Functional Analysis of Histone Post-translational Modifications by the Polycomb Group of Transcriptional Repressors

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Functional Analysis of Histone Post-translational Modifications by the Polycomb Group of Transcriptional Repressors

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Erklärung

Die vorliegende Arbeit wurde zwischen September 2010 und Dezember 2014 unter Anleitung von Dr. Jürg Müller am Max-Planck-Institut für Biochemie in Martinsried durchgeführt.

Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

München, den 19.01.2015

Ana Raquel Pengelly

Erstgutachter: Prof. Dr. John Parsch
Zweitgutachter: Prof. Dr. Angelika Böttger
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Declaration of author's contribution

In the results section of this thesis I present the work that I performed during my PhD thesis from September 2010 to December 2014.

The first part of the results (section **3.1**) has not yet been published and was part of a collaboration with Dr. Alf Herzig. Dr. Herzig cloned the histone transgenes lacking each individual core histone, and injected them into flies. The subsequent stocks containing either the *Df(2L)HisC FRT40A* chromosome or the *yw hs-flp122; hs-nGFP FRT40A* chromosomes in combination with the histone transgenes were generated by myself, as well as the somatic clones, and the stainings.

The second part of the results (section **3.2**) was published in:

Pengelly, A. R., Çopur, Ö., Jackle, H., Herzig, A. & Müller, J. A Histone Mutant Reproduces the Phenotype Caused by Loss of Histone-Modifying Factor Polycomb. *Science* 339**,** 698–699 (2013).

This study was conceived and designed by all authors, the fly strains containing the mutant histones were generated by Dr. Alf Herzig. The deficiency recombinant flies were made by myself and Dr. Ömer Çopur. The generation of somatic clones, the dissections, and the stainings were performed by myself.

The third part of the results (section **3.3**) has not yet been published and is a collaboration with Dr. Reinhard Kalb. Dr. Kalb cloned the *Sce-FLAP* vector and reared the *Sce-FLAP* injected flies. I performed all subsequent crosses to generate Sce^{0} embryos and $Sce^{I48A\ m-;\ z-}$ embryos. All other experiments in this section were performed by myself.

“The important thing is not to stop questioning.

Curiosity has its own reason for existing.”

Albert Einstein

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Abbreviations

Abd-A: Abdominal-A

Abd-B: Abdominal-B

Antp: Antennapedia

ANT-C: Antennapedia complex

ASF1: Anti-silencing function 1

ATM: Ataxia telangiectasia mutated

ATRX: Alpha Thalassemia/Mental Retardation Syndrome X-Linked

Ap: Apterous

BAC: Bacterial artificial chromosome

BSA: Bovine serum albumin

BXC-C: Bithorax complex

Cad: Caudal

CAF1: chromatin assembly factor 1

CBP: CREB-binding protein

CENP-A: Centromere protein A

CHD: Chromatin helicase DNA binding

ChIP: Chromatin Immuno-Precipitation

CID: Centromere identifier

CNS: Central nervous system

Dac: Dachshund

DAXX: Death-Domain Associated Protein

Dfd: Deformed

Dll: Distalless

DNA-PK: DNA dependant protein kinase

Doc2/3: Dorsocross 2/3

Dp: Dp transcription factor

DSHB: Developmental Studies Hybridoma bank

ECL: Enhanced Chemiluminescence

ElB: Elbow B

En: Engrailed

ES cells: embryonic stem cells

Eve: Even skipped

E2F: E2F transcription factor

FACT: Facilitates chromatin transcription

GFP: Green fluorescent protein

Hat1-RbAp46: Histone acetyltransferase Rb-associated protein 46

H2Aub: H2A-K118ub1

HCC: Histone pre-mRNA cleavage complex

HDE: Histone downstream element

HIRA: Histone Cell Cycle Regulation Defective Homolog A

HisGU: Histone gene unit

HRP: Horseradish peroxidase

IC: Immunohistochemistry

IF: Immunofluorescence

INO80: Inositol requiring 80

ISWI: Imitation switch

Lab: Labial

LDS: Lithium dodecyl sulfate

lncRNAs: Long non-coding RNAs

MWCO: Molecular weight cut off

NAP1: Nucleosome Assembly protein 1

NASP: T-nuclear auto antigenic sperm protein

NCP: Nucleosome core particle

Noc: No ocelli

Pb: Proboscipedia

PBS: Phosphate Buffer Saline

PcG: Polycomb group

PhoRC: Pho repressive complex

Pnr: Panier

PNS: Peripheral nervous system

PRC: Polycomb repressive complex

Pros: Prospero

PR-DUB: Polycomb repressive deubiquitinase

PTMs: Post-translational modifications

Rbf: Retinoblastoma family protein

RNAi: RNA interference

SAM: Sterile alpha motif

Sce: Sex combs extra

Scr: Sex-combs-reduced

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SET: Suppressor of variegation 3–9 [Su(var)3–9], Enhancer of zeste [E(z)], and Trithorax [Trx]

SLBP: Stem loop binding protein

snRNA: Small nuclear RNA

SWI/SNF: Switching defective/sucrose non fermenting

TrxG: Trithorax group

Tsh: Teeshirt

TSS: Transcription start site

Ubx: Ultrabithorax

WB: Western blot

WT: Wild-type

3C: Chromatin conformation capture

Nota bene: Polycomb gene abbreviations are listed in **Table 2.**

Abstract

In all eukaryotes, genomic DNA is packaged into chromatin. Nucleosomes, the fundamental units of chromatin, consist of a histone octamer, that comprises two copies of each of the canonical core histones H3, H4, H2A and H2B, around which 147 base pairs of DNA are wrapped in two left handed superhelical turns. Chromatin structure plays a critical role in determining how accessible the DNA template is. This impacts on all the biological processes that require the DNA template, ranging from transcription and replication to chromosome segregation. Chromatin conformation is thought to be regulated by a variety of mechanisms, including covalent post-translational modifications on the unstructured histone tails that protrude from the nucleosome core. The Polycomb group genes encode transcriptional repressors that are involved in proper body patterning during development by maintaining transcriptional repression of developmental regulator genes. Polycomb complexes catalyse two distinct histone post-translational modifications: H3-K27 methylation and monoubiquitylation of H2A-K119 in vertebrates and H2A-K118 in *Drosophila.*

To directly investigate the function of histone post-translational modifications in a metazoan, I used a genetic system that permits the conditional replacement of the endogenous canonical histone proteins with mutated histone proteins in *Drosophila*. I focused on H3-K27 and H2A-K118, the residues that are methylated by the Polycomb Repressive Complex 2 (PRC2) and monoubiquitylatedated by Polycomb Repressive Complex 1 type (PRC1-type) complexes, respectively. I found that cells containing H3-K27R instead of H3-K27 are viable but fail to maintain repression of PRC2 target genes, much like cells that lack PRC2. In addition I found that H3-K27R cells differentiate into adult structures that show homeotic transformations, similar to those seen in PRC2 mutant cells. Unexpectedly, I discovered that repression of Polycomb target genes is maintained in cells containing mutant versions of H2A or the histone variant H2Av that can no longer be ubiquitylated by PRC1-type complexes. This suggests that H3-K27 methylation, but not H2A-K118 monoubiquitylation, is critical for maintaining Polycomb repression in *Drosophila*. However, H2A ubiquitylation is required for completion of embryogenesis and viability. It will now be crucial to continue investigative efforts to determine what functions of H2A ubiquitylation are essential for viability.

1 Introduction

Many transcriptional regulators are enzymes that add post-translational modifications (PTMs) to histones and other proteins. This led to the proposal that PTMs on histones residues, or the combination of particular PTMs creates a "histone code" that specifies particular states of chromatin that impact on transcription and any other DNA based biological process.

Chromatin Immunoprecipitation analyses of PTMs on histones, both at the single gene level and in genome-wide studies, have established correlations between specific histone PTMs and transcriptional activity (Filion, et al., 2010; Kharchenko et al., 2011). Nevertheless, in metazoans, there was no direct experimental evidence showing that histone PTMs were causally linked to a particular transcriptional read-out or any other biological response.

Classic examples of transcriptional regulators that possess histone-modifying activities are Polycomb Group (PcG) protein complexes. Previous studies identified the PRC2 complex as a histone methyltransferase that methylates lysine 27 in histone H3 (H3-K27) (Müller et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Cao et al., 2002). The presence of H3-K27me3 is strongly correlated with Polycomb repression of target genes such as *HOX* genes (Papp and Müller, 2006; Nekrasov et al., 2007; Boyer et al., 2006). Moreover, the catalytic activity of the PRC2 subunit E(z) is critically required for repression of these genes (Müller, et al., 2002). However, other histone and non-histone substrates have been identified for PRC2 and therefore, whether H3-K27 was the *bona fide*, biologically relevant substrate of PRC2, remained unclear (He, et al., 2012; Kuzmichev et al., 2004). In addition, PRC1 has been found to possess a specific E3 ubiquitin ligase activity for H2A-K118 in *Drosophila*, (119 in mammals) (Wang et al., 2004b; Cao et al., 2004). However, although the E3 ubiquitin ligase subunit of PRC1 is required for repression of *HOX* genes, the precise function of H2A ubiquitylation (H2Aub) in Polycomb repression is not yet clear.

1.1 Chromatin structure and function

In all eukaryotes, genomic DNA is packaged into arrays of nucleosomes called chromatin. Nucleosomes, the fundamental structural units of chromatin, consist of a histone octamer that comprises a tetramer of histones H3 and H4 and two dimers of histones H2A and H2B, around which 147 base pairs of DNA are wrapped in two left handed superhelical turns (Luger et al., 1997) (**Figure 1**). An additional histone, linker histone H1, is associated with each nucleosome and plays an important role in arranging nucleosomes into higher order chromatin fibres (Song et al., 2014). In this chapter I discuss how histones and other non-histone based processes can impact on chromatin structure.

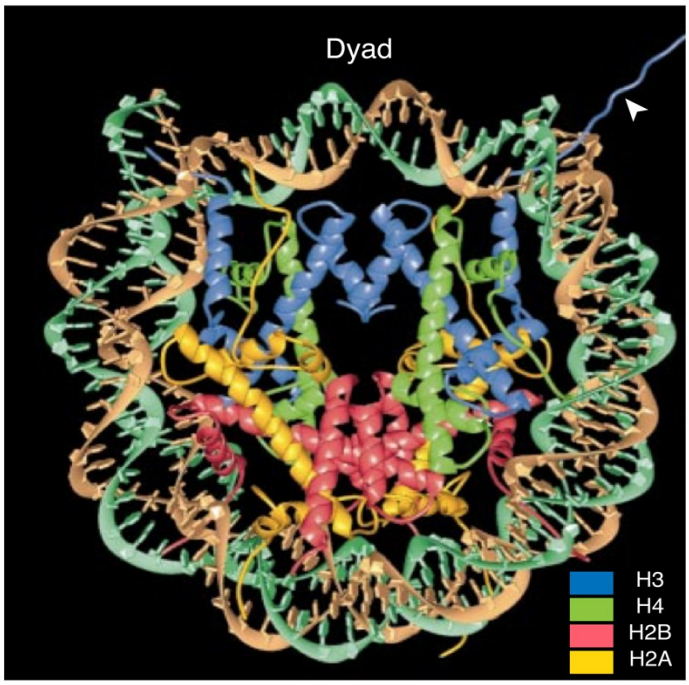

Figure 1. Crystal structure of the nucleosome core particle
Histone H3 is depicted in blue, H4 in green, H2B in red and H2A in yellow, the phosphodiester backbones of the DNA are depicted in brown and turquoise The white arrowhead points to the modelled N-terminal tail of H3 (adapted from Luger et al., 1997).

1.1.1 Histones

1.1.1.1 Canonical Histones

The canonical core histones are H2A, H2B, H3 and H4. Histones are highly conserved positively charged basic proteins that consist of a globular domain and flexible unstructured tails (Luger et al., 1997). During S phase, it is crucial to produce enough histones to package the newly replicated DNA template into chromatin. The histone gene organisation in clusters and the tight regulation of canonical histone gene transcription during S phase contribute to producing the necessary amounts of histones for replication. In addition, histone synthesis must be regulated to avoid toxicity for the cell.

a Canonical Histone gene organisation

Unicellular eukaryotes, like *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* only have one to three copies of each canonical histone gene. However, in multicellular eukaryotes, canonical histone genes are present in multiple copies and are organised in clusters. These clusters can contain tandem repeats of disordered histone genes or gene sets or "units" containing one copy of each of the canonical histone genes or one copy of all of the core canonical histone genes and additionally the linker histone H1. Mammals possess "disordered" histone clusters whereas in the case of *Xenopus*, *Drosophila* and the Cnidarian *Acropora formosa* they are organised in tandem repeat units (Zernik et al., 1980; Miller et al., 1993). In the case of mammals, there are two major histone gene clusters: one cluster that includes linker histone H1 genes, and contains more than 80% of the histone genes, and a smaller cluster that contains the core histones only. To date, the organism with the most histone clusters is *Caenorhabditis elegans,* which has 11 inter spread histone gene clusters (reviewed in Marzluff et al., 2008). However, in *Drosophila melanogaster* histone gene organisation is unique, since the 23 copies of the histone gene units (HisGUs) that contain a single copy of each of the core histone genes: H2A, H2B, H3 and H4 and a copy of the linker histone H1 are present at a single locus on Chromosome 2L (Günesdogan et al., 2010) (**Figure 2**). This unique feature offers the possibility of deleting the whole histone cluster at once.

