletion of these proteins affect the midgut epithelium, I used the intestinal lineage tracing system esg FlipOut (esg F/O) (Korzelius et al., 2014) to drive the expression of hairpin RNA for $CAL1^{RNAi}$, $CENP\text{-}A^{RNAi}$ and $CENP\text{-}C^{RNAi}$ to progenitor cells and their descendant cells. Upon depletion of these factors specifically in the progenitor cells of the intestine, the midgut loses its ability to proliferate and differentiate (Fig. 2.17B-C). The size of the clones was quantified at intervals after knockdown and clone induction by quantifying the number of GFP+ve cells per clone. All the mutant clones were dramatically smaller than the control clones at all time points (Fig. 2.17E).

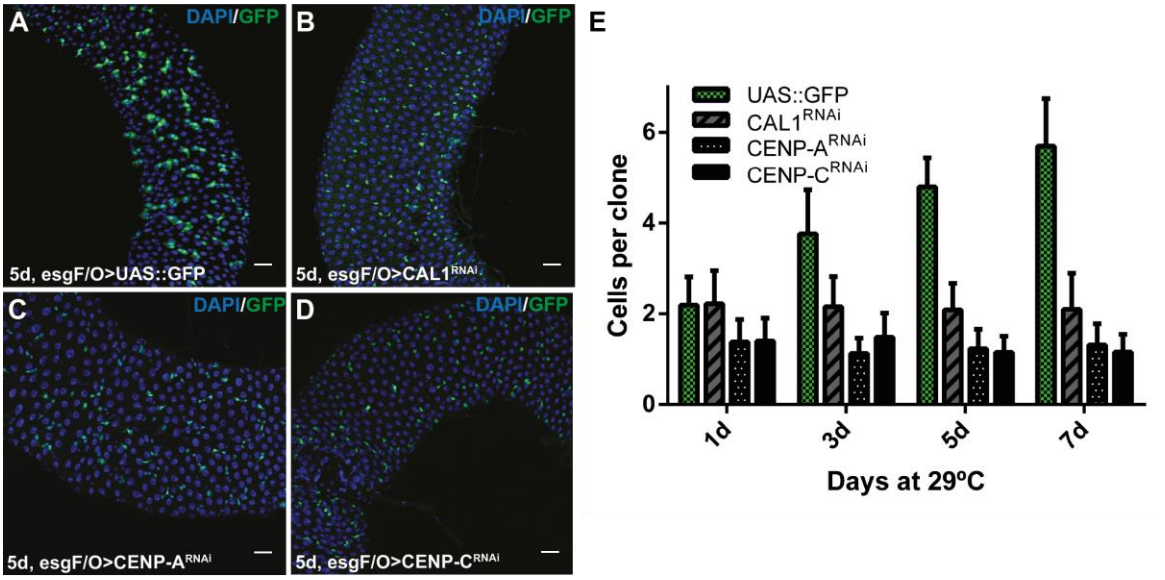

Figure 2.17 The key centromere proteins CAL1, CENP-A and CENP-C are required for proliferation of progenitor cells of the *Drosophila* intestine.

(A-D) Clones generated by the esg F/O system are marked with GFP (green) and were induced for 5 days, nuclei were visualized by DAPI (blue) staining. (A) Control adult midgut, (B) knock down of CAL1, (C) knock down of CENP-A and (C) knock down of CENP-C. The size of clones marked with GFP was significantly reduced after CAL1, CENP-A and CENP-C depletion, resulting in pairs or individual diploid cells. (E) The size of the clones was quantified by counting cell numbers per clone after 1, 3, 5 and 7 days of clone induction. Scale bars, 25 μm.

CAL1, CENP-A and CENP-C seems to be important for ISC proliferation, since they are key player during cell division. Since CAL1 is considered the CENP-A-specific loading factor during mitosis and meiosis (Chen et al., 2014; Dunleavy et al., 2012) and, as already mentioned, all these proteins are co-dependent for their localization, I decided to focus for the rest of this study on understanding better the role of CAL1 in the different cell types of the midgut epithelium.

To further confirm CAL1's function in progenitor cells of the midgut, I investigate the regenerative response after enteric infection. If CAL1 loss abolishes the proliferation and differentiation of the progenitor cells, I assume that it should compromise regenerative growth of the intestine as well. To test this, I exposed the flies to the pathogenic bacterium *Pseudomonas entomophila* (*P.e*) for 2 days. Bacterial infection has a dramatic impact on the gut physiology, that causes a strong stress response that consequently stimulates stem cell proliferation and induces epithelial renewal. Flies expressing CAL1^{RNAi} in progenitor

cells had disorganized midguts without pH3-positive cells compared to the control (Fig. 2.18A). Whereas control midguts showed an almost complete self-renewal of midgut tissue after 2 days of *Pe* infection (based on the ubiquitous expression of GFP), in CAL1^{RNAi} midguts there was little renewal and they had still many GFP negative cells, pointing to an inability to self-renew upon infection. Moreover, these midguts lost ECs and shrank to a small disorganized structure and adult flies suffer a high reduction in their median survival after *P.e* infection compared to control situation (Fig. 2.18B). All these data support that CAL1 and, therefore, very likely CENP-A loading seems to be required for the proliferation of the progenitor cells and their proliferation response to bacterial damage.

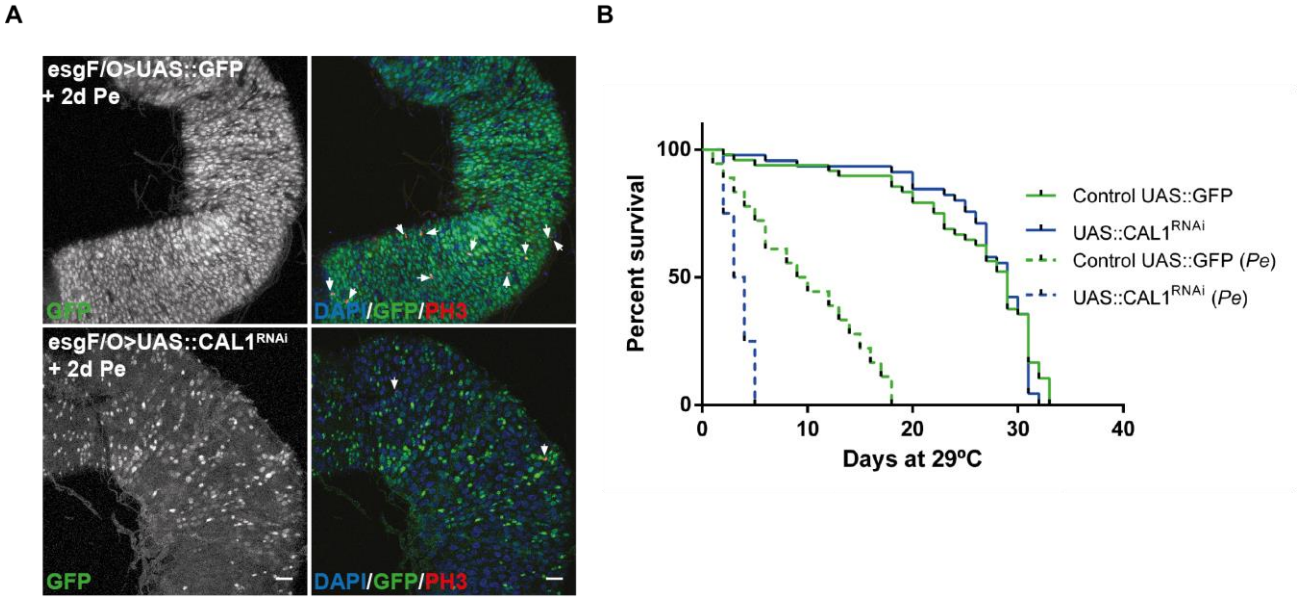

Figure 2.18 CAL1 knockdown in progenitor cells inhibits epithelia regeneration.

(A) Esg F/O midguts expressing UAS::GFP alone (control) or CAL1^{RNAi} 2 days after *P. entomophilae* infection. Samples were stained for pH3 (red, arrows). Scale bars, 25 µm. (B) Kaplan-Meier survival curve of flies with (dotted line) and without (straight line) *P. entomophilae* containing food, n= 50 for each genotype under normal conditions, n=20 for each genotype under *Pe* containing food analysis.

2.2.2 Knockdown of CAL1 and CENP-A in ISCs is detrimental

To validate the role of CAL1 and CENP-A in the proliferation capacity of ISCs, I used an esgGal4 driver combined with Su(H)Gal80 to drive the RNAi constructs exclusively to ISCs. Five days of expressing RNAi against CAL1 and CENP-A led to a loss

of ISCs, measured by quantifying the number of GFP+ve cells (Fig. 2.19E). The loss of ISCs should promote a response in the midgut epithelium to replenish this lost, however I could not observe an increase in the number of mitoses per gut (Fig. 2.19D), since presumably ISCs cannot enter mitosis lacking CAL1 and/or CENP-A. Thus, CAL1 and CENP-A are important for the maintenance and division of ISCs.

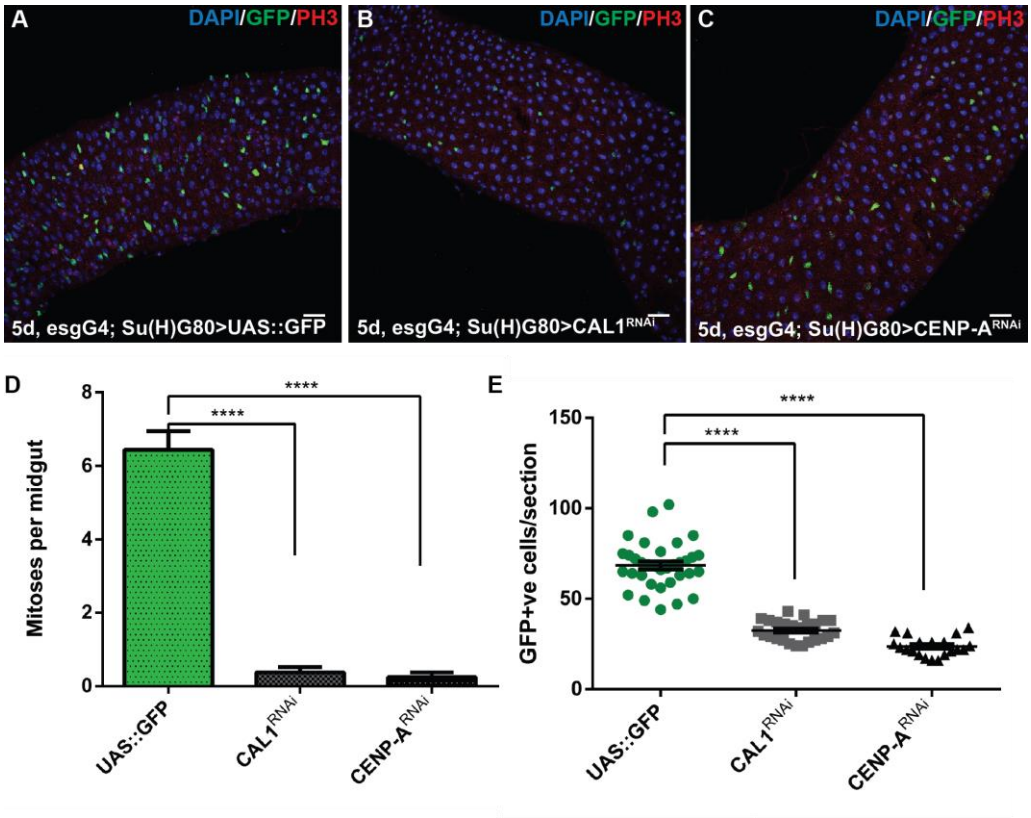

Figure 2.19 CAL1 and CENP-A knockdown in ISCs promotes ISC death.

(A-C) Knockdown of CAL1 and CENP-A in ISCs using the esg^{ts}, Su(H)-Gal80 system. ISCs were marked by GFP (green). Samples were stained for PH3 (red) and DAPI (blue). (A) Control adult midgut, (B) CAL1 depleted midgut after 5 days induction at 29°C, (C) CENP-A depleted midgut after 5 days induction at 29°C. Decrease in the number of GFP positive cells were observed in CAL1 and CENP-A depleted midguts. Scale bars, 25 μm. (D) Quantification of PH3+ cells in adult midguts of the indicated genotype. (E) Quantification of GFP+ cells in adult midguts of the indicated genotype. Statiscal significance was determined by Student's t test (****p<0.0001). Error bars in each graph represent standard error or mean (SEM).

2.2.3 Loading of CENP-A is required in post-mitotic EBs

CENP-A and its loading factor CAL1 have always been studied in the context of cell division. However, according to data from this study CENP-A could play a role in non-dividing cells, since loading of overexpressed CENP-A can be detected in non-dividing differentiated ECs of the midgut epithelium but not of other histones (Fig. 2.4, 2.13 and 2.15) and the mRNA level of CENP-A is higher in transient progenitor EBs than in ISCs (Fig. 2.1B). Furthermore, the depletion of inner kinetochore proteins in progenitor cells seems to stall the differentiation of them, since no EC formation was observed in esg F/O clones depleted either for CAL1, CENP-A or CENP-C (Fig. 2.17).

To check if CAL1 plays a role in non-dividing cells of the midgut epithelium, I use the genetic tools available to drive RNAi constructs specifically to the cell types of my interest. First, I depleted CAL1 under the control of the inducible, enteroblast-specific Gal4 driver (Su(H)-Gal4^{ts}) (Zeng et al., 2010). Three days of expressing RNAi construct against CAL1 led to a complete loss of EBs, measured by quantifying the number of GFP+ve cells (Fig. 2.20B, D). Moreover, when CAL1 was depleted from EBs I could not detect dividing cells marked by PH3 (Fig. 2.20C), since ISCs are the only mitotic cell type in the epithelium, these data support that CAL1 loss in EBs does not have non-cell autonomous functions in regulating ISC proliferation. In conclusion, CAL1 is important for the maintenance and differentiation of EBs, being this a novel discovery of a kinetochore protein having functions outside of mitosis.