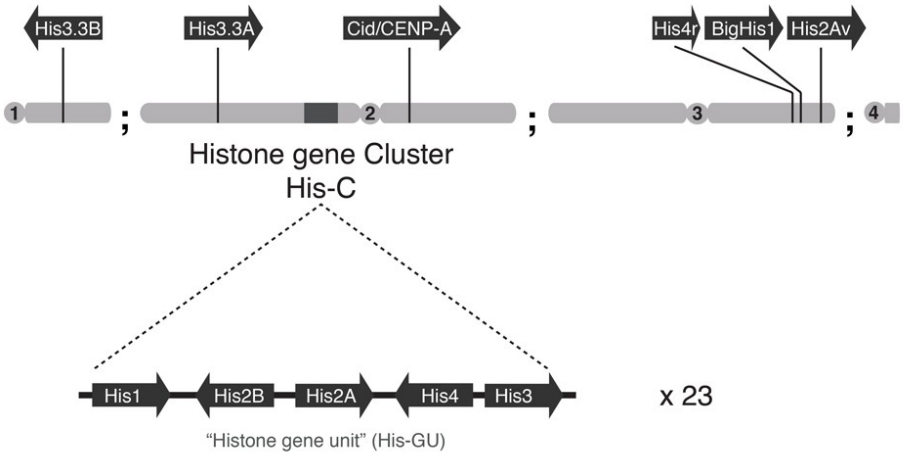

Figure 2. Organisation of histone genes in *Drosophila*
Diagram depicting all four *Drosophila* chromosomes, (their number is indicated at each centromere). The canonical histone gene cluster or "His-C" is represented by a dark grey box on the left arm of chromosome 2. The "histone gene unit" (HisGU) is represented below. On the upper panel, *Drosophila* histone variants are shown at their approximate loci (black lines).

It is likely that the unique organisation of histone genes in clusters contributes to the rapid, high-level transcription required during S phase to produce large amounts of histone proteins to assemble the newly synthesised DNA into chromatin.

b Canonical histone gene transcription

Metazoan canonical histone genes are unique: they are intronless and their message is not polyadenylated. Instead, histone mRNA possesses a stem loop sequence and a "histone downstream element" (HDE) sequence at its 3' end (Birchmeier et al., 1983; Schaufele et al., 1986). During mRNA synthesis, the "Stem Loop Binding Protein" (SLBP) binds to the stem loop and the HDE base pairs with a specific sequence at the 5' end of the single stranded U7 small nuclear RNA (snRNA) (Schaufele et al., 1986; Strub et al., 1986). Together, the SLBP and the histone mRNA specific U7 snRNP recruit the Histone pre-mRNA cleavage complex (HCC) (Sabath et al., 2013; Dominski et al., 2005). This complex interacts with the U7snRNP factor and cleaves the mRNA liberating it from the DNA template (Yang et al., 2012). Next, the histone mRNA is exported to the cytoplasm and translated in a similar manner as polyadenylated mRNAs (Erkmann et al., 2005; Wells et al., 1998).

The levels of Histone proteins are tightly coupled to cell cycle progression. This regulation takes place mainly at the mRNA level. Canonical histone mRNAs start accumulating at the end of the G1 phase to reach their maximum levels in S phase, then remain high during the entire S phase and are rapidly degraded at the end of S phase (Harris et al., 1991). The exact mechanisms of histone gene transcriptional activation at the onset of S phase remain cryptic, probably due to the fact that it is such an essential process that there are compensating mechanisms that mask the effects of removing the components of the major pathways. For example, in *Drosophila* embryos lacking dSLBP, histone mRNAs start to be polyadenylated (Lanzotti et al., 2002).

Interestingly, a study in *Drosophila* suggests that proper canonical histone mRNA processing is regulated by the histone variants H2Av and H3.3 (Wagner et al., 2007). This intriguing finding opens the possibility that nucleosomes containing these histone variants might be present directly at canonical histone genes and thereby promote their transcription. Alternatively, there might be an “in trans” mechanism in which the levels of H2Av protein signals the levels of the chromatin assembly factors required for both incorporation of canonical histones and variant histones.

c Canonical Histone supply regulation during *Drosophila* embryogenesis

In *Drosophila*, the levels of histone protein and mRNA are regulated during embryonic development. Histone mRNA and protein is loaded into the oocyte, and provides the embryo with histones during the rapid first 14 cell cycles of embryogenesis. These first cell cycles of *Drosophila* embryogenesis consist of only S phase and mitosis and occur within the embryonic syncytium in the absence of cytokinesis (reviewed in Kotadia et al., 2010). Subsequently, during S phase of cell cycle 14 zygotic synthesis of histones starts (Lanzotti et al., 2002; Günesdogan et al., 2010). Interestingly, H2A, H2Av and H2B histone proteins have been found to be stored in lipid droplets in *Drosophila* embryos and it has been proposed that this is a means of maternal histone product storage in the embryo, althought there is also evidence that these lipid droplets might function to buffer excess histones that could be toxic for the embryo (Li et al., 2012; Li et al., 2014).

d Canonical Histone deposition during S phase

Once the canonical histones have been produced at high levels during S phase, it is crucial that these histones become incorporated into chromatin behind the replication fork. This mechanism is not only critical to properly package the replicated DNA into chromatin, but it is also crucial to avoid an excess of free histones in the nucleus. An

overabundance of histone products in the cell can cause oversensitivity to DNA damage and chromosome instability (Liang et al., 2012; Meeks-Wagner and Hartwell, 2012). These drastic effects are most likely due to the inappropriate binding of the non-chromatin associated positively charged lysine and arginine rich histones to the negatively charged DNA phosphate backbone.

Specialised multimeric proteins referred to as histone chaperones, are required for histone deposition (Laskey et al., 1977). They bind newly synthesised histones and deposit them specifically onto the replicating DNA. Histone chaperones are specific for different histones: H3-H4 are deposited by a distinct set chaperones than H2A-H2B. Directly after synthesis, in the cytoplasm, new H3-H4 hetero-dimers are first bound by the t-nuclear auto antigenic sperm protein (NASP) complex, subsequently they are handed over to the Histone acetyltransferase Rb-associated protein 46 (Hat1-RbAp46) complex for acetylation, and finally to the anti-silencing function 1 (ASF1) complex for import into the nucleus (Verreault 2000). The H3-H4 dimer is then transferred to the Rtt106 chaperone or the chromatin assembly factor 1(CAF1) complex, which then directly deposit it onto the replicating DNA after the replication fork. Whether the H3-H4 is deposited as a tetramer or by two rounds of a dimer deposition is not known. Next, two H2A-H2B hetero-dimers imported by the Nucleosome Assembly protein 1 (Nap1) chaperone, are assembled onto the so-called H3-H4-DNA tetrasome. How H2A-H2B molecules get incorporated into the replicating DNA is not clear, but it is likely that it involves the histone chaperone complex facilitates chromatin transcription (FACT) since it binds to the replicating polymerase DNA Polymerase I (Hondele et al., 2013). After the nucleosome is assembled, the H1 linker histone is incorporated onto the replicating DNA to form higher order chromatin structures. NASP imports H1 into the nucleus. The precise mechanism by which H1 is deposited onto chromatin is not clear. Variant histones are incorporated by a distinct set of histone chaperones in a replication-independent manner.

Disruption of nucleosomes ahead of the replication fork is also required to allow the passage of the replication machinery. Given that the histone chaperone FACT is capable of displacing H2A-H2B dimers from nucleosomes during transcription, it is likely that FACT displaces the parental H2A-H2B dimers from nucleosomes during replication. However, how the parental [H3-H4] dimers are disassembled remains unknown. In any case, the recycled H2A-H2B dimers and the $[H3\text{-}H4]_2$ tetramers are redeposited onto the replicating DNA. Newly assembled nucleosomes can contain a new and a parental

H2A-H2B dimer, whereas $[H3\text{-}H4]_2$ tetramers are either newly imported or recycled (Dennehey and Tyler, 2014).

Importantly, histone chaperones act collectively with ATP-dependant chromatin remodellers to assemble nucleosomes on to the replicating DNA (Burgess and Zhang, 2013).

1.1.1.2 Histone variants

In metazoans the variant histone genes are polyadenylated, contain introns, and are transcribed in a replication-independent manner, unlike canonical histone genes (section **1.1.1.1**) (reviewed in Henikoff et al., 2004). In addition, histone variant genes are usually present in single copies unlike the clustered canonical histone genes. The major mammalian core histone variants and their *Drosophila* orthologs are listed in **Table 1** (reviewed in Maze et al., 2014). This list is non-exhaustive since recent studies have found many different alternatively spliced versions of some histone variants like mammalian H2A.Z (Bönisch et al., 2012).

Canonical histone	*Drosophila* ortholog	Mammalian histone variant
H2A	H2Av	H2A.Z H2A.X
	-	Macro.H2A H2A.Bbd
H2B	-	H2BE TSH2B
H3	H3.3	H3.3
	CID	CENP-A
H4	H4r	-

Table 1: Major core histone variants in *Drosophila* and mammals

CID (centromere identifier), CENP-A (centromere protein A), TSH2B (testis sperm specific H2B) (reviewed in Maze et al., 2014).

Nucleosomes containing histone variants add to the diversity of cues affecting chromatin structure and DNA accessibility (section **1.1.2.1b**). They are incorporated into chromatin at specific sites in the genome by specialised chaperones that are distinct from the canonical histone chaperones. In mammals, all canonical core histones and the linker histone H1 have histone variants, except H4. Interestingly, the *Drosophila*

genome contains a single copy H4r variant and its protein sequence is identical to canonical H4 (Akhmanova et al., 1996). The H3 variant protein sequences usually vary only in a few amino acid residues compared to the canonical histone H3, with the exception of the centromere specific histone variant CENP-A,(CID in *Drosophila*). For example, H3.3 and canonical H3 only differ by four residues (Hödl and Basler, 2009). H2B variants are present in mammals but to date, no variant has been found in *Drosophila*. H2A variant sequences are the most diverse in sequence of all core histones. At least six H2Av variants have been identified in mammals, but the best-studied ones are H2A.Z and H2A.X. In *Drosophila*, there is only one histone H2A variant, H2Av that is a combination of H2A.Z and H2A.X (**Figure 3**). The histone body of H2Av has a high degree of similarity with the body of human H2A.Z while the most C-terminal residues of H2Av have a high degree of similarity with the C-terminal tail of H2A.X, notably serine 139 on H2A.X that is phosphorylated upon DNA damage, is equivalent to serine 137 in H2Av (**Figure 3B**).

As for the linker histone H1, it has many variants in mammals, but in *Drosophila* only one embryonic specific variant, BigH1, has been identified (Pérez-Montero et al., 2013).

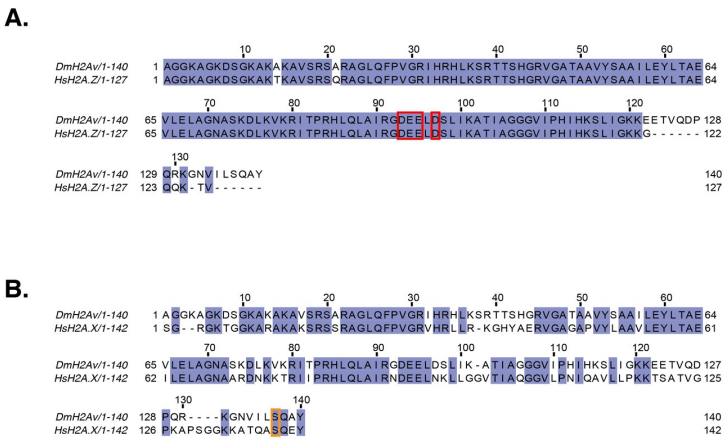

Figure 3. Alignment of *Drosophila* H2Av with human H2A.Z and H2A.X
Alignments generated with Clustal W2 http://www.ebi.ac.uk/Tools/msa/clustalw2/ using default settings. Identical residues are marked in purple. The scale above the alignment corresponds to *Drosophila* H2Av numbering. **A.** Alignment of *Drosophila* H2Av with Human H2A.Z (Uniprot ID number: P08985 and P0C0S5 respectively). The extended acidic patch is boxed in red. **B.** Alignment of *Drosophila* H2Av with Human H2A.X (Uniprot ID number: P08985 and P16104 respectively). Serine 137 in *Drosophila* H2Av and serine 139 in human H2A.X are boxed in orange.

a Histone variants in *Drosophila*

To date, in *Drosophila*, four core histone variants and one linker histone variant have been identified (**Figure 2; Table 1**). Not all of these histone variants are essential for *Drosophila* viability. In the case of *H3.3A* and *H3.3B,* double mutants are viable but sterile (Hödl and Basler, 2009; Sakai et al., 2009). Similarly, *H4r* homozygous mutants are viable and fertile (M. Kuroda personal communication).

Conversely, the recently identified BigH1 embryonic specific linker histone variant is critically required for embryonic development and viability (Pérez-Montero et al., 2013). The centromere specific H3 variant CID is also required for completion of embryogenesis owing to its essential function in proper timing of mitosis (Blower et al., 2006). Lastly, the *H2A.Z* and *H2A.X* ortholog *H2Av* is essential for completion of larval development (van Daal and Elgin, 1992).

In the next sections I will provide a detailed description of the functions of the best-studied histone variants: H2Av and H3.3.

■ H2Av

The mammalian counterpart of H2Av, H2A.Z is deposited by the chromatin remodeller SWR1, outside of S phase, and is displaced from nucleosomes by Inositol requiring 80 (INO80) (Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2011). Genome wide mapping of the yeast ortholog of H2A.Z (Htz1), nucleosomes has shown that these variant nucleosomes localise preferentially in the area surrounding the +1 nucleosome of the TSS (transcription start site) of active genes (Zhang et al., 2005). These features are evolutionary conserved in *Drosophila* (Mavrich et al., 2008).

H2Av has been found to be involved in double strand break repair in the same way as its other mammalian counterpart H2A.X (Madigan et al., 2002). This is consistent with its C-terminal tail sharing a high degree of homology with the terminal tail of H2A.X that is phosphorylated upon DNA damage on serine 139 in mammals (serine 137 in *Drosophila*) by the Ataxia telangiectasia mutated (ATM) and DNA dependant protein kinase (DNA-PK) kinases (**Figure 3;** Burma et al., 2001). Interestingly, in mice, H2A.X is not essential for survival but animals homozygous for an *H2A.X* ^{KO} allele are sensitive to irradiation-induced double strand breaks and males are sterile (Celeste et al., 2002).

In contrast, one of the two H2A.Z genes in mice, H2A.Z.1 is essential for embryonic development (Faast et al., 2001) indicating that despite their similarity (they

differ only by three amino acid residues), H2A.Z2 is not capable of compensating for the lack of H2A.Z1 (Eirín-López et al., 2009).