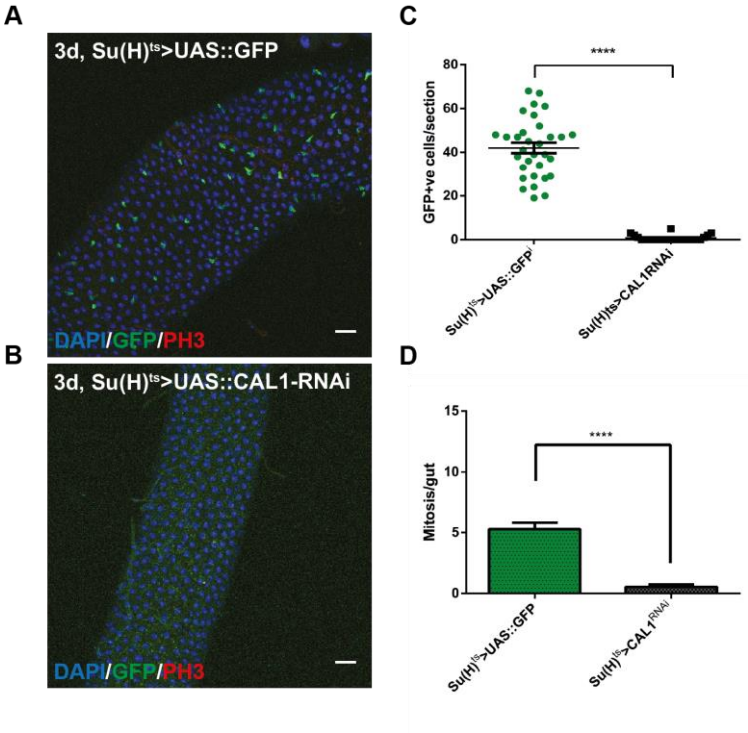

Figure 2.20 CAL1 is required in the non-dividing progenitor cells EBs.

(A-B) Knockdown of CAL1 in EBs specifically using the Su(H)^{ts} system. EBs were marked by GFP (green). Samples were stained for PH3 (red) and DAPI (blue). (A) Control adult midgut, (B) CAL1- depleted midgut after 3 days induction at 29°C. Decrease in the number of GFP positive cells were observed in CAL1 depleted midguts. Scale bars, 25 μm. (C) Quantification of GFP+ cells in adult midguts of the indicated genotype. (D) Quantification of PH3+ cells in adult midguts of the indicated genotype. Statistical significance was determined by Student's t test (****p<0.0001). Error bars in each graph represent standard error or mean (SEM).

2.2.4 CENP-A and its loading chaperone CAL1 are important for endocycling cells

Interestingly, CENP-A was loaded in ECs when the expression of my dual labeling transgene (CENP-A(dt)) was driven by a ubiquitous Gal4 (Fig. 2.4). To test if CENP-A or its loading factor CAL1 are required in ECs as they are in EBs, RNAi was induced by using the inducible EC-specific driver Myo1A^{ts} (Jiang et al., 2009). After depleting CAL1 and CENP-A for three days specifically in ECs, a large and significant increase in ISC proliferation was observed in the whole adult midgut, as indicated by the increased number of PH3+ve cells (Fig. 2.21A, B).

The depletion of CAL1 and CENP-A in ECs affected severely the epithelium morphology, as shown in Fig. 2.21A, these flies had shrunken midguts and were very susceptible to infection, they suffer a high reduce in their median survival after *P.e* infection compared to control situation (Fig. 2.21C). Based on the ubiquitous expression of GFP in all the ECs in the control situation, and the existence of large nuclei lacking GFP signal when CAL1 and CENP-A depletion was induced, I assume that ECs undergo apoptosis after depletion of these factors, though staining with caspase-3 would be required to draw this conclusion. The loss of ECs will also explain the promotion of ISC proliferation. All these data support that CENP-A itself and its loading into chromatin seems to be required for the regulation and maintenance of ECs.

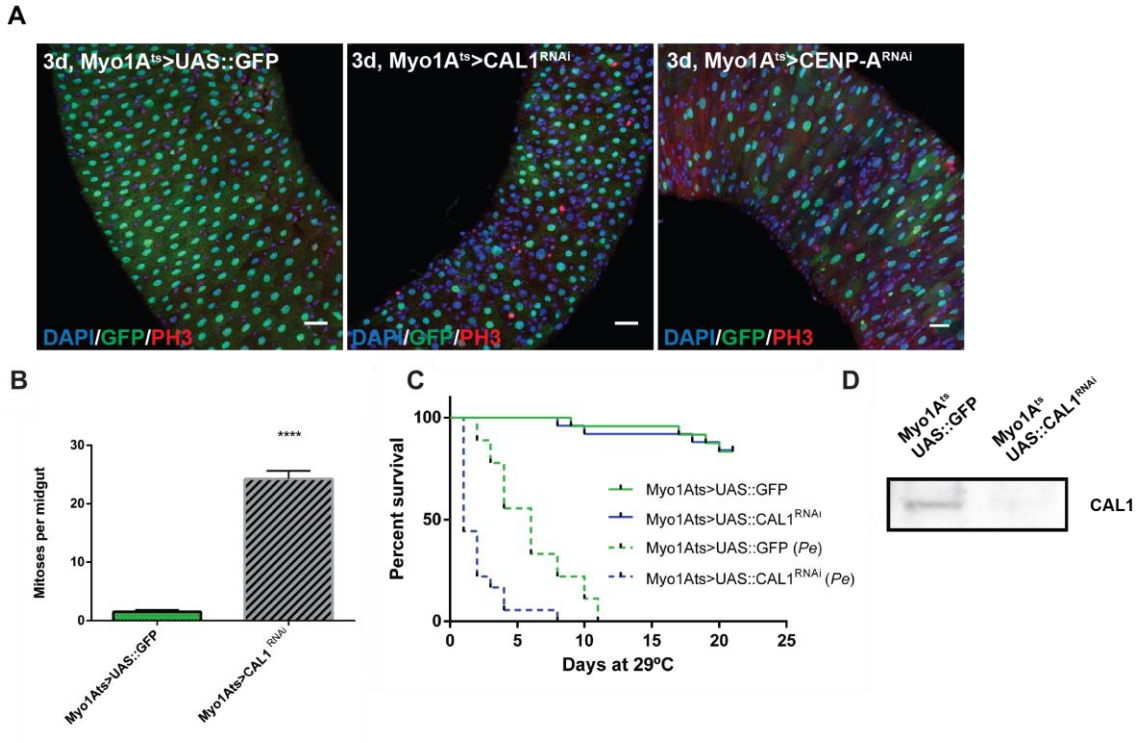

Figure 2.21 CAL1 and CENP-A are important in differentiated endocycling ECs.

(A) Knockdown of CAL1 and CENP-A in ECs specifically using the Myo1A^{ts} system. ECs were marked by GFP (green). Samples were stained for -PH3 (red) and DAPI (blue). Left panel, control adult midgut; middle panel, CAL1 depleted midgut; and right panel, CENP-A depleted gut after 3 days induction at 29°C. Decrease in the number of GFP positive cells were observed in CAL1 and CENP-A depleted midguts. Scale bars, 25 μm. (B) Quantification of PH3+ cells in adult midguts of the indicated genotype. Increase in the number of dividing ISCs (PH3 positive cells) were observed in CAL1 depleted midguts. Statiscal significance was determined by Student's t test (****p<0.0001). Error bars in each graph represent standard error or mean (SEM). (C) Kaplan-Meier survival curve of flies with (dotted line) and without (solid line) *P.*

entomophilae containing food, n= 25 for each genotype under normal conditions, n=20 for each genotype under *P.e* containing food analysis. (D) Whole fly extracts of the indicated genotype after 5 days induction at 29°C. CAL1 was efficiently down-regulated.

Enterocytes make up the majority of the midgut epithelium and one of the main characteristics of this cell type is that they undergo endocycles to increase their size and DNA content (Ohlstein and Spradling, 2006). To test if the previous phenotype of CAL1 and CENP-A depletion in ECs is related with their endocycling ability, I next decided to deplete these factors in other well-characterized endocycling cells in *Drosophila*, the salivary glands of third instar larvae.

Endocycles have mostly been studied in the salivary glands among other tissues of the adult fly, such as follicle and ovarian nurse cells. Remarkably, it has been reported that centromeric regions are under-replicated in salivary chromosomes (Hammond and Laird, 1985; Leach et al., 2000), Depletion of CAL1 was induced in salivary glands by using the ptc-Gal4 driver (Pierce et al., 2004), interestingly salivary glands lacking CAL1 were smaller, as indicated by the significant reduce in the mean nuclear area compared to the control (Fig. 2.22A, D).

To check the chromatin structure of these cells, I performed polytene squashes, but surprisingly the chromatin structure seems to be overall unaffected. HP1 was concentrated at heterochromatin chromocenter in both the cases (2.22B, B') and other histone marks were showing the same band patterning in control and cal1-depleted cells (data not shown).

Endocycles occur by successive S phases taking place without occurrence of cytokinesis, consequently increasing the cellular DNA content (polyploidy) (Edgar and Orr-Weaver, 2001). Next, I decided to test specifically the endocycle rate by EdU incorporation in dissected salivary glands. For salivary glands lacking CAL1, cells failed to progress into S-phase as observed by their reduced EdU incorporation (Fig. 2.22C, C'), as indicated by the dramatic decrease of EdU signal (red) in the salivary glands cells CAL1-deficient compared to control. Salivary glands during *Drosophila* metamorphosis are histolysed 15 hours after puparium formation, as result from a transcriptional switch triggered by ecdysone (Ninov et al., 2007). Remarkably, larvae with salivary glands deficient for CAL1 do not hatch into adult flies (Fig. 2.22E), showing a developmental lethality phenotype upon depletion of CAL1.

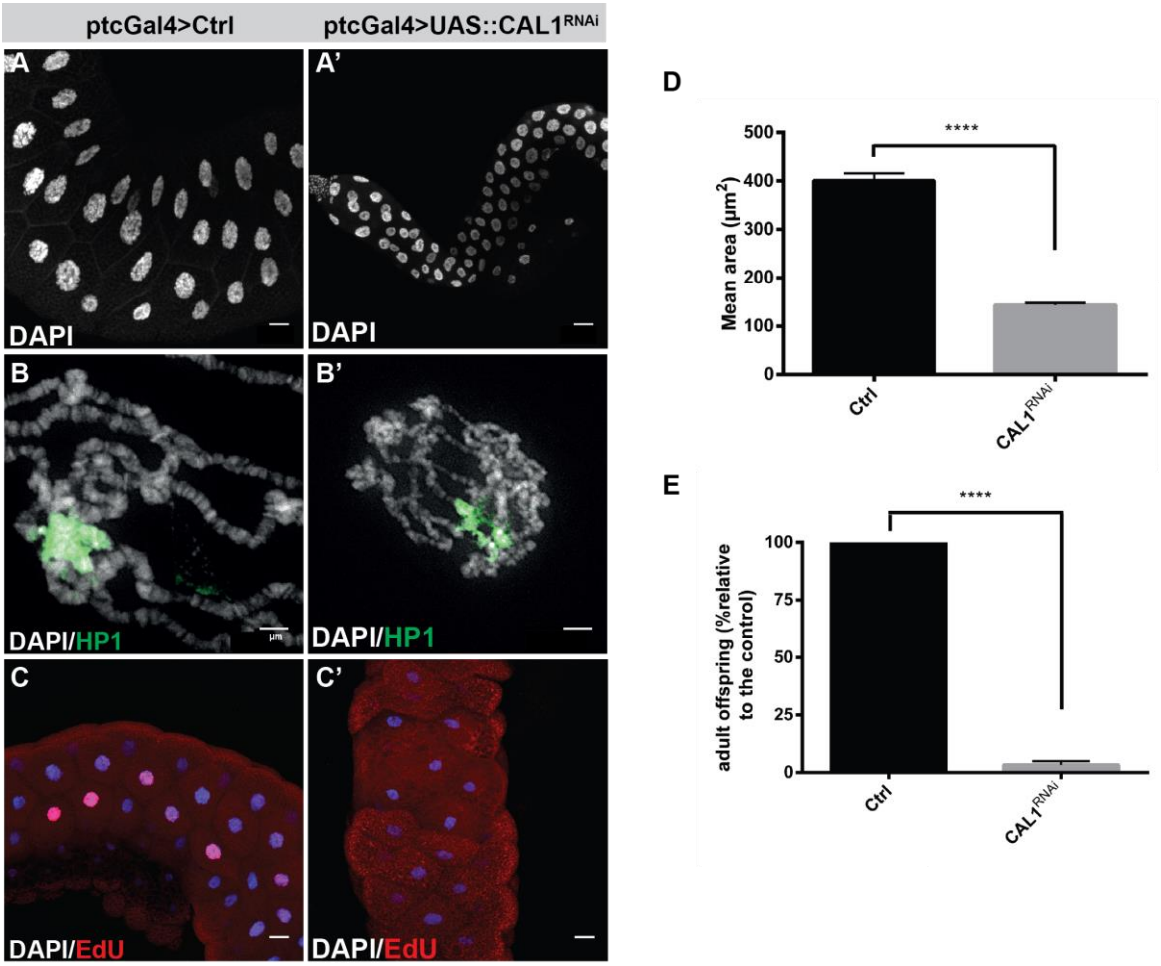

Figure 2.22 CAL1 knockdown impairs endoreplication in salivary glands

(A-C) Knockdown of CAL1 in salivary glands specifically using the ptc-Gal4 driver. (A-A') Salivary gland cells were stained with DAPI. (A) Control L3 salivary gland. (A') CAL1 depleted salivary glands. Decrease in cell nuclear size was observed in CAL1 depleted salivary glands. (B-B') Polytene chromosomes stained with HP1 (green). (B) Control polytene chromosome. (B') polytene chromosome from CAL1 depleted salivary gland. Heterochromatin structure and overall banding pattern seem not to be affected. (C-C') Salivary glands stained for DNA (blue) and incorporated EdU (red). (C) Control and (C') CAL1 depleted salivary glands were dissected and labelled with EdU for 5h. Scale bars, 25 µm. (D) Quantification of mean nuclear area of the indicated genotype. Nuclear size decreases in CAL-1 depleted salivary gland cells. Statistical significance was determined by Student's t test (****$p<0.0001$). Error bars in each graph represent standard error of mean (SEM). (E) Adult offspring frequencies of control genotypes versus CAL1 specifically depleted in salivary glands revealed developmental lethality phenotype. Statistical significance was determined by Student's t test (****$p<0.0001$). Error bars in each graph represent standard error or mean (SEM).

3 DISCUSSION

3.1 CENP-A as a novel epigenetic mark of stem cell identity

The focus of this study was to investigate the distribution of histone H3 variant CENP-A in cells of the *Drosophila* midgut in order to investigate whether this particular histone variant participates as epigenetic factor responsible for maintaining stem cell properties. Differential canonical histone H3 inheritance has been reported in asymmetrically dividing male germline stem cells (GSCs), whereas old histone H3 is selectively segregated to the self-renewed GSC and new H3 is enriched in the differentiating daughter cell (gonialblast, GB) (Tran et al., 2012). Here, I showed that CENP-A is preferentially asymmetrically inherited in cells of the midgut epithelium of the fruit fly, where old CENP-A is retained specifically in ISCs. Remarkably, long-term experiments revealed that CENP-A can persist in ISCs for more than 20 days, it is therefore possible that it is retained for the lifespan of a fly. The stability and persistency of CENP-A supports the idea that CENP-A could act as an epigenetic mark responsible for regulating stem cell properties. Analyzing the distribution of this histone variant in somatic cells provided evidence that the asymmetric distribution of CENP-A is a mechanism specific of stem cells. In contrast to CENP-A, the histone variant H3.3 does not exhibit asymmetry during ISC division.

3.1.1 CENP-A is asymmetrically inherited in ISCs

The proper segregation of genetic information during cell division is crucial to maintain genomic integrity (Kops et al., 2005). Centromeres direct chromosome inheritance, but in multicellular organisms their positions on chromosomes are primarily specified epigenetically rather than by a DNA sequence. The major candidate for the epigenetic mark is chromatin assembled with the histone H3 variant CENP-A (Black and Bassett, 2008).

How centromeres are inherited between somatic cell cycles is well established (Black and Cleveland, 2011). However, the precise timing and loading of CENP-A in

centromeres of stem cells is not known. As other histone variants, CENP-A deposition is uncoupled of DNA replication, therefore during S phase the pre-existing molecules of CENP-A are segregated equally among the two daughter chromatids without filling gaps until the next mitotic cycle. Dunleavy et al. suggested that in humans the gaps are filled by H3.3 variant, which acts as a placeholder until CENP-A loading occurs at late telophase/G1 (Dunleavy et al., 2011). This study has showed that old synthesized CENP-A is retained specifically by ISCs, possible as a mechanism for maintaining stem cell properties (Fig.2.4-2.). This conclusion raises many questions regarding the cell-cycle coupled mechanism of centromere maintenance in this specific stem cell system. In embryonic somatic cells CENP-A loading occurs in anaphase (Schuh et al., 2007), and in the case of neural stem cells of the larvae, this event does not seem to occur until G1 (Dunleavy et al., 2012), but unfortunately nothing is known about the regulation of CENP-A loading in ISCs. The only possible explanation for the CENP-A asymmetry showed in this study would be that the loading of CENP-A in these cells, as in neural stem cells, occurs in G1 phase, once that the two daughter cells have completely separated from each other. Remarkably, in the analysis of CENP-A distribution within ISC-EB pairs (Fig. 2.6), one day after HS induction (before HS the cells should only be expressing CENP-A-GFP, but after HS cells are genetically expressing CENP-A-mKO) the percentage of asymmetric CENP-A distribution is 79% that perfectly correlates to the well-characterized percentage of asymmetry versus symmetry fate division occurring in homeostasis in the gut (80% and 20% respectively) (de Navascues et al., 2012). If the loading occurs as I am proposing once mitotic cycle is over in G1, the two daughter cells produced must differ completely in their centromeric chromatin composition, the daughter stem cell will retain the old synthesized CENP-A nucleosomes, whereas the daughter that continues to mature and differentiate will be deficient for CENP-A nucleosomes and will load the newly synthesized CENP-A. This was confirmed by the staining with Delta antibody that specifically distinguishes ISC retaining old synthesized CENP-A (Fig. 2.6). However, further studies will be required to dissect the timing of CENP-A loading in ISCs, i.e. performing live cell imaging in the adult *Drosophila* midgut. I have tried to establish the protocol for primary culture and live cell imaging of the fruit fly intestine developed by Montagne et al. (Montagne and Gonzalez-Gaitan, 2014), however I did not succeed yet. The peristaltic movements of the alive midgut difficult their imaging and, moreover, as ISCs do not divide in synchrony, the number of ISCs undergoing mitosis in normal condition is very low, making the probability of finding a cell in mitosis very small. Another technique I used to discover when the CENP-A asymmetry is established

during mitosis was immunostaining with pH3. Unfortunately, mitotic ISCs show a very bright pH3 signal due to chromatin compaction at the metaphase plate and in other mitotic phases. This bright signal caused bleed-through into the mKO channel making so impossible to distinguish real signal from not. Next, it will be to establish a protocol for live cell imaging and thus imaging the two hours duration of the mitotic cycle of ISC, and by that understanding the CENP-A dynamics during mitosis in the *Drosophila* midgut.

An asymmetric inheritance of CENP-A during stem cell division implicates the establishment of two asymmetries at cellular and molecular level (as depicted in my working model): (i) an asymmetric cell division of ISCs and (ii) an asymmetric segregation of CENP-A within sister chromatids during replication and in mitosis. Little is known about how is the molecular mechanism underlying the asymmetric cell division of ISCs, but it is important to note that ISCs can divide asymmetrically or symmetrically depending on tissue demand. It has been described that the mode of division can be altered towards a symmetric outcome depending on nutrient availability (O'Brien et al., 2011). The only explanatory mechanism to date by which asymmetric cell division of ISC is established implies the cortical polarization of components of the Par complex and the spindle orientation dependent on tissue polarity of the epithelium, where ISCs attach to the basement membrane via integrins. Loss of integrins from ISCs has been shown to affect their asymmetric division, as well as their proliferation and maintenance. (Goulas et al., 2012). In other stem cells systems, such as GSCs and NBs, the asymmetric cell division of a stem cells is mediated by centrosome polarity (Rebollo et al., 2007; Yamashita et al., 2007). Centrosomes are the main microtubule organizing center in the cell and thus they can influence MT-related processes. It could be that asymmetric mitotic machinery is controlled and regulated by them. However, nothing is known about the role of centrosomes in ISC division, only that MT-related processes are involved in regulating cell fate. Recently it was reported the Sara endosomes containing Notch/Delta go to the spindle and then they are asymmetrically portioned during ISC mitosis (Montagne and Gonzalez-Gaitan, 2014). Investigating the segregation of centrosomes during ISC division would contribute to our better understanding of how the asymmetric distribution of CENP-A is established. Remarkably, there are data suggesting that the centromere-associated inner kinetochore is differentially expressed in ISCs compared to their differentiating progeny (Fig. 1.1). Transcriptome analysis of the different cell types showed that CENP-C and CENP-A mRNA level differs between cell types, being CENP-C lowly expressed in EBs.

Moreover, CENP-C staining in the adult midgut was not observed in all the cells of the epithelium, interestingly it was absent in one of the cells that formed the ISC-EB pair. This could indicate that the inner kinetochore proteins are responsible for orchestrating an asymmetric division by differentially expressing key proteins in kinetochore formation.

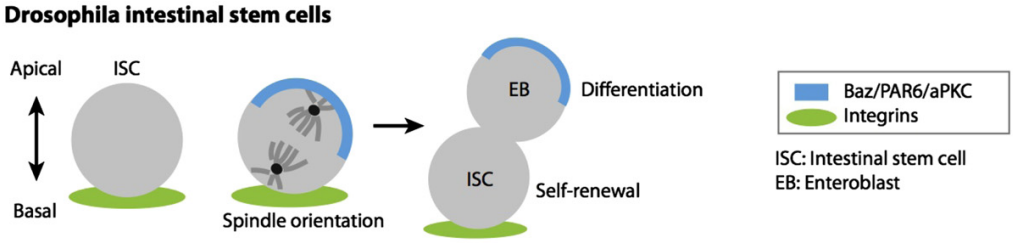

Figure 3.1 Establishment of asymmetric ISC division

Drosophila ISCs have a cell-intrinsic polarity and divide asymmetrically through activity of the Par complex, which is linked to the basement membrane via integrins. The polarization of ISCs results in the restriction of the Par complex (blue in the scheme) towards the apical cortex of dividing ISCs. The Par complex is involved in the positioning of the cleavage furrow and at the end of mitosis is dispatched to the apical daughter cell that will become the future EB. Adapted from (Inaba and Yamashita, 2012).

Apart from the asymmetric cell division of ISCs, an asymmetric segregation of CENP-A within the sister chromatids should be established. A possible explanation of the epigenetic inheritance of intestinal stem cells would be the "silent sister chromatids" hypothesis (Lansdorp, 2007), which claims that sister chromatids bear different epigenetic marks at the centromeric region or at specific genomic loci in stem cells or differentiating cells. The different centromeric epigenetic marks would be required for nonrandom chromatid segregation, whereas the differential pattern of epigenetic marks at specific gene regions would regulate gene expression. Along the same lines, the strand-specific imprinting and selective chromatid segregation (SSIS) model also suggests that epigenetically distinct sister chromatids cosegregate (Klar, 2007), different epigenetic marks at centromeric region of sister chromatids could establish their attachment to a polarized mitotic spindle. Using CO-FISH (chromosome orientation fluorescence in situ hybridization) which allows to distinguish sister chromatids, it was shown in adult skeletal stem cells that sister chromatids segregate asymmetrically being the parental DNA strand containing chromatid retained by the stem cells (Rocheteau et al., 2012). In *Drosophila* GSCs, a biased sister chromatid segregations has also been proposed, but only for sex chromosomes (X and Y) (Yadlapalli and Yamashita, 2013). In the above-mentioned

models, centromeres are hypothesized to be asymmetric to achieve the biased segregation. The results of this study are the first evidence in eukaryotes showing how asymmetric centromeres could be established based on the "age" of the epigenetic mark CENP-A. The retention of old CENP-A could be a mechanism to ensure that stem cells maintain their epigenetic information through division by retention of a particular set of PTMs, though further studies to validate this hypothesis remain to be done. Strengthening this hypothesis, some studies have reported the role of histone modifying enzymes in ISCs maintenance. ISCs mutant for Scrawny, a ubiquitin protease which deubiquitylates histone H2B, are subject to a premature expression of differentiating genes, such Notch (Buszczak et al., 2009). It could be that the set of PTMs inherited together with the "old" CENP-A is responsible for maintaining a specific gene expression pattern for pluripotency. This would be lost in "new" CENP-A and thus the daughter cells enriched with newly synthesized CENP-A could activate specific genes for conducting their differentiation.

An asymmetric CENP-A inheritance in ISCs provides evidence of the role of epigenetic factors contributing to stem cell identity, this is a first step toward the identification of more detailed mechanism that will help to understand how epigenetic information present at centromeric chromatin could be maintained by stem cells. Nevertheless, it is important to stress that these conclusions were drawn out of an overexpression system, by which CENP-A is constitutive express and its expression is not restricted to G2 phase. CENP-A is usually restricted to centromeric chromatin, but is loaded into chromosome arms upon its overexpression, leading so to the formation of ectopic kinetochores and consequently genomic instability which induces organismal lethality (Heun et al., 2006). I carefully controlled that the o/e was not detrimental for the flies by analyzing the mitotic rate and the lifespan of the overexpressing CENP-A(dt) flies in comparison with wt flies (Fig 2. 10). An increased number of mitotic ISCs in the midgut epithelium is an indication of stress or damage to the intestine, in this situation, the number of mitoses per midgut in o/e of CENP-A(dt) was not affected and similar number to wt situation were quantified. Furthermore, no significant difference in the median survival of control flies compared to o/e flies was detected. All these data suggest that the overexpression of CENP-A(dt) has not detectable effect in the adult midgut epithelium or in the viability of the animal, but constitutive overexpression of CENP-A tagged with fluorescent proteins can have unpredictable consequences and can be prone for artifacts.

Therefore, validating these results with a system that limits CENP-A expression to an endogenous level is required.

If, as suggested in this study, CENP-A is asymmetrically distributed and stem cells retain the parental DNA strand containing chromatid enriched with old CENP-A, this implicates that CENP-A should be differentially distributed to one of the two sets of sister chromatids during DNA replication. With the replication fork progression during DNA synthesis, nucleosomes must be disassembled to later be reassembled on newly synthesized DNA. This nucleosome assembly during replication implies the recycling of old histones and the incorporation of newly synthesized ones (Alabert and Groth, 2012). The precise mode of histone incorporation of new and old histones has been a subject of controversy over the past years, however, it remains possible that different types in a multicellular system may use different incorporation modes. The three proposed models for histone recycling at the replication fork are: (i) semi-conservative, (ii) dispersive and (iii) conservative model (Xie et al., 2017). In the model, I propose for asymmetric cell division of ISCs, the reassembly of old histone would follow a conservative model where the tetramer containing old synthesized CENP-A follows a biased incorporation at one of the two strands. I hypothesize that a placeholder mechanism could happen in the other strand deficient for CENP-A tetramers, as with H3.3 in human cells.

Another possible mechanism could be that such differences are established following S-phase by replication-independent differential histone turnover mechanisms at sister chromatids. Sister chromatids having different chromatin states could exhibit differential loading behavior for certain histone variants in G2/M and then be segregated in a sister-chromatid specific manner to the daughter cells during mitosis, creating asymmetry. It is also possible that the asymmetry between two sister chromatids is established on both levels: during S-phase (differential canonical histone deposition) and then additionally during G2 phase or mitosis (differential histone variant deposition).

3.1.2 CENP-A persistency

CENP-A nucleosomes are highly stable at centromeres. This stability is possible through binding with CENP-C, which induces structural changes in the CENP-A containing nucleosomes reshaping these into a more rigid structure (Falk et al., 2015). This changes combine to make CENP-A nucleosomes very long-lived.