H2A.Z has been found to play opposing roles, both in transcriptional regulation of active genes and heterochromatic transcription repression. Indeed, H2A.Z participates in heterochromatin formation and centromere structure formation (Fan et al., 2004; Greaves et al., 2007). In addition, work in mouse ES cells showed that the occupancy of Polycomb group complexes PRC2 and PRC1 correlates with H2A.Z localisation and is disrupted in H2A.Z depleted cells (Creyghton et al., 2008). This is consistent with RING1B, the E3 ubiquitin ligase subunit of PRC1-type complexes monoubiquitylating H2A.Z in human cell lines and mammalian ES cells (Sarcinella et al., 2007; Ku et al., 2012). In a similar way, H2Av has been proposed to be implicated in PcG repression (Swaminathan et al., 2005). However, the authors of this study did not demonstrate a convincing PcG phenotype of H2Av mutants. This data seems to be controversial.

Acetylation of both the mammalian and the *Drosophila* H2A variant is performed by Tip60, a member of the Nu4A complex in mammals (the dTip60 complex in *Drosophila*). In *Drosophila* this acetylation is required for the nucleosomal exchange of phosphorylated H2Av with unmodified H2Av (Kusch et al., 2004). In contrast, in yeast, acetylation of H2A.Z and H4 by the Nu4A complex is required for H2A.Z incorporation into nucleosomes by SWR1 (Altaf et al., 2010).

Genome-wide and gene-specific Chromatin Immunoprecipitation (ChIP) studies in chicken cells and in human cancer cell lines, revealed that acetylated H2A.Z is positioned at the promoters of active genes (Bruce et al., 2005; Valdes-Mora et al., 2012).

Another interesting feature of the H2A.Z variant is the structure of the H2A.Z bearing nucleosome. Early studies in *Drosophila* identified a region in the *Drosophila H2Av* required for viability (Clarkson et al., 1999). This region, buried in the nucleosome core, encodes the acidic patch of H2Av/Z, which is extended in comparison to the canonical H2A acidic patch, and interacts with the H4 N-terminal tail in the nucleosome core particle (NCP) (**Figure 3A**). Moreover, the crystal structure of the NCP with H2A.Z brought to light that although the H2A.Z nucleosome shares similar features with the canonical NCP, it presents a slight destabilisation of the interaction between the H2A.Z-H2B dimer and the $[H3\text{-}H4]_2$ tetramer. In addition, it possesses an altered surface with the potential to interact with nuclear protein partners and to inhibit inter-nucleosomal interactions thereby inducing higher order chromatin structure changes (Suto et al.,

2000; Fan et al., 2002). This is consistent with H2A.Z NCPs facilitating the access of chromatin modifiers to their target genes (Hu et al., 2013).

In summary, H2Av is a critically required histone variant that shares common features with canonical H2A. Notably, both are ubiquitylated by PRC1 complexes. However, unlike H2A, H2Av is located at the +1 nucleosome position, and carries out distinct biological functions that are critically required for viability.

- **H3.3**

In *Drosophila*, there are two H3.3 coding genes, *H3.3A* and *H3.3B.* Their product is identical and differs from the canonical histone H3 by only four amino acid residues. In mammals and in *Drosophila* H3.3 is incorporated into chromatin throughout the cell cycle by the histone chaperones Histone Cell Cycle Regulation Defective Homolog A (HIRA), Alpha Thalassemia/Mental Retardation Syndrome X-Linked (ATRX) and Death-Domain Associated Protein (DAXX) (Tagami et al., 2003; Loppin et al., 2005; Goldberg et al., 2010). This results in a specific distribution pattern at active and inactive genes but also at telomeres in mouse ES cells and neural progenitor cells (Goldberg et al., 2010).

H3.3 is associated with transcriptional activation although it has been shown not to be formally required for transcription in *Drosophila* (Wirbelauer et al., 2005; Hödl and Basler, 2009; Sakai et al., 2009). In line with this finding, H3.3 is not required for viability in *Drosophila*, although genetic studies indicated that it is essential for *Drosophila* male fertility and germ line development (Hödl and Basler, 2009; Sakai et al., 2009). However, a recent report revealed that the canonical *H3* gene expressed under the *H3.3* regulatory sequences can rescue this infertility suggesting that the required feature of H3.3 might not be the amino acid residue differences in H3.3 *per se* but the production of proper protein levels of H3.3 at the right time during the cell cycle, perhaps outside S phase (Hödl and Basler, 2012). Just as its canonical counterpart, H3.3 carries PTMs associated with active transcription at actively transcribed genes (McKittrick et al., 2004). Interestingly, H3.3 has been found to be required for proper establishment of H3-K27me3 repressive chromatin by PRC2 in mouse ES cells, and thereby for maintenance of proper patterns of germ layer development (Banaszynski et al., 2013).

1.1.2 Chromatin structure

In addition to having a function in general packaging of the long DNA fibbers into the nucleus of cells, chromatin is organised in different hierarchical structures that define how accessible the DNA is to the different nuclear machineries. The spacing and overall arrangement of the basic subunits of chromatin dictates the accessibility of the DNA template. It has been proposed that chromatin can go from a "permissive" state in which the nucleosomes are spaced in a relaxed manner, called the "beads in a string" conformation or "primary structure", to a structure in which the DNA is buried within the chromatin, due to the intra-molecular folding of individual arrays for which the linker histone H1 has been proposed to be essential, commonly referred to as the "30 nm chromatin fibre" or "secondary structure", to finally a "tertiary structure": a tightly compacted architectural organisation based on inter-molecular compaction that can be visualised at the metaphase chromosome stage (reviewed in Luger et al., 2012).

Until very recently, despite many attempts, the structure of "30 nm chromatin fibre" had not been solved. New insights on this "secondary structure chromatin fibre" came from a cryogenic electron microscopy structure at an 11-Angstrom resolution (Song et al., 2014). This structure shows that the fibre forms a zigzag like conformation of which the structural unit is a tetranucleosome with a straight linker DNA. Within this unit, two stacks of two nucleosomes are formed. However, clear evidence of the existence of this chromatin architecture *in vivo* is still lacking (Nishino et al., 2012). The tertiary structure of chromatin remains uncharacterised.

The above mentioned chromatin conformations directly affect all the biological processes that occur on the DNA fibre, like transcription, replication and DNA damage repair. How these conformations change from a state in which the DNA is accessible to a state in which the DNA is buried within a higher order structure and *vice versa* has been extensively studied. Diverse mechanisms have the ability to alter chromatin structure. The main ones are described below.

1.1.2.1 Mechanisms of chromatin structure modification

a ATP-dependant chromatin remodellers

ATP dependant chromatin remodellers are multi-subunit molecular machines that actively perform complex rearrangements of chromatin structure via the hydrolysis of ATP. They are highly conserved from yeast to mammals and they all contain a similar ATPase domain, domains or subunits with high affinity for nucleosomes, domains that

recognise histone modifications, and regulator subunits. The latter three kinds of domains are specific to each chromatin remodeller (reviewed in Clapier and Cairns, 2009). They employ complex biophysical mechanisms to achieve chromatin rearrangement, such as nucleosome repositioning, disruption and eviction. These functions are critically required during transcription, DNA replication, and DNA damage repair. Examples of cases when chromatin remodellers are critically required are: nucleosome eviction from the parental DNA to allow the passage of the replication fork during S phase or during DNA damage repair; or proper spacing of nucleosomes after the passage of the replication fork or the transcriptional machinery; or to expose DNA by "sliding" the nucleosomes during transcription for example.

How chromatin remodellers are targeted to specific sites on chromatin still remains an open question. It is likely that the answer lies in their interaction with histone post-translational modifications. The interplay of histone modifications and chromatin remodeller activity is illustrated by the finding that the nucleosome removal activity of RSC is affected by the acetylation status of the nucleosomes at the promoter region of genes (Lorch et al., 2011).

There are four main classes of chromatin remodellers: switching defective/sucrose non fermenting (SWI/SNF), imitation switch (ISWI), chromatin helicase DNA binding (CHD) and INO80.

The SWI/SNF family of chromatin remodellers has the ability to slide and eject nucleosomes. The ISWI family generally regulates nucleosome spacing that can result either in transcriptional repression or activation. Notably, in *Drosophila*, ISWI subfamilies were shown to play a role in the structural maintenance of the male *X* chromosome (Deuring et al., 1999).

The CHD family of remodellers also regulate nucleosome spacing although their precise function remains unclear. However, in *Drosophila*, the CHD family has been found to have a function in transcriptional activation (Srinivasan et al., 2005; Murawska et al., 2008).

The INO80 family not only has a function in nucleosome spacing but it is the only family of remodellers that has a function in H2A.Z deposition by the SWR1 subfamily of remodellers, and H2A.Z eviction by the INO80 subfamily (Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2011). Interestingly, the INO80 ATPase associates with

Pleiohomeotic (Pho), a member of the Polycomb group transcriptional repressors, in *Drosophila* and in mammals (Klymenko et al., 2006; Cai et al., 2007).

b Histone variant incorporation

Differences between histone variants and their canonical counterparts can range from a few amino acid residues, like in the case of canonical H3 and H3.3, to the addition of approximately 20 kDa domain illustrated by the macro-H2A variant (Franklin and Zweidler, 1977; Pehrson and Fried, 1992). Thus, incorporation of these histone variants presumably results in architectural changes at the nucleosome level and at the chromatin level. How does histone variant incorporation affect nucleosome structure?

Since the structure of the canonical nucleosome core particle was first solved, considerable effort has been invested in structural and biophysical studies to detect changes in the structure of nucleosomes containing histone variants.

Probably the most striking example of variant nucleosomes is the centromere specific nucleosome. It seems intuitive that these nucleosomes are unique in structure since they specifically direct kinetochore assembly at centromeres, to which the microtubules attach during mitosis and meiosis. The crystal structure of a human CENP-A containing nucleosome on alpha satellite centromeric DNA, indicated that there is a weakened interaction between the DNA and the variant nucleosome at its entry and exit sites (Tachiwana et al., 2011). Moreover, the CENP-A nucleosome differs from the canonical nucleosome in the L1 loop that protrudes slightly from the NCP and is perhaps a specific binding site for specific chromatin modifying factor. The composition of these nucleosomes has been subject of intense debate. Subjects of discussion range from the number of [CENP-A-H4] dimers (single or double copy) to the height of the nucleosome containing CENP-A. However, recent *in vitro* reconstitution studies complemented with atomic force microscopy (AFM) revealed that CENP-A variant nucleosomes are octameric and exhibit reduced heights compared to canonical octameric nucleosomes (Miell et al., 2013).

c Histone post-translational modifications

Covalent histone post-translational modifications can range from chemical moieties like methylation, to small peptides like in the case of ubiquitylation. PTMs mainly decorate the flexible unstructured tails of histones that protrude from the nucleosome, although recent studies identified PTMs on the globular domains of histones (**Figure 1**) (Xu et al., 2005). A catalogue of reversible histone PTMs has been established,

histones can be: acetylated, phosphorylated, methylated, ubiquitylated, sumoylated, ADP-rybosylated, amongst others (reviewed in Kouzarides 2007). A cohort of histone modifying enzymes carries out these modifications in a highly regulated manner.

These chemical modifications were first discovered in the mid sixties (van Holde, 1989), but it was not until the mid nineties that the first enzymes catalysing these modifications were identified. Since the first histone modifying enzymes to be identified were the histone acetyltransferases in yeast and *Tetrahymena* (Kleff et al., 1995; Brownell et al., 1995; Parthun et al., 1996; Brownell et al., 1996) many studies have been carried out, not only by mutation analysis of these enzymes but also by mutating and deleting the acetylated portions of the histone substrates themselves (Kayne et al., 1988). The later experiments were only possible in unicellular organisms that contain few copies of canonical histone genes, like yeast (section **1.1.1.1a**).

Subsequently came the discovery of the first histone methyltransferase in the murine model and the characterisation of the highly conserved Suppressor of variegation 3–9 [Su(var)3–9], Enhancer of zeste [E(z)], and Trithorax [Trx] (SET) domain (Rea et al., 2000). This led to the postulation of the revolutionary “histone code” hypothesis (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

This hypothesis implied that the combination of different histone post-translational modifications would dictate distinct states of DNA accessibility and thereby distinct transcriptional outcomes.

Histone PTMs have two major functions. Firstly, a structural function, illustrated by acetylation of lysines on histone tails, which neutralises the positive charge of the tail and thereby disrupts its interaction with the negatively charged DNA phosphate backbone. Secondly, histone modifications can serve as anchor sites for chromatin binding proteins and enzymes (reviewed in Grunstein, 1997; Strahl and Allis, 2000). According to the “histone code" hypothesis there are histone PTM “readers" that would have affinity for specific combinations of modifications, and “writers" that could modify histones at individual amino acid residues respectively, and thereby facilitate chromatin conformation changes. This implies that readers can also be writers and once they have bound to a specific histone modification their histone modifying activity can potentially be enhanced or inhibited. Generally readers contain a specific histone modification-binding domain. Many of these domains have been identified, amongst them the specific histone methyl binding domains: the chromodomain, the chromo-shadow domain, the

Tudor domain, the MBT domain and PHD finger; and the bromodomain that binds to acetylated histones (reviewed in Kouzarides, 2007). An example of the stimulation of the activity of a "writer" by binding to histone PTMs, is the recent finding that upon binding to H2A monoubiquitylated nucleosomes, the H3-K27me3 histone methyltransferase activity of the PRC2 complex is enhanced (Kalb et al., 2014).

Ten years after the “histone code" hypothesis was put forth, two studies assigned different combinations of histone PTMs, to different colour coded types of chromatin reflecting their putative function (Filion, et al., 2010; Kharchenko et al., 2011).

However, since many of the enzymes that carry out the histone modifications also have non-histone substrates, studying the direct effect of histone modifications has proven to be a complex task. This has lead to the question if the modifications themselves are causative or if they are a secondary effect of histone modifying enzymes acting on their non histone substrates (reviewed in Henikoff and Shilatifard, 2011). Further *in vivo* functional studies, like the analysis on histone point mutants, complemented with structural and biochemical assays are required to dissect the direct consequences of histone modifications on chromatin.

d Other mechanisms that affect chromatin structure

In addition to the chromatin modifying mechanisms that directly affect histones, there are other mechanisms that have been found to affect chromatin structure and DNA accessibility. An example of these kinds of mechanisms is DNA methylation, which occurs directly on the DNA template at position 5 of CpG dinucleotides, except on the CpG rich regions known as CpG islands, which are predominantly devoid of methylation. CpG methylation is correlated with transcriptional repression, which is thought to be achieved by either preventing transcriptional activators from binding the DNA or by recruiting repressive proteins that bind specifically to methylated CpGs (reviewed in Klose and Bird, 2006).