As already discussed in the previous section, centromere inheritance depends on the retention of CENP-A nucleosomes. Recently, it was discovered that mouse oocytes can retain CENP-A nucleosomes at high levels more than one year after deposition at centromeres (Smoak et al., 2016). In *Drosophila* ISCs, I could detect after 20 days old synthesized CENP-A retained by ISCs (Fig. 2.8). These findings in different model organisms strengthen the idea that CENP-A can serve as epigenetic mark for stemness. The specific retention of pre-existing CENP-A (and its possible modifications) by ISCs could be a mechanism to preserve information important to maintain stem cell properties in this particular niche. In *Drosophila* male GSCs, the retention of pre-existing canonical histone H3 is mediated at least in part by the phosphorylation of H3T3 by haspin. H3T3ph is a mitosis-specific and centromere-enriched epigenetic mark that distinguishes sister chromatids enriched with old H3, establishing so a platform that can be distinguished by the mitotic machinery (Xie et al., 2015). In the following it would be interesting to study post-translational modifications present on directly on CENP-A or other histones present on centromeric chromatin that could be responsible for distinguishing old from newly synthesized CENP-A.

3.1.3 Other histone variants are not asymmetrically distributed

There is increasing evidence indicating that histone variants can influence epigenetic inheritance via a transcription-coupled mechanism (Henikoff et al., 2004). Since CENP-A showed an asymmetric distribution in ISCs, I decided to check the distribution of another histone H3 variant that on top is well-known for its preferential association with transcriptionally active chromatin, histone H3.3. Analysis of H3.3 distribution within the different cell types of the *Drosophila* midgut was not conclusive, but it was clearly no asymmetric. I did not observe a distribution bias for old versus new histone variant H3.3 (Fig. 2.12 and 2.14), from which I can argue that not all the histone variants distribute in the same way during asymmetric ISC division. However, it is important to highlight that the quantification was done comparing H3.3 distribution in ECs and in progenitor cells, that include not only ISCs but also the already committed to differentiate EBs. Including in the quantification EBs could have masked the effect. Thus, optimizing the conditions to specifically identify ISCs by performing a reliable Delta staining remains to be done. This will allow to perform more unbiased types of analysis that specifically points at the distribution between ISCs and their differentiating progeny.

The absence of an asymmetry in this specific histone variant can be thought to be an evidence that could rule out my initial hypothesis that H3.3 is the placeholder mechanism employed by ISCs during S-phase. However, there are several possibilities to explain this: (1) it could be that placeholder mechanism is taking place, but at least the histone variant H3.3 is not responsible of fulfilling the gaps left by the asymmetric segregation of CENP-A towards one of the strands. (2) It could also be that H3.3 indeed acts as a placeholder when the DNA is replicated in S-phase, however due to the low number of centromeric nucleosomes replaced during DNA replication compared to bulk chromatin, the asymmetry cannot be observed. H3.3 would be symmetrically dispersed within the bulk chromatin of both sister chromatids and only asymmetrically segregated at centromeric chromatin.

3.1.4 Working model

Combining the data obtained in this study with current knowledge of the field, I propose the following model (Fig. 3.2). Prior to mitosis, during S-phase, pre-existing CENP-A is differentially distributed to one of the two sets of sister chromatids. This could happen by two possible mechanisms: (i) during S-phase, the reassembly of old histone would follow a conservative model where old synthesized CENP-A containing nucleosomes follows a biased incorporation at one of the two strands: or (ii) after S-phase, following a differential histone turnover mechanism at sister chromatids. It is also possible that the asymmetry between two sister chromatids is established with a combination on both levels: during S-phase (differential canonical histone deposition) and then additionally during G2 phase (differential histone variant deposition).

Then, during mitosis, the mitotic machinery can distinguish the set of sister chromatids enriched for old CENP-A from the other, this could be achieved by a differential pattern of PTMs present in one of the sister chromatids, by an asymmetry on centrosomes or even in the key kinetochore proteins responsible for chromosome segregation. At the end, it results in two daughter cells with an asymmetric distribution of CENP-A. The daughter cell staying as ISC retains pre-existing CENP-A, whereas the daughter that will be committed for differentiation will have a centromeric chromatin more deficient for CENP-A and thus will load the newly synthesized CENP-A in order to continue its path into differentiation, I propose so that the loading of newly synthesis CENP-A in ISCs occurs at G1, as evidenced for neural stem cells (Dunleavy et al., 2012).

Moreover, if CENP-A cannot be efficiently loaded into the cell, the cell will fail to differentiate as it will be discussed in the next section.

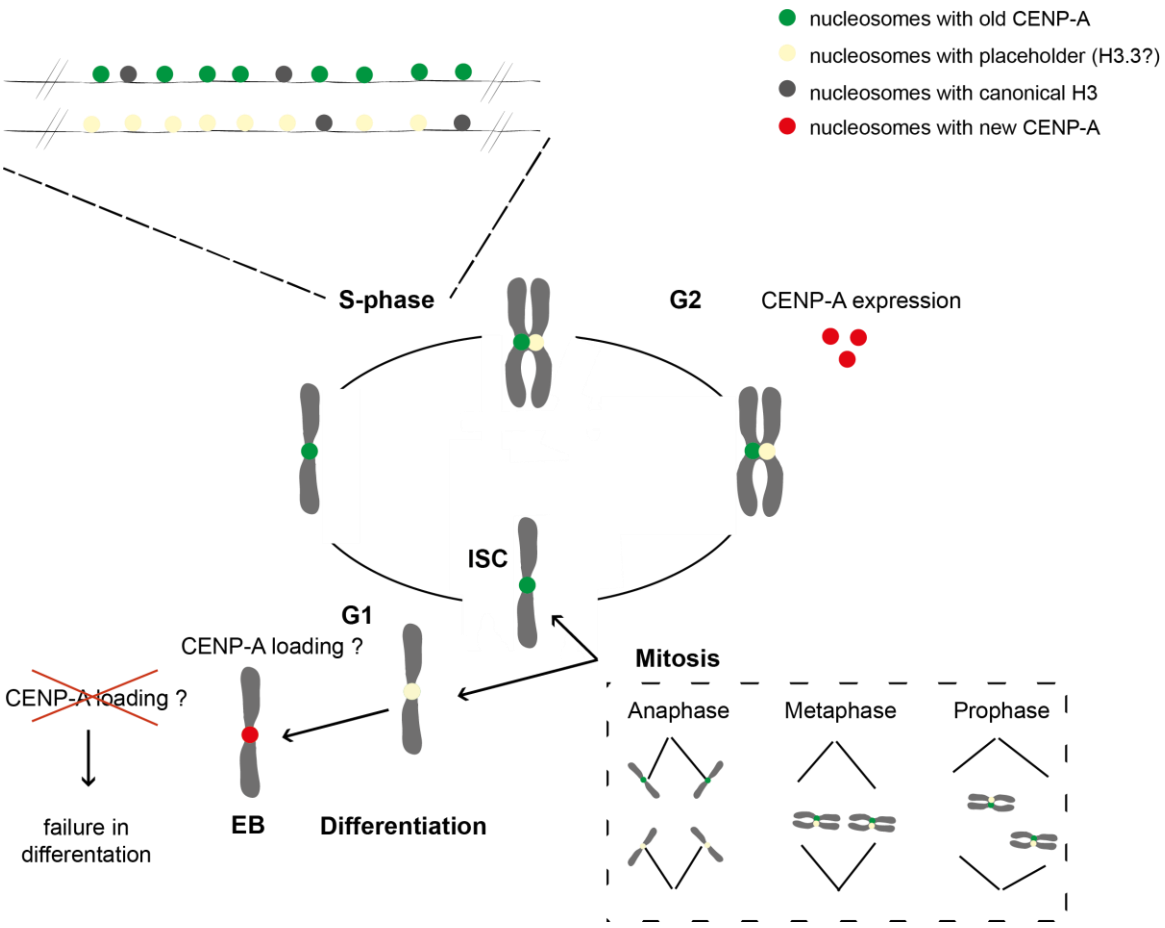

Figure 3.2 Model to explain the asymmetric CENP-A inheritance in ISCs

During S-phase, the old CENP-A could be preferentially segregated in one of the sister chromatids, and since CENP-A loading does not occur during DNA replication, a possible placeholder will be loaded onto the centromeric chromatin of the other sister chromatid. Then during cell division, the sister chromatids with different centromeric chromatin composition will be asymmetrically segregated. The cell containing the old synthesized CENP-A will be stay as ISC and the other daughter cell will exchange the placeholder for newly synthesized CENP-A and continue with a differentiation program.

3.2 Role of kinetochore proteins in non-mitotic cells

CENP-A and its loading factor CAL1 have always been studied in the context of cell division. However, data from this study suggest that CENP-A may play a role in non-dividing cells as well. I could show that the depletion of inner kinetochore proteins in the non-dividing committed progenitor cells leads to the loss of these cell types, indicating that

CENP-A and CAL1 are important for EBs maintenance and differentiation. ECs also seem to be affected by the depletion of kinetochore proteins, ECs undergo endocycles, that are also characteristic for salivary glands, follicle cells and ovarian nurse cells. Cells of salivary glands lacking CAL1 failed to undergo endoreduplication and correct S-phase progression. These surprising results indicate an essential role of the critical centromere components in post-mitotic cells.

3.2.1 Post-mitotic cells in the midgut epithelium are dependent on the presence of kinetochore proteins

Centromeres are epigenetically determined by the presence of the histone H3 variant CENP-A (Sullivan and Karpen, 2001). The main function of centromeres is to serve as foundation for the kinetochore that will allow the attachment of spindle microtubules during chromosome segregation. Therefore, centromeres orchestrate chromosome inheritance. In this study, I found a novel role of CENP-A as a possible determinant of stem cell properties, by using a dual-color method to differentially label preexisting from newly synthesized histones. Noticeably, I could observe exogenous tagged CENP-A not only in the ISCs, that are known to be the only mitotic cells in the epithelium, but also in committed and differentiated cells (Fig. 2.4), which do not undergo mitosis. Therefore, I investigated the role of centromere determining proteins in the different cell types present in the adult *Drosophila* midgut.

The *Drosophila* kinetochore consists of only three KMN proteins: CENP-A (termed also as CID), CENP-C and CAL1 (Przewloka et al., 2007), that are interdependent from each other for their localization and function (Erhardt et al., 2008). First, I depleted individually the three kinetochore proteins from progenitor cells, these includes ISCs and EBs, using the esg F/O system. This tracing system allows the specific evaluation of ISCs and their progeny, since all will be permanently labeled with GFP forming a so called "clone". However, upon depletion of the KMN proteins in the progenitor cells of the intestine, clones failed to form compared to control situation, meaning that the midgut loses its ability to proliferate and differentiate (Fig. 2.18 and 2.19). The lack of KMN proteins and the consequent loss of ISC proliferation and differentiation could be explained by two mechanisms. First, CENP-A, CENP-C and CAL1 are interdependent from each other for localization and function, so the loss of one of them compromises the complete system.

The main characteristic of ISCs is their capacity for dividing and giving rise two self-renewing ISCs and differentiating daughter cells. However, without proper centromere and kinetochore formation, any cell is able to faithfully divide. Secondly, the errors in segregation caused for a deficient kinetochore can lead to aneuploidy. Recently, it was reported that aneuploidy caused by depletion of bub3 in progenitor cells of the midgut epithelium also led to ISC loss (Gogendeau et al., 2015) in the same line as my results, pointing to the possibility that the loss of ISCs ability to proliferate and form clones is reduced is caused by abnormal chromosome number in ISCs. To address more carefully the phenotype, I specifically drive the RNAi constructs against the KMN proteins directly and exclusively to ISCs, and as expected, ISCs were lost upon depletion of CENP-A and other kinetochore components, most likely due to aneuploid ISCs generated. Gogendeau et al. proposed that aneuploidy in ISCs results in premature differentiation. A careful look at my results of depletion of CAL1 and CENP-A in ISCs suggest that there are more differentiated cells (specially ECs) compared to the control, however an exhaustive analysis needs to be done to confirm this hypothesis, such as quantification of DNA content which will show if there is an increase in number of polyploid cells (ECs) or staining with markers that are specifically enriched in differentiated cells (Pdm1 for ECs, and Pros for EEs).

The effect of KMN proteins depletion on ISCs was expected, since they undergo mitotic cycles. Recently, it was reported that CENP-A nucleosomes represent a minority in centromeric chromatin and intriguingly, there are individual CENP-A nucleosomes distributed at low levels throughout the chromatin (Bodor et al., 2014). Therefore, I decided to look specifically at the differentiating cells that are known not to divide any further (Ohlstein and Spradling, 2006), trying to understand whether these proteins have a role outside mitosis. First, I depleted CAL1 I in the transient but committed to differentiate progenitor cells known as EBs. It led to a complete loss of EBs (Fig. 2.20). This could be understood as another evidence for the model presented in the previous section (Fig 3.2 and section 3.1.1), I proposed that the asymmetric distribution of CENP-A during ISC division can be achieved by the daughter cell staying as ISC retaining pre-existing CENP-A, whereas the daughter committed for differentiation having a centromeric chromatin more deficient for CENP-A. Since this daughter cell would need to newly synthesized CENP-A to continue its path into differentiation, the depletion of CAL1 specifically in this cell type will compromise the loading of CENP-A, and thus their appropriate differentiation, leading so to death of the cell. Although the required amount of CENP-A is not known, previously

it has been reported that cell viability in mammals is lost upon reduce CENP-A levels (Black et al., 2007).

If EBs do not fulfill their centromeric chromatin with CENP-A nucleosomes, they seem to fail to complete their differentiation. Next, I checked what happen upon depletion of KMN proteins in already differentiated cells of the midgut epithelium. I drove RNAi constructs against CAL1 and CENP-A specifically to ECs, and remarkably, this promoted ISC proliferation (Fig. 2.21). The increased ISC proliferation rate is usually a sign of stress or injury response (Ren et al., 2010) and indeed it was the case, since the depletion of CAL1 and CENP-A in ECs affected severely the epithelium morphology and the flies suffered a high reduce in their median survival after *P.e* infection compared to control situation. These results evidence that CENP-A itself and its loading into chromatin seems to be required for the regulation and maintenance of ECs, a type of cell that does not undergo division but endoreduplication cycles. Nevertheless, to determine the effect on the onset of enterocyte endoreduplication, a DAPI quantification of cell nuclei remains to be done. Moreover, further analysis on the role of KMN proteins in the other differentiated cell type of the epithelium, the secretory EEs, will be required.

3.2.2 Role of the CENP-A loading factor CAL1 in endoreplication cycles

Depletion of the CENP-A loading factor CAL1 promotes EC loss. Since enterocytes are polyploid and undergo endoreplication, I analyzed if the phenotype caused by CAL1 depletion was consequence of defective endocycles. Little is known about how endoreplication is regulated in ECs. At molecular level, endocycles employ the same machinery as mitotic cycles to regulate the consecutive rounds of DNA replication, including Gap (G) phases between each S phase. In *Drosophila*, most of the knowledge of how endocycles are regulated comes from studies using salivary glands as model of study. Therefore, I also studied the effect of CAL1 depletion in salivary glands cells (Fig. 2.22), intriguingly, though the chromatin structure of the polytene chromosomes was largely unaffected, the endocycle rate assayed by EdU incorporation in dissected salivary glands decreased, suggesting that cells failed to progress into S-phase. Nevertheless, it has been reported that centromeric regions are under-replicated in salivary chromosomes (Hammond and Laird, 1985; Leach et al., 2000), so it could be that the phenotype is caused by other targets of CAL1. To prove that the effect of CAL1 depletion is not an

indirect effect or caused by other targets, but due to the loading of CENP-A itself, a depletion of the histone variant specifically in salivary glands remains to be done.

The two major known regulators of endoreplication are Cyclin E (CycE) and its kinase partner cyclin-dependent kinase 2 (Cdk2) (Zielke et al., 2013), their specific removal from salivary glands causes elimination of endocycles (Zielke et al., 2011). Since the knockdown of CAL1 specifically in endocycling cells also induce an impairment of endocycles, evaluation of CycE and Cdk2 levels upon CAL1 depletion could provide indications to understand the role of CAL1 in the regulation of endocycles. E2F1 is also an important factor for endocycles since it promotes CycE transcription and S phase initiation, but its levels must be suppressed during S phase to achieve continuous endocycles. E2F1 degradation promotes high $APC^{Fzr/Cdh1}$ activity suppressing so geminin accumulation (Zielke et al., 2011). APC/C is not only important for maintenance of endoreplication but also for the switch from mitotic to endoreplication cycles. In mitosis, RCA1 (APC inhibitor) has been shown to be crucial for centromeric localization of CENP-A in *Drosophila* (Erhardt et al., 2008; Goshima et al., 2007). Therefore, APC/C plays a role in CENP-A loading/maintenance (Erhardt et al., 2008) and is important for rereplication control in endocycles (Zielke et al., 2008). I propose that APC/C and CENP-A are interdepent from each other and thus lack of CENP-A caused by CAL1 depletion compromises endoreplication, the same manner as depletion of APC/C activity does. However, further studies will be required to dissect the exact mechanism, such as analysis of APC/C levels in endocycling cells upon CAL1 depletion.

3.3 Open questions and future perspectives

Here, I propose a novel function of CENP-A as an epigenetic factor involved in intestinal stem cell determination. Moreover, centromere determining proteins are important in cells of the fruit fly midgut epithelium that are not diving, giving evidences for a novel role of these proteins outside of mitosis. My results support these two novel findings, but some questions remain to be addressed.

Based on my analysis of CENP-A distribution during asymmetric cell division of ISCs, there is a biased segregation of preexisting CENP-A towards the ISC, whereas the cell committed to differentiate is enriched with newly synthesized. In the following, it would be interesting to study the mode of distribution when the asymmetric division of the ISCs is

compromised towards a symmetric mode. It has been proposed that insulin can altered the mode of division of ISCs increasing the rate of symmetric divisions (O'Brien et al., 2011), feeding flies with insulin and promoting so symmetric ISCs' divisions could reveal whether the asymmetric segregation of CENP-A is a mechanism specifically employed in asymmetric dividing ISCs.

A next step will be to study the precise timing of CENP-A loading during ISC division. Addressing how and when CENP-A is loaded onto centromeric chromatin will be important for our understanding of stem cells division and how they establish differences of stem mother cells and progenitor cells. To further unravel the timing and loading mechanism of CENP-A in ISC in vivo, it would be useful to establish an *in vivo* cell imaging protocol of the intestine. Recent studies have been used *in vivo* cell imaging approaches for analyzing calcium oscillations in ISCs (Deng et al., 2015), and even for studying the dynamics of endosomes during ISC division (Montagne and Gonzalez-Gaitan, 2014).

Importantly, if CENP-A is a stem cell determinant factor, the cell should be able to distinguish pre-existing from newly synthesized protein. Most likely, this would be achieved by specific PTMs present on the old CENP-A that cannot be find in the new one. However, I do not know whether such a modification is present or not. For this purpose, in a first step, making use of the FACS sorting protocol of midgut cells developed by Dutta et al. (Dutta et al., 2015a), ISCs should be isolated, CENP-A purified from them, and by Mass spectrometry the modified residues determined. The function of the corresponding modified residues in establishment a difference from newly synthesized CENP-A could be confirmed by mutating CENP-A at those specific residues and making a transgene for evaluating its distribution in the different midgut cells.

It has become clear that centromeric transcription is crucial for centromeric function. Moreover, non-coding RNAs (ncRNAs) have been associated with significant roles in cell biology, among them modulation of histone modifications. In recent years, researchers are expanding the knowledge of the role ncRNAs in stem cells. Understanding the interactions of centromeric transcripts with DNA or proteins in the context of stem cells has a potential to address different aspects of asymmetric stem cell division establishment. With *Drosophila* as model organism of study, Dutta et al. has established a protocol for transcriptome profiling of individual cells in the fruit fly midgut (Dutta et al., 2015b), that could help to identify ncRNAs specifically enriched in ISCs compared to other cells of the epithelium.

The main advantage of using *Drosophila* midgut as model of study is that the niche is kept intact and therefore all the signals that create a unique environment to regulate ISCs self-renewal and differentiation, facilitating so the dissection of the *in vivo* regulation of the intestine epithelium and their different cell types. Furthermore, the genetic tools available in *Drosophila* open a lot of possibilities for study different processes such as tumor initiation. By suppressing Notch signaling, which blocks differentiation, intestinal stem cell tumors can be generated (Patel et al., 2015; Perdigoto et al., 2011). Since aneuploidy is well established as one of the hallmarks of cancer, elevated CENP-A expression has been associated with cancer development (Tomonaga et al., 2003). In this study, I have discovered a new role of CENP-A and other kinetochore proteins in post-mitotic cells of the midgut epithelium. Depletion of CENP-A and its loading chaperone CAL1 in endocycling ECs induces ISC proliferation, though no tumor initiation was observed in this condition, it could be that CENP-A has a role in regulating alternative cell cycles, and that the unbalance could cause malignancy. Combining the tools available in *Drosophila* as a model organism, together we our knowledge of centromere biology, it could of special interest understanding the role of CENP-A in tumor initiation. Moreover, it would be interesting to analyze CENP-A distribution in tumors. It is known that tumorigenic cells overrun regulation pathways and, in Notch tumors, there is a lack of differentiated cells, it would be expected to find symmetrically dividing cells and thus CENP-A would be equally segregated between both daughter cells.

Based on my results of CAL1 depletion in salivary glands, this compromises endocycle progression, I have proposed that APC/C and CENP-A could be interdependent from each other and thus, lack of CENP-A caused by CAL1 depletion compromises endoreplication, the same manner as depletion of APC/C activity does. To further address the underlying mechanism and proof my hypothesis, analysis of the levels of E2F1, CycE and geminin at the given situation should be performed. It is known that these proteins oscillate during endocycles, peaks or E2F1 protein occurs at G phases, followed by its destruction and increases in CycE accumulation that finally led to geminin accumulation (Zielke et al., 2013).

The discovery of differential CENP-A distribution in intestinal stem cells opens an exciting field for research, and further studies will provide clues about the precise mechanism governing the asymmetric distribution. Furthermore, the identification of

centromere-determining proteins as regulators outside mitosis is also intriguing and opens a new area of research in centromere biology.

4 MATERIALS

4.1 Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich, Merck, Roth, AppliChem, Invitrogen or Roche. A detailed overview of the most important chemicals is shown in the following table.

Chemicals	Provider
2-Propanol	AppliChem
30% Acrylamide solution	Sigma
Agar bacteriology grade	AppliChem
Agorose Ultra Pure	Sigma
Ampicillin	Sigma
APS	AppliChem
β-Mercaptoethanol	AppliChem
BSA	AppliChem
Chloroform	AppliChem
DAPI	AppliChem
Dimethyl Sulfoxide (DMSO)	J. T. Baker
ECL	Thermo Fisher Scientific
EDTA	AppliChem
EGTA	AppliChem
Ethanol absolute	AppliChem
Ethidium bromide (EtBr)	AppliChem
Formaldehyde 37%	J. T. Baker
Glycerol	AppliChem
Heptane	AppliChem
Isopropanol	AppliChem
KCl	AppliChem
KH_2PO_4	AppliChem
Methanol	ZMBH

$MgCl_2$	AppliChem
Milk powder (non-fat, dry)	AppliChem
Mounting medium -aqua/polymount	Polysciences
NaH_2PO_4	AppliChem
NaCl	Sigma
Nonidet P-40	AppliChem
Phenylmethylsulfonylfluoride (PMSF)	Roth
PFA	AppliChem
Rifampicin	Sigma
SDS	Roth
Sodium citrate	AppliChem
Sodium hypochlorit, 12%	AppliChem
Sodium citrate	AppliChem
TEMED	AppliChem
Tris	Roth
Triton X-100	Merck
Tween 20	AppliChem

4.2 Equipment, Hardware and Consumables

Equipment/Material	Provider
0.2 ml PCR reaction tubes, 8-stripes	Sarstedt
10S VoltaLef Halocarbon oil	VWR
1.5 and 2 ml reaction tubes	Sarstedt
15 and 50 ml tubes	Sarstedt
2200 Tape Station Instrument	Agilent
35 mm Glass Bottom culture dishes	MatTek
-80ºC freezer Heraus	Hereaus
Agarose gel trays	Workshop ZMBH
Balance	Sartorius, Kern EG
Bioruptor	Next Gen
Blotting materials	BioRad
Coverslips	ThermoScientific

Deltavision microscope	Olympus/GE Healthcare
Film development system	Dr. Goos Suprema
Fly vials	Gosslein
FUJI Medical X-Ray Film	Fujifilm
Micropipettes	Gilson
Microscopy slides (superfrost plus or polylysine)	Thermo Fisher Scientific
Microwave	Sharp
Nanodrop	A260 Nanodrop
Nitrocellulose membrane (0.2 µm)	Amersham Biosciences
PCR-cycler	BioRad
pH-meter	Sartorius, Kern EG
Petri dishes	Greiner Bio-one
Pipette tips	Sarstedt, TipOne
Power supplies	Biorad, EMBL PS143
Protein gel equipment	BioRad
PVDF transfer membrane	GE Healthcare
SDS-PAGE glassplates	BioRad
Stereomicroscope	Zeiss
Tabletop centrifuges	Eppendorf
TCS SP5 confocal microscope	Leica
Trans-Blot Turbo Transfer System	BioRad
Vortex	Scientific industries
Waterbath	Memmert
Whatman Paper	Roth

4.3 Buffers

4.3.1 Agarose gel electrophoresis

50x Tris-acetate-EDTA	242g/l Tris-HCl 18.6g/l EDTA pH 7.7 adjusted with acetic acid

4.3.2 Biochemical buffers

Separation gel (10.5%)	0.375M Tris-HCl (pH 8.8) 10.5% acrylamide/bisacrylamide 30:0.8% 0.1% SDS 0.05% APS 0.05% TEMED
Stacking gel	0.123M Tris-HCl (pH 6.8) 4.4% final acrylamide concentration made of acrylamide/bisacrylamide 30:0.8% 0.1% SDS 0.03% APS 0.1% TEMED
4x Laemmli sample loading buffer	50mM Tris-HCl pH 6.8 10% Glycerol 2% SDS 0.5% β-Mercaptoethanol 0.02% Bromphenolblue
1x SDS gel running buffer	25mM Tris 190mM Glycine 0.1% SDS
Transfer buffer	25mM Tris 192mM Glycine 0.1% SDS 20% Methanol
10x TBS	30g/l Tris 88g/l NaCl 2g/l KCl pH 7.5
Blocking buffer	1x TBS 0.1% Tween-20

	5% Milk powder
Washing buffer	1x TBS 0.1% Tween-20
Ponçeau	0.2% Ponçeau 3% TCA
Mild stripping buffer	15g/l Glycine 0.1% SDS 1% Tween-20 pH 2.2
DNA lysis buffer	100 mM Tris-HCl pH 7.5 100 mM EDTA pH 8 100 mM NaCl 0.5% SDS
LiCl/KAc solution	142.5 µl 6 M LiCl 57.5 µl 5 M KAc
TE buffer	0.01 M Tris-HCl pH 8 0.001 M Na_2EDTA

4.3.3 Immunofluorescence buffers

Cohen's buffer	10mM $MgCl_2$ 25mM $C_3H_7Na_2O_6P$ (sodium glycerophosphate) 3mM $CaCl_2$ 10mM KH_2PO_4 0.5% NP-40 30mM KCl 160mM sucrose
PBS blocking solution	1x PBS 0.1% Triton X-100 2.5% BSA 10% FBS

PBST, permeabilization solution	1x PBS 0.1% Triton X-100
Ringer's solution	7.2 g/l NaCl 0.17g/l $CaCl_2$ 0.37g/l KCl pH 7.3-7.4
TBST (TBS-Tween)	0.2M Tris-HCl 17%NaCl 1% Tween-20