Another chromatin modifying mechanism is performed by a subset of long non-coding RNAs (lncRNAs). The best studied examples are the lncRNAs involved in dosage compensation of the sex chromosomes: *Xist* RNA in mouse or the *roX* RNAs in *Drosophila*. In the case of *Xist*, it spreads along the inactive X chromosome and has been proposed to induce histone modifications and DNA methylation and thereby trigger transcriptional repression (Reviewed in Gendrel and Heard, 2014). To further understand the function of lncRNAs it is necessary to perform loss of function analyses.

However these experiments are not so straight forward to perform and interpret since deleting the lncRNA, not only abolishes the transcription of the lncRNA but also removes its corresponding DNA coding sequence, and it is difficult to distinguish between the RNA itself being required for its function or just the act of transcription (Bassett et al., 2014).

Nuclear compartment localisation has also emerged as potentially playing an important role in transcriptional regulation (Fraser and Bickmore, 2007). Many studies have determined distinct functional topological domains of chromatin in the nucleus. This was triggered by the establishment of the chromosome conformation capture (3C) branch of techniques (Dixon et al., 2012; Nora et al., 2012). However, the order of events has been a difficult problem to solve. Does localisation to nuclear domains take place before or after transcriptional repression or activation? In addition, how mechanistically the localisation in the nucleus affects transcription also remains an open question.

To achieve proper chromatin structure regulation, it is essential to have a concert of distinct mechanisms that affect chromatin. This is illustrated by the tight coupling between histone variant deposition by chromatin remodellers and regulation of chromatin remodellers by histone post-translational modifications. In addition, the binding of transcription factors (Cirillo et al., 2002) to nucleosomes might also impact on the overall structure of chromatin.

1.2 Polycomb/Trithorax transcriptional regulators

During development, distinct cells differentiate to become functionally and phenotypically specialised to generate a whole organism. A fundamental question in biology is how, after differentiation, genetically identical cells can achieve so diverse phenotypes and functions and maintain their cellular identity throughout the life of an organism. It seems intuitive that these divergences arise from many different levels of regulation. The first level of regulation most likely is at the time of transcription. Early in *Drosophila* development the gap and pair rule gene products dictate the transcriptional status of developmental regulator genes that should be transcriptionally ON or OFF in different cells, however, the gap and pair rule gene products are only present transiently at the beginning of embryogenesis (Müller and Bienz, 1992; Zhang and Bienz, 1992; Shimell

et al., 1994). When the gap and pair rule signal dissipates, these developmental regulator genes are maintained in the OFF state by the Polycomb group (PcG) of transcriptional repressors in those cells where they should be transcriptionally silent. In contrast, Trithorax group (TrxG) proteins maintain these genes in a transcriptionally active state in cells where they should be expressed. The main targets of the PcG and the TrxG proteins are the *HOX* genes that are transcription factors responsible for proper body patterning along the anterior-posterior axis of metazoans (Lewis, 1978). Most PcG and TrxG factors are essential for viability, and in the same way as *HOX* genes, are conserved in metazoans and plants. Strikingly, PcG proteins have also evolved to fulfil a variety of functions, ranging from *X* chromosome inactivation in mammals to vernalisation regulation in plants (Plath et al., 2003; Silva et al., 2003; Gendall et al., 2001).

1.2.1 The Polycomb group of transcriptional repressors

Historically, Polycomb group repressors were initially identified in *Drosophila melanogaster* due to the defective body patterning phenotype of adults and embryos mutant for Polycomb group genes. Notably, the first gene that was discovered was *Pc,* owing to the particular phenotype of mutations at this gene that result in the appearance of ectopic sex combs on the second and third leg in males. This was particularly visible in the adult males by scoring the number of sex combs on the second and third pair of legs, as normally they only have sexcombs in the first pair of legs (Lewis, 1978).

In the last fifty years, major progress in our understanding how PcG proteins function has been made. Starting by the identification of many novel PcG genes and subsequently progressing in the advancement of the understanding of their molecular function.

1.2.1.1 PcG target genes

The classical PcG target genes are the *HOX* genes, however, the PcG system also regulates other target genes (Dura and Ingham, 1988; Busturia and Morata, 1988; Oktaba et al., 2008; Gutiérrez et al., 2010).

a *HOX* genes

This set of highly conserved transcription factors was first identified in *Drosophila* in the late 19th century owing to the phenotype of mutants of these genes, which typically involve transformations of distinct portions of the body into others along the anterior-posterior axis, referred to as homeotic transformations (Lewis 1978). In *Drosophila,*

HOX genes are organised in two distinct clusters: the *Antennapedia* complex (*ANT-C*) and the *Bithorax* complex (*BX-C*). The former contains the *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sexcombs reduced* (*Scr*) and *Antennapedia* (*Antp*) genes, and the latter contains the *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (**Figure 4**). Subsequently, in the mid 1980s it was discovered that these genes are expressed in specific domains along the anterior-posterior body axis that follow an order analogous to their genomic position. This characteristic is referred to as spatial collinearity (**Figure 4**) (Harding et al., 1985; Akam, 1987; Duboule and Morata, 1994).

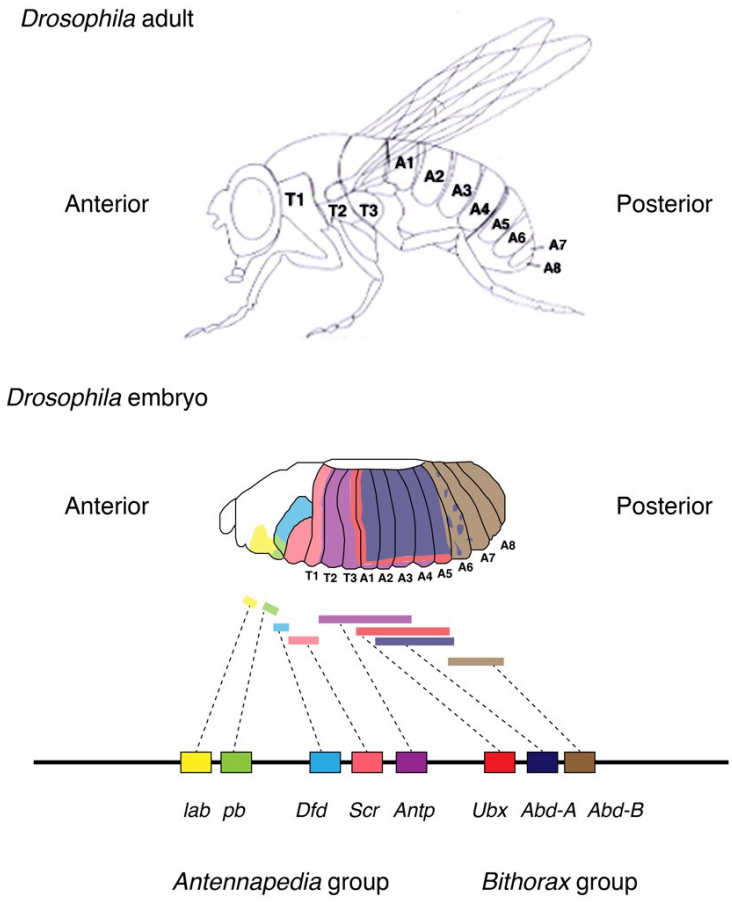

Figure 4. *HOX* genes in *Drosophila*
Lower panel shows the *Drosophila HOX* genes on DNA (black line), and their expression domains are indicated above with a line of the corresponding colour. The expression domains of each gene in the embryo in each segment are depicted above. The corresponding segments in the *Drosophila* adult are represented in the upper panel.

It was also observed that *HOX* genes have the ability to control each other. The most posterior gene products can transcriptionally repress the anterior genes (Hafen et al., 1984; Struhl and White, 1985). Around the same time, it was discovered that *HOX* genes contain a specialised domain referred to as the homeobox, which encodes the homeodomain (McGinnis et al., 1984; Scott and Weiner, 1984). This specialised domain confers specific DNA binding activity to HOX proteins, which allows them to bind to their target genes.

The first piece of evidence that a Polycomb group gene product could regulate proper body patterning was found in 1978 (Lewis, 1978; Struhl 1981). But it was not until 1986 that *in situ* hybridisation experiments brought to light the first molecular direct evidence showing that *Pc* mutant *Drosophila* embryos misexpress *HOX* genes outside their normal expression patterns (Wedeen, et al., 1986).

The discovery that these genes are conserved in mammals did not come until later, with the identification of the four *HOX* clusters in mouse: Hoxa, Hoxb, Hoxc and Hoxd; each of them containing the ortholog sub-clusters of ANT-C and BX-C (Duboule and Dolle, 1989; Graham et al., 1989).

b Other target genes

Besides the classical *HOX* target genes, novel target genes in *Drosophila* were identified through the genome wide studies of PcG binding sites (Schwartz et al., 2006; Nègre et al., 2006; Tolhuis et al., 2006; Oktaba et al., 2008; Kwong et al., 2008). Notably these genes include developmental regulator genes like *engrailed (en), apterous (ap), teeshirt (tsh), panier (pnr), Distalless (Dll), even skiped (eve), caudal (cad), Dorsocross2/3 (Doc2/3)* and interestingly also include cell cycle regulator genes like *Cyclin B, Retinoblastoma family protein (Rbf), E2F transcription factor (E2F),* and *Dp transcription factor (Dp).* In addition, many other genes were found to be bound by PcG proteins, but still remain to be confirmed as actual functional PcG target genes.

An additional set of target genes was discovered in further genome wide binding patterns of PRC1 (Polycomb repressive complex 1) subunits (section **1.2.1.1b**), complemented with functional assays. PcG target genes can be divided into two different categories. Class I that require *Ph, Psc-Su(z)2*, *Pc* and *Sce* (**Table 2**) to be stably repressed, and include: *HOX* genes, *elbowB (elb), no ocelli (noc*) and *en.* In turn, the class II genes require *Psc-Su(z)2* and *Ph* but not *Pc* or *Sce* to be stably repressed, include: *Doc2/3*, *dachshund (dac)*, *prospero (pros)*, *eve* and *cad* (Gutiérrez et al., 2012).

1.2.1.2 PcG proteins form multimeric complexes

Biochemical purification of PcG proteins revealed that most of the identified Polycomb group proteins form multi-subunit complexes (reviewed in Müller and Verrijzer, 2009). These complexes are Polycomb Repressive Complex 1 (PRC1), Polycomb Repressive Complex 2 (PRC2), Pho Repressive Complex (PhoRC) and Polycomb Repressive Deubiquitinase (PR-DUB). Currently, Super sex combs (Sxc) is the only PcG protein that is not known to be part of a multimeric complex. Sxc/Ogt is an O-GlcNAc (β-linked *N*-acetylglucosamine) transferase for the PRC1 subunit Ph (Polyhomeotic) (Gambetta et al., 2009). O-GlcNacylation of Ph is critical for Ph repressor function and represents the major biological function of Ogt in *Drosophila* (Gambetta and Müller, 2014).

Three out of four of the core PcG complexes have histone modifying activities, PRC1, PRC2 and PR-DUB, and only one of the complexes, PhoRC, has the ability to bind directly to specific sequences on DNA (reviewed in reviewed in Müller and Verrijzer, 2009). This will be discussed below (section **1.2.1.2d**.).

a The PRC1-type complex family

As its name indicates, the first Polycomb repressive complex to be purified was the PRC1 complex (Shao et al., 1999; Saurin et al., 2001). The purification revealed that the core PRC1 complex contains stoichiometric amounts of Polyhomeotic (Ph), Posterior sex combs (Psc), Pc and Sex combs extra (Sce), and substoichiometric amounts of Sex comb on midleg (Scm) (**Table 2**) (reviewed in Simon and Kingston, 2009). Mammalian orthologs were also found to form a stable core PRC1 complex (Levine et al., 2002). Subsequently, it was shown that PRC1-type complexes have two main functions: an enzymatic activity that monoubiquitylates the C-terminal tail of H2A and a non-enzymatic ability to compact nucleosomal arrays (Wang et al., 2004b; Cao et al., 2004; Francis et al., 2004). This led to the hypothesis that the main function of PRC1 complexes in PcG repression was to monoubiquitylate H2A. Interestingly, it was later discovered that Psc and Sce are members of other complexes. In *Drosophila* at least two individual PRC1-type complexes have been identified, the canonical PRC1 complex and the dRing Associated Factors (dRAF) complex. dRAF does not contain Pc, Ph or Scm but contains Sce/Ring, Psc and the lysine (K) specific demethylase 2 (dKDM2) demethylase as well as additional cofactors. *In vitro* E3 ligase reactions on oligonucleosomes showed that dKDM2 enhances the E3 ligase activity of the complex. This was also demonstrated *in vivo,* since a depletion of dKDM2 resulted in a reduction

in bulk H2A ubiquitylation (Lagarou et al., 2008). This led to the idea that dRAF is the main E3 ligase for H2A in *Drosophila* . Interestingly, dKDM2 also has demethylase activity specific for H3-K36me2, a modification associated with active transcription. This lead to the idea that dRAF regulates a “trans-histone” pathway by at the same time ubiquitylating H2A and demethylating H3-K36me2. However, a recent study showed that dKDM2 is not essential in *Drosophila*, suggesting that canonical PRC1 is able to compensate for the lack of dKDM2 (Zheng et al., 2014). In addition, it cannot be excluded that canonical PRC1 could also efficiently ubiquitylate H2A *in vivo*.

Drosophila Polycomb group protein	Abbreviation	Human ortholog	PcG complex	Domains	Functional features
Polycomb	Pc	CBX2, CBX4, CBX6, CBX7, CBX8	PRC1	Chromodomain and Chromoshadow domain	Interacts with H3-K27me3
Polyhomeotic distal and Polyhomeotic proximal	Ph-d, Ph-p	PH1, PH2	PRC1	SAM domain, C_2C_2 zinc finger	unknown function
Posterior sex combs	Psc	NSPc1/PCGF1, MEL18/ PCGF2, PCGF3, BMI1/ PCGF4, PCGF5, MBLR/ PCGF6	PRC1	RING finger	Chromatin compaction, cofactor for E3 ligase activity of Sce
Suppressor of Zeste 2	Su(z)2	NSPc1/PCGF1, MEL18/ PCGF2, PCGF3, BMI1/ PCGF4, PCGF5, MBLR/ PCGF6	PRC1	RING finger	Chromatin compaction, cofactor for E3 ligase activity of Sce
Sex combs extra/ RING	Sce	RING1A (RING1) and RING1B (RING2, RNF2)	PRC1	RING finger	E3 ubiquitin ligase activity for the C terminus of H2A
Sex combs on midleg	Scm	SCML2, SCML4	PRC1 (substochiometric)	SAM domain, two MBT domains, zinc finger domain	interacts with PhoRC via dSfmbt
Lysine (K)-specific demethylase 2	dKDM2	KDM2B	PRC1/dRAF	CXXC-type zinc finger, F-box domain, JmjC domain	Demethylase for H3-K36me2
Ring and YY1 Binding Protein	RYBP	RYBP	PRC1 (substochiometric)	zinc finger RanBP2-type	Stimulates the activity of Ring1B in mammals
Enhancer of zeste	E(z)	EZH1 & EZH2	PRC2	Two SANT domains, a SET domain, CXC domain, WD40 domain	Mono-, di-, and trimethylation of H3-K27
Suppressor of Zeste 12	Su(z)12	SUZ12	PRC2	C_2H_2 zinc finger, VEFS-Box	Stimulates H3-K27 methylation by E(z), nucleosome binding
Extra sex combs and Extra sex combs like	Esc, Esc-l	EED	PRC2	WD40 repeats	Stimulates H3-K27 methylation by E(z), binds to methylated nucleosomes
Nucleosome remodelling factor 55/CAF1	Nurf55	RBBP4 (RBAP48), RBBP7(RBAP46)	PRC2	WD40 repeats	Nucleosome binding
Polycomb-like	Pcl	PHF1(PCL1), PHF19(PCL3), MTF2(PCL2)	PRC2 (substochiometric)	Tudor domain, two PHD fingers	Stimulates H3-K27 trimethylation is thought to recuit PRC2 to target genes
Jumonji, AT rich interactive domain 2	Jarid2	JARID2	PRC2 (substochiometric)	AT-rich interaction domain (ARID), zinc finger domain (C_5HC_2 type), jumonji N (JmjN) domain, and a JmjC domain	Stimulates histone methyl transferase activity of PRC2, and has DNA binding activity
Jing	Jing	AEBP2	PRC2 (substochiometric)	Three C_2H_2-type zinc fingers	Stimulates histone methyl transferase activity of PRC2
Additional sex combs	Asx	ASXL1, ASXL2, ASXL3	PR-DUB	Two LXXLL motifs and a PHD finger (C_4HC_3 zinc finger)	Stabilises Calypso protein
Calypso	Caly	BAP1	PR-DUB	UCH domain	Ubiquitin carboxyl-terminal hydrolase for H2Aub
Pleiohomeotic	Pho	YY1, YY2	PhoRC	Four C_2H_2 zinc fingers	Binds to specific sequences in Polycomb Response Elements on DNA
Pleiohomeotic like	Phol	YY1, YY2	PhoRC	Four C_2H_2 zinc fingers	Binds to specific sequences in Polycomb Response Elements on DNA
Scm-related gene containing four MBT domains	dSfmbt	MBTD1, LMBL2	PhoRC	Zinc finger, four MBT domains, SAM domain	Binds to mono- and dimethylated lysines on histones

Table 2: Polycomb group subunits in *Drosophila* and humans

Abbreviations: CBX (ChromoBox protein homolog); SAM domain (Sterile Alpha Motif); PCGF (Polycomb Group RING fingers); EED (Embryonic Ectoderm development); NSPc1 (Nervous System Polycomb 1); MEL18 (Melanoma Nuclear Protein 18); BMI1 (B lymphoma Mo-MLV insertion region 1); MBLR (Mel18 And Bmi1-Like RING Finger) ; RING (Really Interesting New Gene); RNF2 (Ring Finger Protein 2); MBT (Malignant Brain Tumour); SANT(switching-defective protein 3 [Swi3], adaptor 2 [Ada2], nuclear receptor co-repressor [N-CoR], transcription factor [TFIIIB]); AEBP2 (Adipocyte Enhancer-Binding Protein); PHD (Plant homeodomain); PHF (PHD finger protein 1); MTF (metal response element binding transcription factor 2); UCH (Ubiquitin C-terminal Hydrolase).

The mammalian counterpart of the dRAF complex is the BCOR complex (Gearhart et al., 2006) (**Table 2**)**.** In addition, a novel Lethal (3), malignant brain tumour (L3MBTL2) complex was found in mammals, containing the Sce orthologs RING1A and RING1B, the Psc ortholog MBLR, E2F and CBX3 an ortholog of Pc (Trojer et al., 2011). This complex was found to also ubiquitylate H2A and L3MBTL2 alone was shown to compact nucleosome arrays *in vitro*. Moreover, murine Rybp stimulates H2A ubiquitylation by Ring1B *in vitro,* and knock down of RYBP in human cell lines leads to a decrease in bulk H2A ubiquitylation levels (Gao et al., 2012).

Yet more complexes have been identified by comprehensive purifications in human cell lines that showed that each of the orthologs of Psc referred to as PCGF (Polycomb group RING fingers), forms a distinct PRC1-type complex. Thus, there are six PRC1-type complexes in mammals. The canonical PRC1-type complexes are referred to as PRC1.2 and PRC1.4 (Gao et al., 2012). Besides, dRAF, canonical PRC1, and the PRC1.6 complex, these complexes have not been identified in *Drosophila,* although orthologs of most individual protein components are present (Alfieri et al., 2013).

The function of H2A ubiquitylation in Polycomb repression has been subject of debate. Following the identification of Sce/Ring as the major E3 ubiquitin ligase for H2A and the observation that depletion of Sce/Ring in *Drosophila* cells resulted in the up-regulation of the *HOX* gene *Ubx*, H2Aub was proposed to be required for transcriptional repression of Polycomb target genes (Wang et al., 2004b). However, a study in murine ES cells showed that in catalytically inactive mutants of Ring1B (one of the two mammalian orthologs of Sce/Ring), proper repression of *HOX* genes was maintained. This lead to the proposal that H2A ubiquitylation was not required for repression of PcG target genes. However, in these experiments, the Ring1A paralog of Ring1B, was still present in the analysed cells and they contained residual levels of H2Aub, which in turn brought this idea into question (Eskeland et al., 2010). Interestingly, this study also indicated that complete KO of Ring1B causes degradation of the canonical PRC1 complex most likely owing to destabilisation of the complex. Moreover, further studies in mouse ES cells lacking both Sce/Ring orthologs Ring1A and Ring1B but expressing a catalytically inactive version of Ring1B suggested that chromatin compaction still takes place at *HOX* genes independently of H2A ubiquitylation (Endoh et al., 2012). In addition, the same study reported that H2A ubiquitylation is required for proper repression of non-*HOX* target genes.

Analysis in *Drosophila* showed that KO of Sce/Ring leads to misexpression of *HOX* genes and other developmental regulator genes outside their normal expression domains. The same study reported that in larval tissues depleted for Sce by RNA interference, the levels of most *Drosophila* PRC1 subunits were not affected, suggesting that perhaps in *Drosophila* the stability of the PRC1 complex might be independent of the presence of Sce (Gutiérrez et al., 2012).

Interestingly, recent tethering experiments in mouse ES cells established that H2A ubiquitylation is sufficient for PRC2 recruitment and H3-K27me3 chromatin establishment (Blackledge et al., 2014; Kalb et al., 2014). The study of H2A ubiquitylation has been hindered by the lack of antibodies specifically recognising the modification in ChIP (Chromatin Immuno-Precipitation) experiments, and therefore the inability to pin point the genomic location of the modification. However, recent studies in have shown specific H2Aub patterns by ChIP in mammalian cells (Tavares et al., 2012). More recently, a new H2Aub antibody has been shown to work in *Drosophila* cell lines (Schaaf et al., 2013). Currently, the function of H2A ubiquitylation in Polycomb repression remains an open question.

Canonical PRC1 was also found to have the ability to compact polynucleosomes independently of histone tails *in vitro*. The first evidence for this came from electron microscopy experiments in which nucleosomal arrays were incubated with individual PRC1 subunits and scored for compaction (Francis et al., 2004). In addition, Psc regions required for compaction *in vitro,* were found to be required for transcriptional repression *in vivo,* in *Drosophila* (King et al., 2005). Furthermore, not only does the PRC1 complex have the ability to compact nucleosomal arrays but also this ability persists when the arrays contain tailless histones. This characteristic comes hand in hand with the aptitude of PRC1 components to inhibit ATP dependant chromatin remodelling on nucleosomal plasmids or arrays (Shao et al., 1999; Francis et al., 2001). This suggests that PRC1-type complexes might contribute to gene repression through chromatin compaction, independently of H2A ubiquitylation.

b The PR-DUB complex

The Calypso protein was identified in a genetic screen for *Drosophila* mutants exhibiting a PcG phenotype. It was subsequently purified from *Drosophila* embryos (Gaytan de Ayala Alonso et al., 2007; Scheuermann et al., 2010). The purification identified the Polycomb repressive deubiquitinase (PR-DUB) complex formed by the ubiquitin C-terminal hydrolase Calypso and the PHD finger containing, Additional sex

combs (Asx). Analysis of the deubiquitinating enzymatic activity of PR-DUB showed that its substrate is H2Aub nucleosomes *in vitro* and *in vivo.* Mutants for the PR-DUB components show a considerable increase in the bulk levels of H2Aub, and Calypso and Asx are required for the repression of a subset of PcG target genes during embryogenesis and development.

The mammalian counterpart of Calypso is BRCA1-associated protein 1 (Bap1), and its biochemical purification from human cell lines and mouse spleen followed by mass spectrometry analysis, identified the Asx orthologs: Asxl1 and Asxl2, amongst other proteins as Bap1 interactors (Machida et al., 2009; Dey et al., 2012). Another study also found BAP1 to interact with YY1 and to act as a transcriptional activator in human cell lines (Yu et al., 2010).

The discovery of this complex and the fact that its activity is required for PcG repression, poses an apparent contradiction, since the H2A E3 ubiquitin ligase, Sce/Ring, is also required for the repression of PcG target genes (Gutiérrez et al., 2012). Interestingly, *Calypso* and *Asx* are required for only a subset of the genes that require *Sce* for repression, but a synergy was observed between *Sce* and *Asx*, in which the phenotype of animals mutant for both genes show more severe misexpression of *HOX* genes and homeotic transformations than the individual mutants. Several hypotheses were proposed to solve this conundrum (Scheuermann et al., 2012) and will be discussed in section **4.3**.

c The PRC2-type complex family

The core *Drosophila* PRC2 (Polycomb repressive complex 2) complex contains the histone methyltransferase Enhancer of Zeste (E(z)), Suppressor of Zeste 12 (Su(z)12), Extra sex combs (Esc) and its paralog Esc-like, and Nucleosome remodelling factor 55 (Nurf55/Caf1) (Czermin et al., 2002; Müller et al., 2002) (**Table 2**). Early studies demonstrated not only that PRC2 has a specific methyltransferase activity for H3-K27, but also that PRC2 can mono-, di-, or trimethylate H3-K27. In addition, the methyltransferase activity of E(z) is required for repression of Polycomb target genes *in vivo* (Müller et al., 2002).

The catalytic activity, the specificity and the composition of core PRC2 was also shown to be conserved in mammals (Cao et al., 2002; Kuzmichev et al., 2002) (**Table 2**). It was further shown in human cell lines that H3-K27me3 is also associated with transcriptional repression of PcG target genes (Kirmizis et al., 2004).

Interestingly, in one of the PRC2 purifications from human HeLa cells, the AEBP2 zinc finger protein was identified as part of the PRC2 complex (Cao et al., 2002). Further biochemical analysis showed that it indeed forms part of a recombinant PRC2 complex by direct interaction with other subunits, and that it stimulates the histone methyltransferase activity of PRC2 *in vitro* (Cao and Zhang, 2004).

Subsequently, a tandem affinity purification (TAP) strategy of the Polycomb like (Pcl) protein in *Drosophila* showed that it forms a distinct complex with the core PRC2, although in sub stoichiometric amounts. Importantly, the same study showed that Pcl is required for high levels of H3-K27me3 selectively at Polycomb target genes (Nekrasov et al., 2007).

Later studies in human cell lines demonstrated the ability of the Pcl ortholog PHF1 to form a distinct complex with core mammalian PRC2 and to confer specificity to trimethylate H3-K27me3 at PcG target genes is conserved in mammals (Sarma et al., 2008; Cao et al., 2008). How Pcl confers high levels of H3-K27me3 specifically at PcG target genes is not clear, however, the explanation might lie in the ability of Pcl to interact with target gene chromatin perhaps through its PHD finger and thereby generate high levels of H3-K27me3.

Moreover, the purification of the EZH2 in human cell lines, yielded the identification of a novel subunit of the mammalian PRC2 complexes: JARID2 (Li et al., 2010; Son et al., 2013). Subsequent studies in *Drosophila* also found that Jarid2 interacts with the *Drosophila* PRC2 complex (Herz et al., 2012). Jarid2 has the ability to bind DNA and stimulates PRC2 methyltransferase activity *in vitro*. In addition, depletion of Jarid2 results in the transcriptional up-regulation of a subset of PRC2 target genes in mammalian ES cells (Peng et al., 2009).

Interestingly, it was shown that Esc and its mammalian counterpart EED binds to arrays of methylated polynucleosomes *in vitro,* via its WD40 domain. This led to the idea that it facilitates the propagation of the H3-K27me3 mark (Margueron et al., 2009). In addition, gene specific and genome wide chromatin immunoprecipitation (ChIP) assays revealed that the H3-K27me3 mark occupies target gene chromatin in broad domains that range from the upstream regulatory gene region to the end of the coding region and sometimes throughout several genes (Nekrasov et al., 2007; Modencode project, Kharchenkoet al., 2011; Schwartz et al., 2006). This suggests that perhaps

during the establishment of H3-K27me3 domains, Esc might facilitate the binding of the PRC2 complex to H3-K27me3, thereby ensuring its spreading.