4.4 Enzymes

Enzymes	Provider
Benzonase	Sigma
Expand™ Long Template PCR System	Roche
Gibson Assembly® Master Mix	New England Biolabs
Pfu x Polymerase	Jena Biosciences
Quick Ligase	New England Biolabs
Restriction Enzymes	New England Biolabs
T4 DNA Ligase	New England Biolabs
2x Taq Master Mix	Fermentas
5x Red Load Taq Master	Jena Biosciences
ƛ-Phosphatase	New England Biolabs

4.5 Commercial Kits

Kit	Provider
Click-iT® Plus EdU Alexa Fluor 647 Imaging Kit	Thermo Fisher
CloneJeT PCR Cloning Kit	Thermo Fisher
GeneJET™ Gel Extractin Kit	Fermentas

LightCycler™ 480 SYBR Green I Master	Roche
NucleoSpin Plasmid-Purification	Macherey-Nagel
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel
QIAquick® PCR Purification Kit	Qiagen

4.6 Antibodies

4.6.1 Primary Antibodies

Antigen	Species	Dilution	Provider
β-Galactosidase	mouse	1:1000	DSHB
CAL1	rabbit	1:5000	Erhardt lab
CENP-A	chicken	1:200	P. Heun
CENP-A	rabbit	1:500	Active Motif
CENP-C	guinea-pig	1:500	G. Karpen
Delta	mouse	1:1000	DSHB
H3	rabbit	1:1000	Abcam
H3T3P	rabbit	1:1000	Merck Millipore (#05-746R)
HP1	Mouse	1:1000	DSHB
Prospero	mouse	1:250	DSHB
PH3	rabbit	1:1000	Merck Millipore (#06-570)
V5	mouse	1:1000	Invitrogen

4.6.2 Secondary Antibodies

Antigen	Species	Dilution	Provider
Alexa Fluor 488 goat IG	chicken	1:500	Invitrogen
Alexa Fluor 488 goat	mouse	1:500	Invitrogen

IG			
Alexa Fluor 546 goat IG	mouse	1:500	Invitrogen
Alexa Fluor 647 goat IG	mouse	1:500	Invitrogen
Alexa Fluor 647 goat IG	rabbit	1:500	Invitrogen
Alexa Fluor 647 goat IG	guinea pig	1:500	Invitrogen
Goat polyclonal IgG-HRP	rabbit	1:5000	abcam
Goat polyclonal IgG-HRP	mouse	1:10000	abcam

4.7 DNA vector constructs

Plasmid name	Source
AAV_CAG_smRuby-Myc	Looger Lab
pBS-FRT-Sv40PolyA-FRT	Chen Lab
pCDF3-dU63	Boutros Lab
pMT-CENP-A-GFP	Erhardt Lab
pFCKSA_smGFP-FLAG	Looger Lab
pUASp	Edgar Lab
UASp-H3(dt)	Chen Lab

4.8 Primers

Primers were ordered and synthesized by Sigma Aldrich.

Primer name	Sequence **(5' – 3')**
Cloning CENP-A(dt)	

CENP-A_GFP FG_fwd	GGG GTC GGC AAT TTA TGG TGA GCA AGG GCG
CENP-A_mKO FG_fwd	GGG GTC GGC AAT TTA TGG TGA GTG TGA TTA
NheI_CENP-A	ATC GCT AGC ATG AGC AGA GCC AAG AGA
GFP_CID FG_rev	CGC CCT TGC TCA CCA TAA ATT GCC GAC CCC
mKO_CENP-A_rev	TAA TCA CAC TCA CCA TAA ATT GCC GAC CCC
Seq_M13_fwd	GTA AAA CGA CGG CCA G
Seq_M13_rev	CAG GAA ACA GCT ATG AC
SphI_GFP _rev	ATC GCA TGC TTA CTT GTA CAG CTC GTC C
pUASp_fwd	CCG GGT ACC CGG GGA TCT TGA AGT T
pUASp_rev	ATG GCG CTA TTA ACA AGT ATT CTT C
XbaI_CENP-A FG_fwd	AGC TCT AGA ATG AGC AGA GCC AAG
qRT-PCR	
act5dqpcr_fwd	TGG CAC CGT CGA CCA TGA AGA TC
act5dqpcr_rev	TTA GAA GCA CTT GCG GTG CAC
GADPH1_fwd	GCT CCG GGA AAA GGA AAA
GADPH1_rev	TCC GTT AAT TCC GAT CTT CG
GFP_fwd	GAA CCG CAT CGA GCT GAA
GFP_rev	TGC TTG TCG GCC ATG ATA TAG
Cloning for C-tagging endogenous CENP-A by CRISPR	
CAP_NheI_smGFP_fwd	TAA GCA GCT AGC TGG ACT ACA AGG ACG ACG AC
CAP_XhoI_smGFP_rev	TGC TTA GAG CTC TTA TTT ATC ATC GTC GTC TTT
CAP_EcoRV_smRuby_fwd	TAA GCA GAT ATC TGG AAC AGA AAC TTA TCT CAG AG
CAP_ApaI_smRuby_rev	TGC TTA GGG CCC CTA CAA ATC CTC TTC AGA GAT GAG T
FRTfragment_gRNA39_fwd	GAA GTT TAG TTA CCC GGG GAT CTT GAA GTT CCT AT
FRTfragment_NcoI_ gRNA39_rev	CTA AAC TAA GAC CCA GCT CCA TGG TCA AAA GCG CTC T

gRNA_Target37_fwd	GTC GTG GAC TAA AAT TGC CGA CCC
gRNA_Target37_rev	AAA CGG GTC GGC AAT TTT AGT CCA
gRNA_Target38_fwd	GTC GCT AAG CCT AAA CTT CTC TTT
gRNA_Target38_rev	AAA CAA AGA GAA GTT TAG GCT TAG
gRNA_Target39_fwd	GTC GTT AGT CCA AAA GAG AAG TTT
gRNA_Target39_rev	AAA CAA ACT TCT CTT TTG GAC TAA
HR_CID_fwd	TGT AAA ACG ACG GCC AGT ATG CCA CGA CAC AGC AGA
HR_CENP-A Modifying PAM gRNA 37_rev	TGC GGC GCC CTA TAG TGA GTC GTA TAA ATT GCC GAC CCC GTT CGC AGA TGT AGG CC
HR_CENP-A Modifying PAM gRNA 38_rev	TGC GGC GCC CTA TAG TGA GTC GTA TAG ACT AAA CTT CTC TTT TGA AAT TGC CGA CCC CGG TCG C
HR_CENP-A Modifying PAM gRNA 39_rev	TGC GGC GCC CTA TAG TGA GTC GTA TTA AAC TAA GAC TAA ACT TCT CTT TTG GAC ACA AAT TGC CGA CC
HR_smFPtag_fwd	CAA TCG AAA AAG CAA CGT ATG CCA CGA CAC AGC AGA
HR_smFPtag_rev	AGA ATT ATT TAA CCT TAT AAA TGA GAC AGA ACT GTT GCA GTC TC
Lin_pBS_fwd	ATA CGA CTC ACT ATA GGG CGA A
Lin_pBS_rev	ACT GGC CGT CGT TTT ACA AC
Lin_pDsRed_fwd	CAT TTA TAA GGT TAA ATA ATT CTC ATA TAT CAA G
Lin_pDsRed_rev	ACG TTG CTT TTT CGA TTG
Seq_pCFD3_fwd	ACC TAC TCA GCC AAG AGG C
Seq_pCFD3_rev	TGC ATA CGC ATT AAG CGA AC

4.9 *E.coli* strains

Name	Genotype
DH5α	F- Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)poa supE44 lambda-thi-1

4.10 Fly strains

Name	Genotype	Source
Balancer Line	If/CyO; Sb/TM3, Ser	Erhardt Lab
CAL1 TRiP	y sc v; P{TRiP.GL01832}attP40	BDSC stock 55730
CAL1 TRiP	y sc v; P{TRiP.HMS02281}attP2/TM3,Sb	BDSC stock 41716
CENP-A(dt)	UAS::FRT-CENP-A-GFP-FRT-CENP-A-mKO/ UAS::FRT-CENP-A-GFP-FRT-CENP-A-mKO	This study
CENP-A GFP	w^{1118}; P{GFP-cid.H}8-10	BDSC stock 25047
CENP-A TRiP	y sc v; P{TRiP.HMS02160}attP2	BDSC stock 40912
UAS::CENP-A-V5		
CENP-C TRiP	y sc v; P{TRiP.GL00689}attP2	BDSC stock 38917
CENP-C TRiP	y v; P{TRiP.HMJ21500}attP40/CyO	BDSC stock 54806
Delta-lacZ^{05151}	If/CyO; Dl-lacZ/TM6,B	Edgar Lab
esg^{ts}	esg-Gal4/CyO; tub-Gal80^{ts} UAS-GFP/TM6B	Edgar Lab
esg F/O	esg-Gal4 tubGal80^{ts} UAS-GFP/CyO; UASflp>CD2>Gal4/TM6B	Edgar Lab
esg^{ts}; Su(H)-Gal80	esg-Gal4-UAS-2xeYFP; Su(H)GBE-Gal80, tub-Gal80^{ts}	Edgar Lab
hsp70-Gal4	w; P{Gal4-Hsp70.PB}2	Erhardt Lab
hsFLP	w-; sco/CyO; hsFLP, MKRS/TM6,Tb	Lohmann Lab
H3 (dt)	w;; UAS::FRT-H3-GFP-FRT-H3-mKO/	Chen Lab

	TM6B	
H3.3 (dt)	w;; UAS::FRT-H3.3-GFP-FRT-H3-mKO/ TM6B	Chen Lab
OregonR	+/+	Erhardt Lab
Myo1A^{ts}	Myo1A-Gal4^{NP0001}/CyO; tub-Gal80^{ts}; UAS-GFP/TM6B	Edgar Lab
Su(H)^{ts}	Su(H)GBE-Gal4, UAS-CD8-GFP/CyO; tub-Gal80^{ts}/TM6B,Tb	Edgar Lab
SNAP-CENP-A	y, w; attP40 P{pAttBBB-SNAP-CENP-A}	Dr. Pauleau, Erhardt Lab
ub. Gal4	ub. Gal4/CyO	Erhardt Lab

4.11 Software

- Adobe Photoshop CS6 Adobe
- Adobe Illustrator CS6 Adobe
- EndNote Clarivate Analytics
- Fiji NIH
- GraphPad Prism GraphPad
- Lasergene GATC biotech
- Microsoft excel Microsoft
- Microsoft powerpoint Microsoft
- Microsoft word Microsoft

5 METHODS

All methods listed here are standard protocols used in the Erhardt lab unless otherwise specified.

5.1 Fly culture

Flies stocks were kept on standard fly food [0.72% (w/v), 7.2% (w/v) maize, 2.4% molasses, 7.2% (w/v) malt, 0.88% (w/v) soya, 1.464% (w/v) yeast and acid mix (1% propionic acid + 0.064% orthophosphoric acid)] at 18°C. To prevent mite contamination, food vials were exchanged every 3-4 weeks.

5.2 Virgin collection

Females virgins were collected within 8-15 hours after the culture had been cleared of adults. To speed up development, the vials were kept at 25°C during the day. Virgins were selected based on their light body color, the dark spot in their translucent abdomen and/or their unfolded wings. The flies were held back for 4-5 days to check for larvae in the holding vial. Confirmed virgins were used in matings schemes.

5.3 Fly husbandry

Flies were grown on vials of standard fly food on 12-hour day-night cycle. For each cross, around 15 females and 5-8 male flies were used. Flies stocks were maintained at 18°C or 25°C and crosses were generally maintained at 25°C.

5.4 Generation of transgenic flies

After the generation of the transgene for the dual labelling of CENP-A, its injection into the w^- strain for P-element mediated germ line transformation was performed by Katrine Weischenfeldt (A. Teleman laboratory, DKFZ Heidelberg).

5.5 Transgenes expression

The expression of transgenes in the adult midgut was achieved by using the UAS-GAL4 system (Duffy, 2002). Crosses were set up at 25°C.

5.5.1 Heat shock scheme

In order to prevent the random flippase-induced recombination of the UAS-FRT-CENP-A-GFP-FRT-CENP-A-mKO, the flies were raised at 18°C. Adult flies at 5-7 days after eclosion were heat-shocked in a 37°C water bath for 90 minutes, flipped to fresh vials and then kept at 29°C for the desired period of time before dissection. Guts were analyzed at different timepoints after heat shock.

5.5.2 Induction of RNAi in flies

As the binary UAS/GAL4 system allows targeted gene expression, it was the technique of choice to induce RNAi in flies. Depending on the driver, RNAi is switched on in a certain tissue.

5.6 Feeding and survival assays

5.6.1 Bacterial infection

For gut infections, *Pseudomonas entomophila (P.e)* glycerol stocks were used. *Pseudomonas entomophila* cultures were grown in conical flasks at 30°C for 48 hours in Luria Broth (LB) supplemented with 100 µg/mL rifampicin (Sigma) for selection. Cultures were spun down at 2500 rpm at 4°C for 25 minutes. Bacterial pellets were resuspended in 7 mL of 5 % sucrose. Flies were fed with 0.5 mL of the concentrated bacterial suspension on Whatman filter paper and yeast paste for oral infection. Flies fed on 5% sucrose and yeast paste were used as mock control.

5.6.2 Survival experiments

Crosses were set up at 18°C, adult flies were shifted to 29°C for 2 days before infection and flies were infected with P.e as detailed before. In the case of survival on normal food, adult flies were just shifted to 29°C. The number of loving flies were counted every 24 hours. Equal numbers of control flies were maintained at similar conditions along with the experiments.

5.7 Temperature shift experiments

In this study, centromere proteins were specifically depleted in post-mitotic cells using a temperature sensitive inducible UAS-GAL4 system. Crosses were set up and maintained at 18°C, until eclosion. Flies were transferred to new vials every 4-5 days. Three days after eclosion, flies were shifted to 29°C for transgene expression and flies were shifted to new food every 2 days. Time of transgene expression at the restrictive temperature varied from 3-7 days and is indicated for each experiment.

5.8 Clonal analysis

The esg F/O lineage tracing system (Jiang and Edgar, 2009) uses the temperature-inducible expression of a FLPase which will activate a constitutive Act>STOP>Gal4 driver by removing the STOP cassette flanked by FRT sites. Crosses were set up and cultured at 18°C until eclosion and eclosed flies were transferred to new food vials every 5-6 days.

5.9 Immunohistochemistry protocols

5.9.1 *Drosophila* midgut immunostaining

Only the female guts were analyzed in our experiments because of larger size and ease in handling. Guts from adult flies were dissected in 1x PBS and fixed for 30 minutes in 1x PBS with 4 % paraformaldehyde at room temperature while shaking at 750 rpm. Fixative was removed and samples were rinsed twice with PBST (0.1%TritonX-100 in 1x PBS). Guts were blocked in 2.5%BSA/0.1%TritonX-100/10% FBS in PBS for 30 minutes

at room temperature. After blocking, samples were incubated with primary antibodies dilutions in blocking solution overnight at 4°C on a rotator (except for Delta antibody, that usually was incubated for at least 48h at 4°C). Subsequently, samples were washed three times with PBST (15 min each wash) and incubated thereafter with secondary antibodies in blocking solutions for 2 hours at room temperature while shaking in the dark. Following antibody incubation, samples were washed three times in PBST and incubated with DAPI for 10 minutes, rinsed twice with PBS and mounted on a glass slide using Aqua-Poly/Mount mounting medium (from Polysciences). Slides were sealed with nail polish.

5.9.2 Labelling proliferative cells in the midgut and in salivary glands with the Click-iT® EdU Plus Kit

Flies or larvae were dissected in Ringer's solution. The dissected tissue was incubated for 4-5h in 10µM EdU diluted in Ringer's solution. Afterwards, samples were rinsed twice in PBS and fixed for 30 minutes in 1x PBS with 4% paraformaldehyde at room temperature. Fixative was removed and samples were rinsed twice with PBS and incubated for 20 min in PBST to allow permeabilization. The Click-iT® Plus reaction cocktail was freshly prepared according to the manufacturer protocol and the tissue was incubated in this solution for 30 minutes at room temperature protected from light. Samples were shortly washed twice with 2.5% BSA in PBS and incubated with DAPI for 10 minutes, washed twice again in 2.5% in PBS and mounted on a slide in Aqua-Poly/Mount mounting medium (from Polysciences).

5.9.3 Immunostaining of *Drosophila* polytene chromosome squashes from salivary glands

Salivary glands were dissected from third instar larvae in Cohen medium. The excised glands were incubated in that medium with detergent for 8-10 minutes to allow the dissolution of cytoplasmic membrane structures. Next, the glands were excised in acetic acid/formaldehyde squashing solution (45% acetic acid, 3.7% formaldehyde) for 10 min, then they were also squashed in this solution. Once the spread was satisfactory, slides were submerged in liquid nitrogen. The coverslip was removed and slides were washed several times in cold TBST with gentle agitation. Slides were incubated with primary antibody

dilutions for 90 min at room temperature in a humidity chamber. Once the primary incubation was complete, slides were washed in cold TBS-T three times for 5 min each, followed by incubation with secondary antibody for 1 h. Afterwards, slides were again washed in cold TBS-T, incubated with DAPI for 10 min and mounted by placing a drop of mounting medium on the squash and then a coverslip over it.

5.10 DNA methods

5.10.1 Molecular cloning

All the molecular cloning methods were carried out according to Sambrook and Russel, 2001, unless otherwise stated.

5.10.2 Plasmid DNA isolation from E. coli

The transformed *E.coli* having a cloned plasmid was cultured in LB medium supplemented with 50 mg/L Ampicillin at 37°C. A small quantity of plasmid DNA was isolated form 5 mL of cultured *E.coli* cells by plasmid purification kit (NucleoSpin Plasmid-Purification Kit). For the isolation of a large amount of plasmid DNA, 100 mL of *E.coli* cells was cultured and the plasmid was isolated by plasmid purification kit (NucleoBond® PC 100).

5.10.3 Genomic DNA isolation from *Drosophila* adult flies

About 5 flies were place in an ice-cold 1.5 ml reaction tube, which was then transferred to -20°C for 15 min. The frozen flies were homogenized in 200 µl of lysis buffer using a plastic pestle and incubated at 65°C for 30 min. 400 µl of LiCl/KAc solution was added, mixed by inversing the tube several times and incubated for 10 min on ice. Samples were centrifuged at 14000 rpm for 15 min at room temperature, supernatant was then transferred to a new tube. 300 µl isopropanol was added and the tubes were inverted several times, then centrifuged at room temperature for 10 min to pellet the genomic DNA. The pellet was washed in cold 70% ethanol and centrifuged again for 10 min, air dried and dissolved in 75 µL TE buffer.

5.10.4 Gel electrophoresis

The agarose gels used to check DNA digest or PCR reaction contained 1% agarose dissolved in 1x TAE and 1:10000 ethidium bromide of the stock solution. All the samples and the corresponding DNA ladder were loaded on the gel and separated in 1x TAE at 150 V for 30 min.

5.10.5 Quantification of DNA

The isolated DNA (plasmid DNA, digested DNA, PCR fragments, genomic DNA from flies or PCR reactions clean-up) was quantified spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies) by measuring light absorption at 260 nm.

5.10.6 DNA sequencing

DNA sequencing from PCR products or cloning events was performed with the sequencing company GATC (www.gatc-biotech.com).

5.10.7 Polymerase chain reaction (PCR)

The PCR method was used to generate gene specific constructs which wew used to generate transgenic flies. The different reactions were carried out using Taq or Pfu DNA polymerase according to the supplier's recommendations. Each PCR consisted of 35-40 cycles and the annealing temperature was set depending on the annealing temperature of each primer pair.

Following conditions were used for a general reaction:

Step 1 (initial denaturation):	95°C	-	3 min
Step 2 (denaturation):	95°C	-	10 sec
Step 3 (annealing):	50-72°C	-	30 sec

(depending on the annealing temperature of each primer pair)

Step 4(elongation):	72°C	-	1 min/Kb to be amplified

Step 5 (repetition of cycles)	Steps from 2 to 4	-	35-40 cycles
Step 6 (final elongation):	72°C	-	5 min
Step 7 (hold):	4°C	-	∞

5.10.8 CRISPR gRNA design and cloning

The sequence for gRNAs was designed using the E-CRISP online tool (www.e-crisp.org) and cloned into the pCFD3-dU63 expression vector using the protocol available at Crispr Fly Design (www.crisprflydesign.org). Oligonucleotides were ordered from Sigma Aldrich.

The annealed gRNA sequence fragments were ligated to linearized pCDF3 (Bbs1) vector using T4 DNA ligase for 1 hour at room temperature. Ligation reactions were transformed into chemically competent DHFα E.coli cells using the heat shock method. Clones were probed for presence of the plasmid by colony PCR. Colony PCR was performed according to the DramTaq™ DNA Polymerase protocol. Positive clones were cultured overnight at 37°C and plasmid DNA was isolated as described before.

5.11 Biochemical Techniques

5.11.1 Protein extract preparation from adult *Drosophila* intestine

For each sample, 10-15 guts from female flies were dissected in PBS. Each sample was homogenized in 200 µl of cold lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM NaF, 1% NP-40, 10% glycerol, 1.5 mM EDTA pH 8, supplemented with protease inhibitors) using a pestle and subsequently subjected to 15 cycles (30s ON, 30s break, level 5) of sonication (Bioruptor). Lysates were incubated with 125U benzonase for 15 min at room temperature and then cleared by centrifugation for 30 min at 13200 rpm, 4°C. 30 µl of the extracts were mixed with Laemmli-buffer and denatured at 95°C for 5 min before loading onto the SDS gel.

5.11.2 SDS PAGE and Western blot analysis

Sodium dodecylsulfate (SDS) poly-acrylamide gel electrophoresis (PAGE) was performed in 10.5% gels according to Sambrook et al. (2001). The gel was run at 60V until samples reached the separating gel an then set to 120V for 1-2h. Separated proteins were transferred onto a nitrocellulose membrane using a Borate transfer buffer with 20% methanol by wet blotting at 400 mA for 1 hour. To confirm the transfer membrane was briefly stained with Ponçeau. Nitrocellulose membranes were blocked for 30 min with 5% milk powder in washing buffer. Primary antibodies were incubated O/N at 4°C in the blocking solution. After 3 washes, secondary antibodies were incubated for 2 h at RT. Secondary antibodies were coupled to horseradish peroxidase (HRP) and the signal was detected by chemiluminescence (HRP/ ECL solution).

5.12 Microscopy and data analysis

5.12.1 Microscopy and image processing

Flies were sorted and dissected using a binocular dissection microscope (Stereomicroscope, Zeiss) with external light source.

Images of adult midguts were acquired on a Leica TCS SP5II confocal microscope using and a HCX Plan APO 40x/1.30 Oil Cs objective. Serial Z-sections were taken at 1 µm distance. Shown are the projections of the maximal intensity. Images of polytene squashes of salivary glands were acquired on a widefield Deltavision Core system (Applied Precision). Images were acquired with an Olympus UplanSApo 60x objective at bining 2x2. They were taken as serial z-section of 0.2-0.5 µm distance. All Deltavision images were deconvolved (enhanced aggressive, 10 cycles, high noise filtering) and maximum projected.

The color intensity of most images has been enhanced equally for all images within the same experiment using only linear adjustments. microscope Images were processed in ImageJ Fiji and Adobe Photoshop.

5.12.2 Quantifications of histone distribution in the different cell types

The number of total cells, progenitor cells and ECs was determined from the DAPI channel of the analyzed image using Fiji and the "Analyze Particles" function. For ECs size was set from 300 to 1000000 (Pixel units) and circularity 0.25-1.00. For progenitor cells (ISCs + EBs) size was set from 100 to 300 pixel units and circularity 0.5-1.00. The fraction of GFP, mKO or GFP/mKO positive cells was determined manually using the "Cell counter" plugin.

The number of ISCs was easily determined in the case of CENP-A distribution by using the Delta-lacZ reporter, β-Galactosidase +ve cells were quantified using the wand tool in Fiji and adding them to the ROI manager. The fraction of GFP, mKO or GFP/mKO positive cells was determined manually using the "Cell counter" plugin.

5.12.3 Quantifications of mitotic index

Mitotic indices were determined by counting the number of Phospho Histone 3 (PH3) positive cells from >10 whole female midguts from three independent experiments. Counting of PH3 positive cells was performed manually on the Leica SP5. Analysis was done in blind.

5.12.4 Statistical data analysis

Kaplan-Meier survival was assessed using the following statistics ($p>0,0001$, Log-rank (Mantel-Cox Test), 3 x 20 animals/genotype. $PH3^{+}$ cell quantifications were analyzed using Student's t-test with Welch's correction.

6 APPENDIX

6.1 Slow CENP-A turnover

In this study, it was observed that old synthesized CENP-A (GFP labeled) can be retained in midgut cells and be detectable 20 days after its synthesis (Fig. 2.8). This was surprising, since it is known that it takes 12 days for flies fed on normal food to completely renew the posterior midgut epithelium (Jiang et al., 2009). However, CENP-A nucleosomes are very stable and it has been reported recently that they can remain at high levels even 1 year after their deposition at mouse centromeres (Smoak et al., 2016). To test CENP-A turnover using a different system, I overexpressed CENP-A tagged with V5 using a hsp70Gal4 driver and checked if CENP-A-V5 was detectable days after its induction. CENP-A-V5 was visualized in midgut epithelium 7 days after (Fig. 6.1A) and it could be detected in whole fly extracts at all the timepoints tested without changes in the V5 detectable levels. This points to the stability of CENP-A as the responsible factor and excludes the idea that the stability could be caused by the tag, strengthening the hypothesis of CENP-A could be an epigenetic factor regulating stem cell properties.

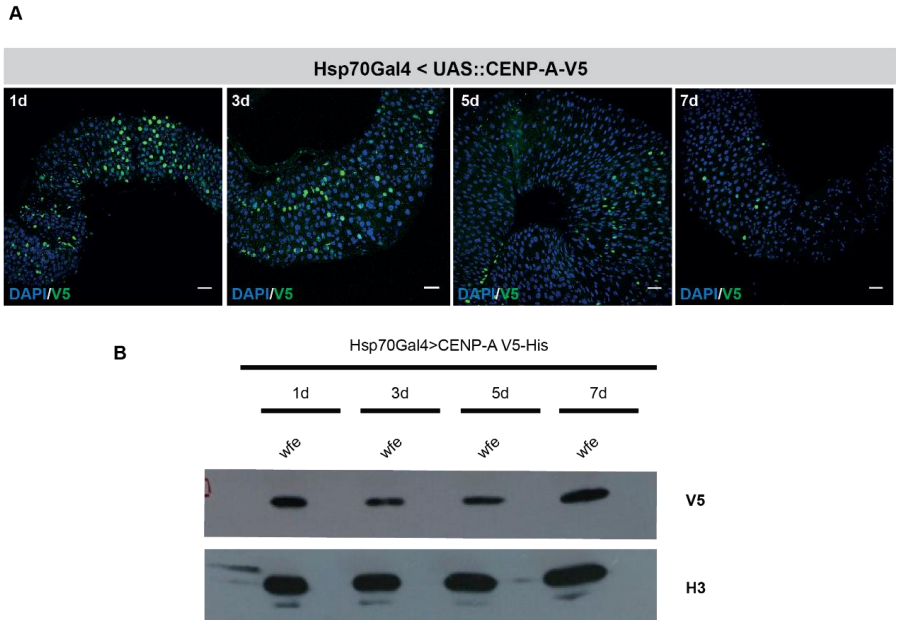

Figure 6.1 CENP-A persists in the midgut epithelium 7 days after its induction

(A) Overexpression of CENP-A-V5 was driven by hsp70-Gal4 and induced by a heatshock at 37°C for 90 minutes. Fixed and stained female fly guts show how CENP-A stays in the epithelium even after 7 days of its induction. Samples were stained with DAPI (blue) and V5 (green). Scale bars, 25 μm. (B) Whole fly extracts (wfe) of flies overexpressing CENP-A-V5 (hsp70Gal4>UAS::CENP-A-V5) at different timepoints after induction. CENP-A-V5 could be detected even 7 days after its induction.