It is clear that the histone methyltransferase activity of the PRC2 complex family is crucial for the maintenance of PcG transcriptional repression. This has lead to the hypothesis that H3-K27 is the biologically relevant target of PRC2 complexes. However, studies in mammals indicate that PRC2 complexes have other histone and non-histone substrates. The linker histone H1 has been shown to be methylated by a PRC2 variant complex containing a specific splice version of EED (the ortholog of *Drosophila* Esc) (Kuzmichev et al., 2004). Furthermore, a recent analysis showed that the transcription factor Gata4 is methylated by PRC2 in mouse foetal hearts and that this inhibits its transcriptional activator function (He et al., 2012).

d The PhoRC complex

Biochemical purification of the DNA binding PcG protein Pho (Pleiohomeotic) from *Drosophila* embryos, revealed that it forms two distinct dimeric complexes, one with a novel PcG protein: Scm-related gene containing four MBT domains (dSFMBT), this complex is known as Pho repressive complex (PhoRC); and another complex with the chromatin remodeller INO80 (Klymenko et al., 2006). Importantly, the PhoRC complex is the species that is bound at specific sites on Polycomb response elements (PREs). PREs are DNA elements in *cis* of Polycomb target genes that regulate their expression (Müller and Bienz, 1991; Simon et al., 1993) (section **1.2.1.3**). Mutation of the Pho binding sites on PREs results in displacement of PhoRC and PRC1 subunits (Klymenko et al., 2006).

Interestingly, amongst the core Polycomb group proteins only Pho and Phol (Pho like) have been found to bind DNA in a sequence specific manner (Brown et al., 1998; Brown et al., 2003). Indeed, both proteins have been shown to act redundantly, and double mutants for both *Pho* and *Phol,* show more severe misexpression of *HOX* genes outside their normal expression pattern than individual mutants (Brown et al., 2003). The mammalian counterparts of Pho and Phol are Ying Yang 1 (YY1) and Ying Yang 2 (YY2) (Brown et al., 2003). However, mammalian PREs have yet not been identified, and given the differential distribution of YY1 and other PcG proteins on chromatin, it seems unlikely that YY1 has the same recruiting function as Pho in *Drosophila.* However, some PRC2 subunits co-localise with YY1 at some target genes (Reviewed in Simon and Kingston, 2013; Squazzo et al., 2006).

1.2.1.3 Targeting of PcG complexes

Recruitment of PcG complexes to target genes is achieved in *Drosophila via* PRE binding. *In vivo* reporter gene assays showed that PREs bear a silencing ability that is dependant on the PcG system (Sengupta et al., 2004). In addition, PREs have been shown to be nucleosome depleted (Kahn et al., 2006; Mohd-Sarip et al., 2006; Mishra et al., 2001). This is consistent with the fact that most PcG proteins have been found to co-localise at PREs and not over target gene bodies (Papp and Müller, 2006). However, as mentioned in section **1.2.1.2d**, only Pho and Phol have been found to stably anchor to specific sequences on PREs, making PhoRC the only complex that directly binds to PREs (Oktaba et al., 2008).

How exactly the non DNA binding PcG complexes are recruited to target genes remains unclear, although an interaction between the PhoRC subunit dSFMBT and the PRC1 member Scm has been described, linking PhoRC to PRC1 targeting (Grimm et al., 2009).

A longstanding model posits that there is a “hierarchical recruitment" of PcG complexes. First, Pho/Phol binding complexes, bound to PREs would recruit E(z) containing complexes to trimethylate H3-K27 and this in turn would facilitate the binding of Polycomb via its chromodomain and thereby recruit PRC1 (Wang et al., 2004a). However several lines of evidence argue against such a model: first, the dissociation constant (Kd) of the interaction between the Polycomb chromodomain and H3-K27me3 is unusually high for a recruitment mechanism (Fischle et al., 2003), second, PhoRC interacts directly with the PRC1 subunit Scm without the need for PRC2 (Grimm et al., 2009), third, PRC1 has been shown to be recruited to a subset of target genes in the absence of H3-K27me3 in mouse ES cells (Tavares et al., 2012), and fourth, three recent independent studies in mouse ES cells and in *Drosophila* embryos demonstrate that H2Aub is sufficient to recruit PRC2 type complexes (Kalb et al., 2014; Blackledge et al., 2014; Cooper et al., 2014).

1.2.1.4 Mechanisms of transcriptional repression by PcG complexes

In order for a gene to be fully transcribed, it must go through a full transcription cycle: first there must be assembly of a pre-initiation complex to allow proper initiation of transcription, followed by elongation and termination. How and at which step do PcG complexes affect this mechanism?

The first clues indicating that PcG proteins could act by directly blocking the transcriptional machinery came from the finding that PRC1 interacts directly with members of the general transcription factor TFIID (Saurin et al., 2001). Further reporter transgene analyses, showed that although RNA polymerase II and Tata binding protein (TBP) occupy Polycomb silenced chromatin, RNA polymerase II cannot initiate transcription, suggesting that transcription initiation is blocked on PcG repressed chromatin (Dellino et al., 2004). One study suggested that loss of Sce/Ring orthologs Ring1A and Ring1B results in the release of RNA polymerase II and therefore transcriptional elongation on Polycomb target genes in mouse ES cells (Stock et al., 2007). Recent global run-on sequencing (GRO-seq) studies also suggest that there is an overall increase of paused RNA polymerase II in mutants for PRC2, indicating that PRC2 somehow inhibits RNA polymerase II pausing on target genes (Chopra et al., 2011). These results are in agreement with PcG complexes obstructing the transcriptional machinery.

Another way PcG complexes could mediate transcriptional repression are chromatin modifications. The discovery that three out of four of the PcG complexes are histone-modifying enzymes, gave rise to the idea that chromatin modifications could be responsible for transcriptional repression of target genes. The main histone post-translational modifications carried out by the PcG system are: H3-K27 methylation, monoubiquitylation of H2A-K118 (K119 in mammals) and deubiquitylation of H2A-K118ub.

High levels of H3-K27me3 decorate nucleosomes on PcG target genes. H3-K27me3 blankets broad domains on target genes, encompassing regulatory sequences and the entire coding regions (Papp and Müller, 2006; Schwartz et al., 2006). In contrast, most PcG proteins are locally bound at PREs, with the exception of Pc, which not surprisingly, mirrors the distribution of H3-K27me3. The fact that most PcG proteins can be visualised at PREs unlike the H3-K27me3 modification lead to the proposal that a looping mechanism takes place, in which the Polycomb subunit binding to H3-K27me3 would facilitate spreading of H3-K27me3 and transcriptional repression. However, it might also be that the PcG system only interacts transiently with the body of target genes and therefore is undetectable by ChIP experiments at gene bodies (Kahn et al., 2006). Many studies have shown that removing the enzymes responsible for H3-K27me3 and H2Aub results in derepression of PcG target genes. However, these analyses remove the enzymes themselves and not their substrate. In order to directly show that the modification itself is responsible for PcG repression it is crucial to also mutate the histone amino acid residue substrate. Before this thesis was started there

was no direct evidence that histone modifications themselves were responsible for transcriptional repression by PcG complexes. In addition how exactly these modifications contribute to the repression of transcription remains unclear. An additional mechanism that is key to PcG repression of chromatin is the chromatin compaction ability of the PRC1 complexes (Francis et al., 2004; Grau et al., 2011).

A likely scenario is that PcG complexes achieve transcriptional repression by a combination of the distinct mechanisms mentioned above.

1.2.2 The Trithorax system

The TrxG of protein complexes counteracts transcriptional repression by PcG proteins. They were first discovered because of their homeotic transformation phenotype, which is exactly the opposite of the PcG phenotype: the more posterior body segments are transformed into more anterior body segments (Ingham, and Whittle, 1980). TrxG proteins can be sorted into two distinct categories: the general facilitators of transcriptional activation and the specific PcG antagonists. The general activators, like for example the TrxG gene *Brahma*, that encodes an ATPase subunit of a SWI/SNF complex, is involved in facilitating general transcription probably through making chromatin more accessible to the transcriptional machinery (Armstrong et al., 2002). The specific PcG antagonists, directly hamper the action of PcG complexes, this family includes the chromatin modifying enzymes:

-Trithorax (Trx) is one of the three histone methyltransferases that specifically trimethylates H3-K4 and ortholog of human Mixed Lineage Leukaemia 1 and 2 (MLL1/MLL2);

-Absent, small or homeotic discs 1 (Ash1) the ortholog of the mammalian ASH1L, that has a histone methyltransferase activity for H3-K36;

-Ubiquitously transcribed tetratricopeptide repeat, X chromosome (Utx), that also has a mammalian ortholog of the same name, a histone demethylase for H3-K27me3 (Copur and Müller, 2013);

-Kismet (kis) the ortholog of the mammalian CHD chromatin remodeller family (reviewed in Schuettengruber et al., 2011).

Interestingly, in PcG/TrxG double mutants, there is still misexpression of PcG target genes, which implies that TrxG proteins are not required for transcriptional activation but rather to counteract PcG repression (Klymenko and Müller, 2004).

1.3 Aim

As discussed in the previous chapter, it is commonly accepted that histone PTMs affect chromatin conformation and the transcriptional status of genes. This has been inferred from the mutant phenotypes of enzymes responsible for the catalysis of the modifications themselves. However direct functional analysis of the histone residues that are subject to PTMs has only been possible in single cell eukaryotes that contain only few histone coding genes. This kind of analysis has not been possible in metazoans due to the complex organisation of their numerous histone-coding genes at distinct genomic loci. Nevertheless, in *Drosophila melanogaster* all the histone genes are at the same genomic cluster, therefore making it a suitable model to genetically address the function of specific histone modifications.

The Polycomb group of multimeric complexes are highly conserved transcriptional repressors that maintain the transcriptionally repressed state of developmental regulator genes. Three out of four of these complexes have histone modifying activities, however the exact function of these modifications remained unclear. Therefore, the aim of this thesis is to directly investigate the function of histone residues that are post translationally modified by PcG complexes by performing histone genetics in *Drosophila melanogaster*.

2 Materials and Methods

2.1 Recombinant DNA techniques

2.1.1 Site directed mutagenesis of "gateway plasmids"

Site directed mutagenesis was carried out using the following PCR reaction mix:

- 5 µl 10x *PfuUltra* High-fidelity DNA polymerase buffer
- 100 ng of DNA
- 1 µl Forward primer at 10 µM concentration
- 1 µl Reverse primer at 10 µM concentration
- 1 µl 10 mM dNTPs
- 1 µl *PfuUltra* High-fidelity DNA polymerase (Agilent Technologies)
- ddH_2O to 50 µl

As template DNA each of the following constructs described in (Günesdogan et al., 2010) were used: *pENTR221-HisGU.WT, pENTRL4R1-HisGU.WT* and *pENTRR2L3-HisGU.WT.*

The PCR cycling conditions were the following:

Cycle step	Temperature	Time	Cycles
Initial denaturation	95ºC	2 min	1
Denaturation	95ºC	1 min	18
Annealing	60ºC	1 min	
Extension	68ºC	16 min	
Final extension	68ºC	18 min	1

Table 3: PCR cycling conditions for site directed mutagenesis

After the PCR reaction, the template construct was digested by adding 20 units (1 µl) of *DpnI* (cuts G^{6m}ATC sequence, hence eliminating the template plasmid and leaving only the PCR product) directly to the PCR mixture and incubating 1 hour at 37ºC. 2-4 µl of the mixture were transformed into chemically competent cells (DH5 alpha from New

England Biolabs [NEB] [NEB 5 alpha]). As a control, to assess the efficiency of the DPNI digestion, 2 to 4 µl of the PCR reaction without primers was also transformed into DH5 alpha.

The following mutagenesis primers were used in this study to mutate the H2A lysines: K117, K118, K121 and K122 into arginines:

Name	Sequence (5' to 3')	Codons changed
H2A-K117R/K118R/K121R/K122R-F	CCCGTCGTACCGAGCGTCGTGCCTAAACGTTTC	AAGAAG into CGTCGT twice (two Lysines into two arginines)
H2A-K117R/K118R/K121R/K122R-R	GAAACGTTTAGGCACGACGCTCGGTACGACGGG	

Table 4: Mutagenesis primers used to generate the *H2A*$^{4K->4R}$ transgenes.

To verify the sequence of the constructs the following primers were designed:

Name	Sequence
HisC-R1	AAATTGAAACGTTCATCCCC
HisC-F1	AATTAATAATAGACGCTTCTTTCAGA
HisC-R3	TTGGAGGAGACACCAATCTTC
HisC-F2	AACGTCCGCTTCCCCAGT
HisC-R4	TTCTCTTGCAACGGAACG
HisC-F3	GCCTTCTTAGCTTTGGGCTT
HisC-R5	GCGTCTCGTCTAGCTCACTACA
HisC-F4	TTACATTGCATGTTGGTCGC
HisC-F5	GATGGCATAGCTCTCCTTCCT
HisC-R6	TCACTTTACACACGGAACACG
HisC-F6	CAGGCGCTCCAGTTTACCTA
HisC-R7	TGTAGGTTTTTAGCTTAGCCTTTGA
HisC-F7	CACCACGATGTGATGCTGTA
HisC-R8	TACCCCTATTAAGCAATCGGTC
HisC-F8	AGGCTTCGTGATACCTTGGA
HisC-R9	TCTCTCGGGTTTTTGTCGTT
HisC-R10	CCGTCTTAAAGTCCTGAGCG
HisC-F9	CAAGAGTGCTCCAGCCACC
HisC-R11	TAAATGTTGAGCTGACAATTAAAA
HisC-F10	TCTATTATTTATGGCGCAAACG

Table 5: Primers used for verification of *H2A*$^{4K->4R}$ transgenes

2.1.2 Gateway cloning of the *pfC31-attB-3xHisGU* rescue constructs

pfC31-attB-3xHisGU.H2A-K117R/K118R and the *pfC31-attB-3xHis-GU.H2A-K117R/K118R/K121R/K122R* constructs were generated by using the Gateway ® LR Clonase ® II Plus enzyme to catalyse the "LR recombination reaction" between

the corresponding pENTRs: *pENTR221-HisGU.WT, pENTRL4R1-HisGU.WT, pENTRR2L3-HisGU.WT* and the *pDESTR3R4-fC31attB* (as in Günesdogan et al., 2010).