6.2 CRISPR/Cas9-mediated C-terminal targeting of the endogenous CENP-A locus

CENP-A is usually restricted to centromeric chromatin and its loaded into chromosome arms upon its overexpression, leading so to the formation of ectopic kinetochores and consequently genomic instability which induces organismal lethality (Heun et al., 2006). One of the main conclusions of this study was that CENP-A is asymmetrically distributed in adult midgut cells, where ISCs retained the old synthesized CENP-A possible as an epigenetic mechanism to maintain the stem cell properties. However, this conclusion is drawn out of data where CENP-A was overexpressed and not only restricted to centromeres. I carefully controlled that the o/e did not affect flies' viability (Fig 2. 10), but constitutive overexpression of CENP-A can have unpredictable consequences and can be prone for artifacts. Thus, to strengthen our hypothesis, I tried to generate dual labeling CENP-A flies using CRISPR/Cas9 and by so tagging the endogenous *CENP-A* locus and perform the analysis at endogenous level, maintaining physiological expression levels and gene regulation.

For this experiment, I designed a new tag where the sequence encoding the fluorescent proteins instead of being the conventional, it was replaced by the so-called "spaghetti monster" fluorescent proteins (smFPs) coding sequence. The peculiarity of this smFPs is that they have 10-15 copies of single epitope tags (in my case, either FLAG or Myc) inserted into the fluorescent protein scaffold (Viswanathan et al., 2015), which opens an enormous variety of techniques to use the tag with, not only for assessing the *in vivo* asymmetric distribution of CENP-A, but for unravelling the underlying mechanism using other methods as FACS or ChIP.

Using the online tool E-CRISP (www.e-crisp.org), I chose between multiple guide RNAs that would target the C-terminal end of the endogenous *CENP-A* locus, I proceeded

with the most promising three based on their proximity to the C-end and their lack of off targets (target region and sequences in Fig. 6.2, upper panel).

As a first step, I generated the guide RNA (gRNA) sequences by annealing DNA oligonucleotides *in vitro* and cloned them into the pCDF3-dU63 expression vector (protocols available at http://www.crisprflydesign.org/). When injected into transgenic Cas9 fly embryos, the vector will produce the respective gRNA that targets Cas9 nuclease to a region downstream of CENP-A, there it will introduce a double-stranded break. At this point, is where the donor plasmid plays its role by providing a DNA fragment containing the dual-labeling tag with suitable homology arms, and therefore the sequence of interest will be inserted at the desired position (strategy 2 of Fig. 6.2). Another possibility is to use the gRNA vector to generate transgenic flies that will be crossed with transgenic Cas9 flies. In this case, the donor vector encoding the tag was injected into the embryonic progeny of this cross.

To screen for CRISPR edited events, I performed PCR against smGFP and CENP-A using as template genomic DNA isolated from embryos. In total, I screened 172 flies generated by following the first strategy and 226 from the second. Out of the 398 flies screened, 37 were sterile (9,29%) and 361 negative for the edited event of having inserted the dual labeling tag in the endogenous locus.

Tag: FRT-smGFP (FLAG)-FRT-smRFP (Myc)

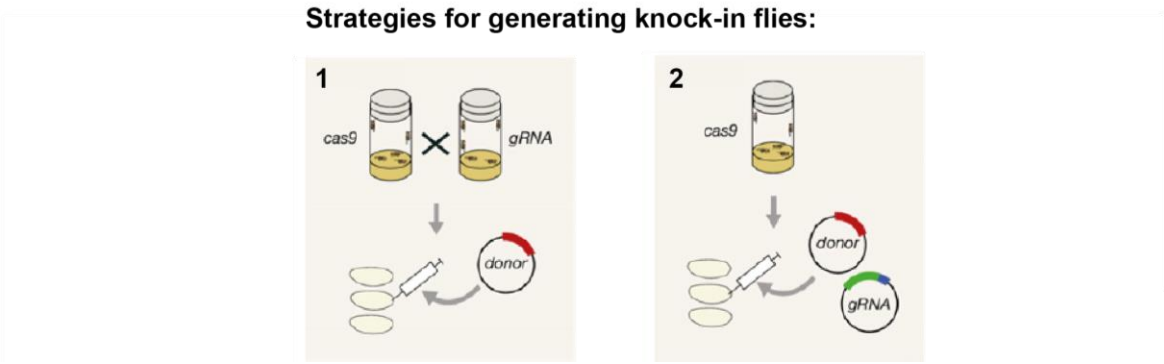

Figure 6.2 Strategies used to C-tag endogenous CENP-A locus

CDS of CENP-A and the different gRNAs targets that were used. The C-tag I wanted to knock-in was FRT-smGFP(FLAG)-FRT-smRFP(Myc). Out of the three different available possibilities to generate edited events in flies, I used the following: (1) having transgenic flies for the gRNA and crossed them to nos-cas9 female flies, a donor plasmid encoding the tag was injected into the embryonic progeny (injections and selection of gRNA transgenics flies was performed by the Microinjection Service of Cambridge University); and (2) donor and gRNA plasmids were injected into nos-cas9 embryos (embryo injection was performed by Sandra Miller, AG Teleman, DKFZ). The scheme with the strategies for generating knock-in flies was adapted from (Port et al., 2015).

6.3 SNAP-CENP-A in flies

Since I could not generate dual labeling CENP-A flies using CRISPR/Cas9 and perform the analysis at endogenous level, I decided to use instead SNAP-CENP-A flies (generated by Dr. Pauleau, Erhardt lab).

The SNAP-tag protein labeling system enable a specific covalent binding of any molecule to the protein of interest, in my case CENP-A. The SNAP-tag is a small protein and it is modified so that it specifically binds *para*-substituted benzyl guanine compounds. Its substrates are small fluorophores that allow the visualization of the protein and are coupled to benzyl guanine (BG) holding the ability to react with SNAP-tag (Fig 6.3).

Flies were fed with 0.2mg/mL of commercial SNAP-Block (NEB), thus the old existent SNAP-CENP-A was quenched using a nonfluorescent compound that blocks the reactivity of the SNAP-tag (Fig. 6.3 upper panel). After the efficient blocked of the old synthesized CENP-A, flies were flipped onto normal food and newly synthesis of SNAP-CENP-A was allowed. For the detection of newly synthesized SNAP-CENP-A, samples were labeled using fluorescent SNAP-SiR-647 (Fig. 6.3 lower panel).

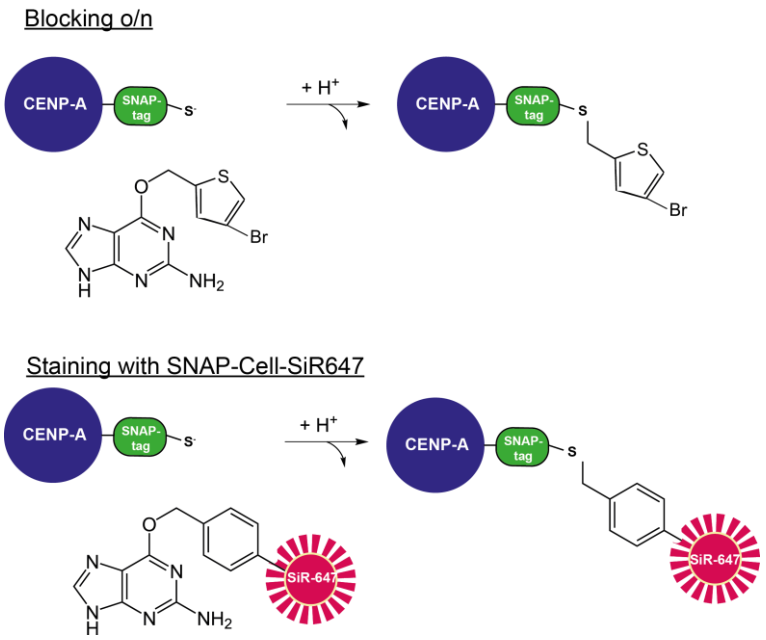

Figure 6.3 SNAP-tag reaction schemes

Schematic of the different labeling strategies, modified from https://en.wikipedia.org/wiki/SNAP-tag. The CENP-A-SNAP fusion protein can be quenched using SNAP-Block, a nonfluorescent BG. The CENP-A-SNAP fusion protein can also be detected and visualized using fluorescent SNAP-SiR-647.

Noticeably, SNAP-CENP-A flies are also an overexpression system, they were generated by site-directed insertion, so they not only have the endogenous locus of CENP-A but also the transgene encoding for SNAP-CENP-A inserted as a single copy in the second chromosome (2L, position 25C7). However, I check for the expression level, and as indicated in Fig. 6.4B, centromeric foci can be detected in progenitor cells of the midgut epithelium to a level comparable to endogenous (Fig. 2.1)

The outline of the experiment is depicted in Fig 6.4A. First, newly eclosed SNAP-CENP-A flies were starved for 1 hour to facilitate the feeding of the SNAP-Block. The block was fed overnight and samples were collected after the blocking procedure to check for the lack of SNAP-CENP-A signal (negative control, Fig. 6.4C). Nothing is known about the CENP-A loading cycle in stem cell, thus I decided to make a long timepoint to be able to detect signal for newly synthesized CENP-A. Seven days after blocking all the old synthesized SNAP-CENP-A, newly synthesized could be detected (Fig. 6.4D). These are preliminary data, but encouraging to validate our hypothesis of how CENP-A is asymmetrically distributed in progenitor cells of the *Drosophila* midgut.

Next I will optimize the timeline for analyzing SNAP-CENP-A, and I will include Delta staining to be able to specifically detect ISCs and by so, I will be able to analyze how CENP-A is distributed at endogenous level.

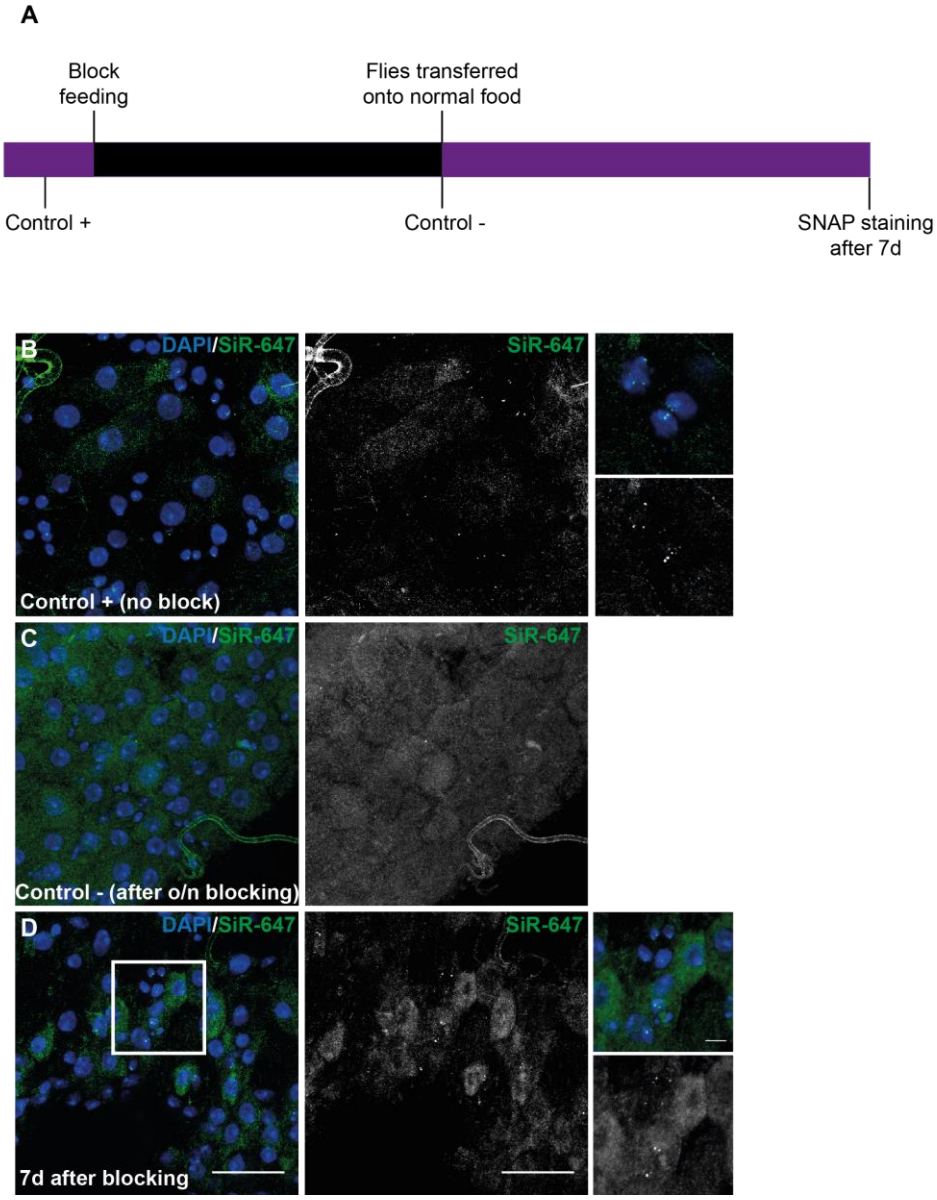

Figure 6.4 Newly deposited CENP-A can be detected in midgut cells 7 days after blocking

(A) Timeline of SNAP-CENP-A flies handling prior to imaging. The existent SNAP-CENP-A is quenched by the addition of SNAP-Block (bromothenylpteridine, BTP) and SiR-647 will allow the labelling of newly synthesized SNAP-CENP-A. (B) Representative images from positive control flies stained with SNAP-SiR-647, all progenitor cells of the midgut epithelium show signal for SNAP-CENP-A. (C) Representative images from negative flies blocked overnight by feeding them with BTP and stained with SNAP-SiR-647. No SNAP-CENP-A signal can be detected. (D) Representative images from flies 7 days after the overnight block. Newly synthesized CENP-A can be detected by staining with SNAP-SiR-647. Scale bars, 25 μm.

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