The reaction was set up according to the manufacturer's instructions "MultiSite Gateway ® Three Fragment Vector Construction Kit" (Life Technologies). Because of the presence tandem repeats in out final vector, the reaction mixture was transformed into chemically competent OmniMAX cells (Life technologies) with the manufacturer's instructions.

Final *pfC31-attB-3xHisGU* clones were verified by restriction digest with 5 units of *SacII* (that cuts CCGC**I**GG) and *XbaI* (that cuts T**I**CTAGA) from New England Biolabs in NEBuffer4 supplemented with 100 µg/ml Bovine Serum Albumin (BSA) at 37ºC for 1 hour reactions were then ran on a 0.7% agarose TAE (40 mM Tris, 20 mM acetic acid, 1mM EDTA) gel supplemented with SYBR® Safe (Life Technologies). The sequence of clones showing a correct digest (two DNA bands of 15,456 bp and 7,322 bp), was determined by sequencing using the same primers as described for the gateway plasmids . Constructs were then injected into the germ line of *Drosophila melanogaster* by phiC31-mediated site directed transgenesis (Bischof et al., 2007). The specific landing site stocks used are mentioned in (section **2.3.1**, **Table 9**).

2.1.3 Cloning of the *pW35-H2Av* for the generation of the *H2Av^{KO}* deletion allele

Using the oligonucleotides 5'-CGACTCTAGAGGATCCTGAATAGACTGACCTGACGT-3' and 5'-TATCCCTAGGGGATCCACACGTTGGAGGCGACAC-3' as primers for PCR, the *H2Av* downstream region from 3R: 22694919 to 3R: 22699800 was amplified from *W1118* genomic DNA, cloned into the "P element" *pW35* (Gong and Golic, 2003) digested with *BamHI-HF* from NEB, using the 'In-fusion HD Plus EcoDry kit' from Clontech according to manufacturer's instructions leading to the *pW35-H2Avdownstream* vector that was then verified by DNA sequencing. Subsequently the H2Av Upstream region from 3R: 22688946 to 3R: 22692767 was amplified from *W1118* genomic DNA using the oligonucleotides: 5'-TAATGTACCGCGGCCGCCAATACAACCAACGTGTGTAG-3' and 5'-GGGGCATGCGCGGCCGCGAACGTGACTCTGGGCACT-3' and cloned into *pW35-H2AvDownstream* digested with *NotI-HF* from NEB leading to *pW35-H2Av* that was then verified by DNA sequencing and transformed into the germ line of *Drosophila melanogaster* (*W1118*) by standard methods (Rubin and Spradling,

1982).The *pW35-H2Av* P element contains the *white* gene, and the up and downstream regions of H2Av mentioned above, flanked by I-SceI recognition sequences and *FRT*s.

2.1.4 Generation of the *H2Av^{KO}* deletion allele

The ends-out recombination strategy was used to disrupt *H2Av* and replace its entire coding region with a *mini-white* marker gene following the strategy described previously (Gong and Golic, 2003). In brief, transheterozygous embryos on chromosome two for the *pW35-H2Av P element* and for a chromosome bearing the *hs-FLP and the hs-I-SceI* transgenes encoding for the flipase and the I-SceI enzymes respectively, under the heat-shock (*hsp70*) promoter; were heat-shocked one hour at 37ºC three times at 24, 48 and 72 hours post egg laying. When these embryos reached adulthood, they were individually crossed to *H2Av^{810}*/*TM6B* adults as a complementation test. Approximately 300 individual crosses were set up. Several independent targeting events were isolated that failed to complement the lethality of *H2Av^{810}*. One of these alleles, *H2Av^{KO}*, was selected for in-depth analysis by PCR using primers (5‘ to 3’): GACCTTGGAGCGACTGTC and CACCAAACTTCAACTACTG; ACTCGTGCT-GACGACCTGAAC and CACATTGTTCAGATGCTCGG; GCGCAGGTAGAAGTGCATC and CACGGCTGCAGTGGCTC. The entire *H2Av^{KO}* genome region was also verified by sequencing.

To verify the sequence of the *pW35-H2Av* constructs the primers shown in **Table 6:** were used. To verify the correct disruption of the *H2Av* gene, the following primer pairs were used for diagnostic PCR:

Primer pair Q1, for downstream verification:

H2AvKO-F2	GACCTTGGAGCGACTGTC
Downstream-1R	CACCAAACTTCAACTACTG

Primer pair Q2, for upstream verification:

Upstream-1F	ACTCGTGCTGACGACCTGAAC
H2AvKO-R1	CACATTGTTCAGATGCTCGG

Control primer pair for negative verification:

Up-seq-6F	GCGCAGGTAGAAGTGCATC
up-1R	CACGGCTGCAGTGGCTC

Name	Sequence
H2Av-Up-1-F	ACTCGTGCTGACGACCTG
H2Av-Up-2-F	CGTGTGAGATACAGATAATG
Upstream3-F	AGGCGTCCTATATGAGATG
up-seq-1-R	TTCGAGTTCAATATATGCGG
up-seq-1-F	TGAGACAAGCTGCCACAT
up-seq-2-R	AAGAAGAAGAATTGCTGCG
up-seq-2-F	TGCGAGAGAGCCAAGTTG
up-seq-3-R	AATCGTGTCCACCGAATCG
up-seq-3-F	TGACGCCACGGATGCTG
up-seq-4-R	ACACCAGCCGTCACTGTG
up-seq-4-F	CTATCGAATTCAGTAAATGTA
up-seq-5-R	GATTTCATGAAGTATGTCTCA
up-seq-5-F	AGTCGAGATCTCCGTTGTTT
up-seq-6-R	AGAACTACGATGCTGTGGTC
up-seq-6-F	GCGCAGGTAGAAGTGCATC
upstream-3-R	TACGAACGTGACTCTGGG
upstream-2-R	AGCGAGATGCGCTGCC
Upstream1-R	CACGGCTGCAGTGGCTC
Downstream-1-F	GTAATCATTTCTGTGCGCCA
Downstream32-F	TGAATAGACTGACCTGACGT
Downstream-seq-1-R	GTTACTACTTGCTTCCTTAATC
Downstream-seq-1-F	GAATAGCGAGCATTTACACA
Downstream-seq-2-F	GTGCATCTGGCAGATGCG
Downstream-seq-2-R	GAGCGCGAGAATGAGATTG
Downstream-seq-3-R	GATCTGCAGTAGTCATCTT
Downstream-seq-3-F	GAATGTGGCGCGCAGATA
Downstream-seq-4-F	CGTGATGCAGTGTCCAAG
Downstream-Seq-4-R	GATTTGCAAGGTCAACTGTG
Downstream-seq-5-R	TTACCGTTATGCCATTCTAG
Downstream-seq-5-F	CTAGATGAGATCCGTTACTA
Downstream-seq-R-6	CTGTATCGTAGAAGAATGCC
Downstream-seq-6-F	CTACAGCTCCGTGATTTATG
Downstream-seq-7-F	GAGGAGTGTCTACAATTA
Downstream-1-R	CCACCAAACTTCAACTACTG
Downstream-seq-R-7	TCGGCACTGGCTATCAGC
Downatream2-R	GCAGGCACCTAGCTTTCG
Real-up-R	AGACGCCGTCAGCGGAC

Table 6: Primers used to verify sequence of the *pW35-H2Av* constructs

2.1.5 Cloning of the *pH2Av-attB* rescue constructs

The *pH2Av-WT-attB* construct was generated from *pUAST-attB* (Bischof et al., 2007) by replacing a BamHI fragment with the entire *H2Av* genome region and 231 bp of the 5'UTR of *Ball* and 423 bp of the 3'UTR and the second exon of *BM-40-SPARC* (from 3R:22692299 to 3R:22695382) amplified with primers: 5'-TTATGCTAGCG-GATCCCCGCTCGAAATGCCGGGCCATC-3' and 5'-GACGCGAGTTGTTGGTCTCCG-GATCCACTAGTGTCG-3' from the BAC *CH322-97I07* (BACPAC) using the 'In-fusion HD Plus EcoDry kit' from Clontech according to manufacturer's instructions.

The *pH2Av-K120R/K121R-attB* was generated by first constructing the *pBSIIsk(+)-H2Av-WT* by digesting *pBSIIsk(+)* with *SalI* and *SpeI* and cloning in the *H2Av* genome fragment amplified with primers 5'-CCGCTCTAGAACTAGTGGAGAC-CAACAACTCGCGTC-3' and 5'-CCCCCTCGAGGTCGACAATCCACCAGGTACGCCT-GC-3' from the BAC *CH322-97I07* (BACPAC) with the In-fusion HD Plus EcoDry kit'

from Clontech according to manufacturer's instructions. Site directed mutagenesis was carried out as previously described (section **2.1.1**) on *pBSIIsk(+)-H2Av-WT* using oligos: 5'-CAAGTCGCTGATCGGCCGTCGTGAGGAAACGGTGCAG-3' and 5'-CTGCAC-CGTTTCCTCACGACGCCGATCAGCGACTTG-3' as mutagenesis primers containing a two codon substitution: AAA AAG into CGT CGT generating the construct *pBSIIsk(+)-H2Av-K120R/K121R.* Then, the *pH2Av-K120R/K121R-attB* was generated employing the same strategy as for *pH2Av-WT-attB,* except *pBSIIsk(+)-H2Av-K120R/K121R* was used as a template instead of BAC CH322-97I07 (BACPAC).

To verify the sequence of the constructs the following primers were used:

Name	Sequence
pAttB-H2Av-3UTR-F	GACGCGAGTTGTTGGTCTCCGGATCCACTAGTGTCG
pAttB-H2Av-5UTR-4-R	TTATGCTAGCGGATCCCCGCTCGAAATGCCGGGCCATC
H2Av-Primer 1-F	GGAGACCAACAACTCGCGTC
Se-H2Av-1-F	CGTCCGATTGCCGACTGGCT
Se-H2Av-1-R (Do-1-F)	GTAATCATTTCTGTGCGCCA
Se-H2Av-2-F	GTGTGGTGGAATATGCTCGT
Se-H2Av-2-R	ACGAGCATATTCCACCACAC

Table 7: Primers used to verify sequence of the *pH2Av-WT-attB* and the *pH2Av-K120R/K121R-attB* constructs

2.1.6 Cloning of the *pSce-attB* rescue transgenes

The *pSce-WT-attB* construct was generated as *pH2Av-WT-attB*, but the BamHI fragment from *pUAST-attB* was replaced with the entire *Sce* genome region including 1,717 bp upstream of the *Sce* annotated 5'UTR and 223 bp downstream of the annotated 3'UTR (from 3R:23505930 to 3R:23509468) amplified with primers: 5'-TTATGCTA-GCGGATCCTACCTGCTATGCCAGGTACT-3' and 5'-CGACACTAGTGGATCCCA-CATTCTACTAAACCCACAG-3' from *w1118* genomic DNA.

The *pSce-I48A-attB* vector was constructed as *pH2Av-K120R/K121R-attB* by first generating the *pBSIIsk(+)-Sce-WT* in the same way as *pBSIIsk(+)-H2Av-WT,* by amplifying the same genomic region as for *pSce-WT-attB* with primers: 5'-CCGCTCTAGAAC-TAGTTACCTGCTATGCCAGGTACT-3' and 5'-CCCCCTCGAGGTCGACCACATTC-TACTAAACCCACAG-3' from the *Sce-FLAP* vector[1]. Then site directed mutagenesis was performed using *pBSIIsk(+)-Sce-WT* as a template with primers: 5'- GAACTGAT-

1The *Sce-FLAP* vector was cloned by Reinhard Kalb from the Max Planck Institute of Biochemistry, Martinsried, Germany

GTGTCCCGCCTGCCTGGACATGCTG-3' and 5'- CAGCATGTCCAGGCAGGCGG-GACACATCAGTTC -3' replacing ATC with GCC and generating *pBSIIsk(+)-Sce-I48A,* that was then used as a template instead of *w1118* genomic DNA to produce *pSce-I48A-attB*.

To verify the sequence of the constructs the following primers were used :

Name	Sequence
gSce, 527 to 546, F	TCGATCTGTGCGCTCTGCTG
gSce, 1027 to 1044 F	TCACCGCTCGTTCAAACG
gSce, 1482 to 1503 F	GGAAATTGAGGTGTTGACAGAG
gSce, 1962 to 1985 F	CATTTGTTGTTAGGCCTTACTCTG
gSce, 2459 to 2479 F	CAGGAGAAGGTGATGGCTAAG
gSce, 2983 to 3002 F	GGAGTCCGAGTCGGATTCAC
gSce, 3505 to 3529 F	TCTGAAATGGAAATATGTCTAGCTG
gSce, 3633 to 3658 F	CCACAGATCTATATTCCTTCGTCAAC
gSce, 346 to 328, R	TCGGCCAACGTCTATTGAC
gSce, 843 to 822, R	CACACCAAATTGCTGTCTCATG
gSce, 1816 to 1796, R	TTATGTCCACTTGCCATTCAC
gSce, 2323 to 2305, R	GGTCACAATGCAGTCGGAG
gSce, 2808 to 2791, R	TTCGAGGCGCTCGTTATG
gSce, 3320 to 3301, R	GGATAACCAACTGCTGTGGC
gSce, 3803 to 3783, R	CTAAACCCACAGAAGAGCCAC

Table 8: Primers used to verify sequence of *pSce-WT-attB* and *pSce-I48A-attB* constructs

2.2 *Drosophila* DNA techniques

2.2.1 DNA extraction from adult flies

Approximately 15 adult flies per genotype were snap frozen in liquid nitrogen. Flies were homogenized at 4ºC by 40 strokes with a micro-pestle (Eppendorf) in 400µl Buffer A (1M Tris-HCl, pH 7.5; 500mM EDTA, pH 8; 4 M NaCl, 10% SDS). Flies were incubated 30 minutes at 65ºC. 800µl of 5M KAc, 6M LiCl solution was added and mixed

by several inversions. Mixture was incubated on ice for 10 minutes, and then spun 15 minutes at 12,000 rpm at room temperature. 600µl of isopropanol was added to the supernatant and mixed several times by inversion. After spinning at 12,000 rpm for 20 minutes at room temperature, the pellet was washed with 500 µl of cold 70% ethanol. The DNA was then re-spun at 12,000 rpm for 10 minutes. The supernatant was removed and the pellet was air dried for 1 hour. DNA was resuspended in 75 µl of auto-claved, ddH_2O overnight at 4ºC and stored at -20ºC.

2.3 Fly genetics and stocks

2.3.1 Existing fly stocks used for this study

Genotype
w;+/+;+/+ (w1118)
yw; FRT2A E(z)^{731}/TM6C
yw hs-flp122; y+ hs-nGFP FRT2A
yw hs-flp122; FRT40A hs-nGFP
yw; hs-I-SceI Sco/CyO
w; FRT82B cu sr SceΔ/ TM6C
yw hs-flp122; FRT82B hs-nGFP
w; His2Av^{810}/TM3, Sb^{1}
yw hs-flp122; FRT40FRT42 hs-nGFP
yw P{y[+t7.7]=nos-phiC31int.NLS}X; M{3xP3-RFP.attP}ZH-86Fb,
yw P{y[+t7.7]=nos-phiC31int.NLS}X; M{attP}ZH-86Fb,
yw P{y[+t7.7]=nos-phiC31int.NLS}X; M{3xP3-RFP.attP}ZH-68E
yw P{y[+t7.7]=nos-phiC31int.NLS}X; PBac{y[+]-attP-3B}VK00033
yw P{y[+t7.7]=nos-phiC31int.NLS}X , PBac{y[+]-attP-3B}VK00037
yw M{vas-int.Dm}ZH-2A; M{3xP3-RFP.attP}ZH51C

Table 9: Existing fly stocks used in this study

2.3.2 Fly stocks generated during this study*

Table 10: Generated fly stocks

Genotype
w; Df(2L)HisC FRT40A/CyO twi::Gal4 UAS::GFP; 6xHisGU-WT/6xHisGU-WT
w; Df(2L)HisC FRT40A/CyO twi::Gal4 UAS::GFP; 6xHisGU-H3K27R/6xHisGU-H3^{K27R}
w; Df(2L)HisC FRT40A/CyO twi::Gal4 UAS::GFP; 6xHisGU-H2A$^{4K->4R}$/6xHisGU-H2A$^{4K->4R}$
w; Df(2L)HisC FRT40A; 6xHisGU-WT/ SM5^TM6B
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU-WT
yw hs-flp122; y+ FRT40A; 6xHisGU-WT
w; Df(2L)HisC FRT40A; 6xHisGU-H3^{K27R}/ SM5^TM6B
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU-H3^{K27R}
yw hs-flp122; y+ FRT40A; 6xHisGU-H3^{K27R}
w; H3.3A^{0} , Df(2L)BSC104 FRT40A ; 6xHisGU-H3^{K27R}/ SM5^TM6B
yw H3.3B^{0} hs-flp122; hs-nGFP FRT40A; 6xHisGU-H3^{K27R}
w; Df(2L)HisC FRT40A; 6xHisGU-H2A$^{4K->4R}$/ SM5^TM6B
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU-H2A$^{4K->4R}$
w; Df(2L)HisC FRT40A; 6xHisGU-H2A$^{4K->4R}$, His2Av^{KO}/ SM5^TM6B
yw hs-flp122; H2Av^{WT}, hs-nGFP FRT40A, H2Av$^{KK->RR}$; 6xHisGU-H2A$^{4K->4R}$, His2Av^{KO}/ SM5^TM6B
w; FRT82B His2Av^{KO}/ TM6B
w; His2Av^{WT}; FRT82B His2Av^{KO}
w; His2Av$^{KK->RR}$; FRT82B His2Av^{KO}/ TM6B
yw hs-flp122; y+ FRT40A
(y)w; UAS-flp,Sce^{KO}/TM6C
w; SceFLAP; nos-gal4-VP16, Sce^{KO}
(y)w; Sce^{I48A}; UAS-flp, Sce^{KO}/TM6B
w; Sce^{I48A}, SceFLAP/CyO; nos-gal4-VP16, Sce^{KO}
w; Sce^{WT}; FRT82B Sce^{KO}/TM6B
w; Sce^{I48A}; FRT82B Sce^{KO}/TM6B
yw hs-flp122; FRT40FRT42 hs-nGFP
w; FRT40FRT42 Caly^{2} , Sce^{I48A} /CyO; FRT82B Sce^{KO}/ TM6B
yw hs-flp122; FRT40FRT42 hs-nGFP ; FRT82B Sce^{KO}/SM5^TM6B
*w; Df(2L)HisC FRT40A; 6xHisGU$^{\Delta H2A}$ */ SM5^TM6B*
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU$^{\Delta H2A}$
*w; Df(2L)HisC FRT40A; 6xHisGU$^{\Delta H2B}$ */ SM5^TM6B*
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU$^{\Delta H2B}$
*w; Df(2L)HisC FRT40A; 6xHisGU$^{\Delta H3}$ */ SM5^TM6B*
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU$^{\Delta H3}$
*w; Df(2L)HisC FRT40A; 6xHisGU$^{\Delta H4}$ */ SM5^TM6B*
yw hs-flp122; hs-nGFP FRT40A; 6xHisGU$^{\Delta H4}$

* The transgenes lacking the individual core histones, marked with an asterisk, were constructed by Ufuk Günesdogan, Christian Reiter, Herbert Jäckle and Alf Herzig in the Department of Molecular Developmental Biology at the Max Planck Institute of Biophysical Chemistry, Göttingen, Germany.

2.4 *Drosophila* microscopy methods

2.4.1 Fluorescent Immnunostaining of imaginal discs

15-30 third instar larvae were selected and then dissected by splitting them in half, turning the interior inside out, and the gut, salivary glands and fat bodies were removed leaving the larval cuticles with attached imaginal discs and brains. Cuticles were fixed with 1ml of 4 % Formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 20 minutes in agitation. At least 6 washes were performed with BBT (1% BSA, 0.1% Triton X-100 in PBS) within 30 minutes, in agitation. Cuticles were incubated in 100 µl of primary antibody diluted in BBT, over night at 4ºC in mild agitation. Cuticles were washed at least 6 times in BBT within 1 hour in agitation and then incubated with 100 µl of fluorescently labelled secondary antibody and Hoechst 33342, at a 50 ng/ml concentration, diluted in BBT and over night at 4ºC (or for at least 3 hours in agitation at room temperature). Two washes with BBT and four with PBT (0.1% Tween 20 in PBS) were performed during the morning, in agitation under a black cover to protect from light and imaginal discs were transferred to a glass well and dissected in PBT. The discs were then transferred in PBT to a microscopy slide and most of the liquid was removed and mounting was performed by adding 15µl of Fluoromount-G (Southern Biotech) and placing a coverslip on top. Samples were kept at 4ºC.

Samples were then visualised and images were acquired using one of the following confocal laser scanning microscopes:

- Leica TCS AOBS SP2
- Leica TCS AOBS SP8
- Zeiss LSM 780

In all cases an average of four acquisitions of the same focal plane were taken.

2.4.2 Immunohistochemical analysis of embryos

Appropriate fly strains were crossed and allowed to lay eggs on apple agar plates in a small cage for three days. Then embryos were collected and dechorionated with 100% sodium hypochlorite for two minutes and washed extensively with ddH_2O. Fixing was performed in 15 ml glass vials under gentle agitation for 20 minutes in 2.03 ml fixing solution (1800 µl PEM [100 mM Pipes pH 7, 0, 2 mM $MgSO_4$, 1mM EGTA pH 8.0] and 230 µl formaldehyde 35 %) and 6 ml heptane. The fixing solution was carefully

removed, and then most of the heptane was removed. Remnants of formaldehyde were removed by washing two times with heptane. Devitellinisation was carried out in 3 ml heptane plus 6 ml methanol by vigorously shaking the suspension. Devitellinised embryos would sink to the bottom into the methanol phase. Heptane was removed and embryos were washed four times with methanol. The methanol was removed and embryos were washed four times 5 minutes each with BBT. Blocking was carried out by washing the embryos in BBT at least five times, for at least one hour. The embryos were incubated in primary antibody diluted in BBT overnight at 4ºC. At least 8 washes were performed for at least 1 hour with BBT and embryos were then incubated in appropriate secondary biotin-conjugated antibody diluted in BBT overnight at 4ºC (or at least three hours at room temperature). Four washes with BBT and eight washes with PBT were carried out and in the mean time, a mixture of 10 µl reagent A, 10 µl reagent B (reagents A and B from Vectastain "Elite" ABC kit, *Vector Laboratories, Inc.*) and 980 µl PBT was prepared and incubated under mild agitation for 30 minutes (during this incubation each avidin molecule should bind to on average three biotin-Horseradish Peroxidase H molecules, leaving one binding site free for the biotinylated secondary antibody). The embryos were incubated in this mixture for 30 minutes under mild agitation. At least eight PBT washes were performed in agitation during at least 30 minutes. Embryos were then transferred into a glass well and immersed in 1 ml PBT with 12 µl 1%$CoCl_2$/$NiCl_2$ and 25 µl 20 mg/ml 3,3'-diaminobenzidine (DAB) (Note: DAB is carcinogenic, therefore care was taken not to contaminate working area, surfaces were covered with bench-coat and DAB-containing waste was inactivated by oxidation in sodium-hypochlorite overnight). Most of the liquid was removed and substituted by 1 ml PBT with 12 µl 1% $CoCl_2$, $NiCl_2$, 25 µl DAB and 2 µl 3 % H_2O_2. Staining reaction was observed under the binocular while gently stirring. When reaction got close to the optimal intensity, several quick washes with PBT were performed. To completely stop the reaction, embryos were transferred into an Eppendorf tube and washed sequentially with 30 %, 50%, 70 % and 100 % ethanol (the last wash in 100% ethanol was repeated 3 times to remove any remaining water). Embryos were then immersed in a mix of ethanol: methyl salicylate (1:1) and subsequently in 100% methyl salicylate. At this point, the preparation could be stored at 4°C. Mounting was carried out by transferring selected embryos onto a microscope slide containing a drop of Durcupan™ (44610 FLUKA). On both sides of the embryos, coverslips (thickness no. 1.5) were placed as spacers, and a larger coverslip was put over the embedded embryos and the spacer-coverslips. Slides were stored at -20°C to maintain the viscosity of Durcupan for adjusting the embryo's position for imaging purposes. Samples were visualised using a Zeiss

Axio Scope.A1 microscope using the Nomarsky settings and pictures were taken using the Zeiss AxioCam MRm.

2.4.3 Microscopic analysis of embryonic cuticles

Appropriate fly strains were crossed and allowed to lay eggs on apple-agar plates in a small cage for three days. Embryos sorted using a green fluorescent protein (GFP) marker were collected 24-28 hours after laying and dechorionated with 100% sodium hypochlorite for 2 minutes. Extensive washes with ddH_2O were carried out. Batches of four to six embryos or non-hatched larvae were placed on a dried drop of "Scotch tape Magic"-heptane solution (1 roll of "Scotch tape magic" dissolved in 100 ml of heptane) on an empty petri dish and a drop of water was placed on top. Very carefully, using a fine capillary or a blunt end needle the vitelline membrane was removed. The embryonic cuticles were transferred to a drop of Hoyer's medium (15 g of gum Arabic in 25 mL of ddH_2O, heated to 60°C, and stirred overnight, to which 100 g of chloral hydrate was successively added) with lactic acid (1:1) to digest away the fat, on a microscopy slide and covered with a coverslip. The slides were incubated at 65ºC for 30 minutes and then a weight was added on top of the cover slip and left over night at 65ºC for the cuticles to be flat. Samples were visualised using a Zeiss Axio Scope.A1 microscope using the Dark Field setting and pictures were taken using the Zeiss AxioCam MRm.

2.4.4 Microscopic analysis of adult cuticles

Selected adult flies were put on ice for a few minutes to anaesthetise them. They were then transferred to a piece of Parafilm "M" on a CO_2 block and the appropriate fragments of the thoracic or abdominal segments were dissected. The adult portions were then transferred to a drop of Hoyer's medium with lactic acid (1:1) on a microscopy slide and covered with a coverslip. The slides were incubated at 65ºC for 30 minutes and then a weight was added on top of the cover slip and left over night at 65ºC. Samples were visualised using a Zeiss Axio Scope.A1 microscope using the Bright Field Nomarsky settings and pictures were taken using the Zeiss AxioCam MRm.

2.5 Biochemical methods

2.5.1 Soluble nuclear extracts from *Drosophila* embryos

Appropriate fly strains were crossed and allowed to lay eggs on apple-agar plates in a medium sized cage for three days. The plate was changed and the adults were let

to lay eggs over 12 hours. Typically each nuclear extract was made from six consecutive 12 hour collections that were kept at 4ºC. After collection, embryos were dechorionated with undiluted bleach for 2 minutes and washed extensively with ddH_2O. Embryos were directly placed in a 25 mL glass dounce containing 4 mL of buffer NU1 (15 mM Hepes pH8, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA pH8, 0.5 mM EGTA pH8, 350 mM sucrose, 1 mM DTT, 1x Halt phosphatase inhibitor cocktail (Thermo Scientific), 1x Complete protease inhibitor cocktail (Roche), 1 mM AEBSF (Sigma-Aldrich)**,** kept on ice for 10 minutes**,** to avoid an osmotic shock, and homogenized by 30 strokes with an electric homogeniser at 4ºC. Nuclei were filtered through two layers of Miracloth (Merk Millipore 475855) and pelleted at 4,000 rpm for 10 minutes at 4ºC. Nuclei were washed in 800 µL of low-salt buffer (15 mM Hepes pH 8, 20 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA pH 8, 20% glycerol, 1 mM DTT, 1x Halt phosphatase inhibitor cocktail, 1x Complete protease inhibitor cocktail, 1 mM AEBSF) and lysed by 40 manual strokes with a loose plastic pestle in equal volumes of low salt buffer and high salt buffer (15 mM Hepes pH 8, 800 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8, 20% glycerol, 1 mM DTT, 1x Halt phosphatase inhibitor cocktail, 1x Complete protease inhibitor cocktail, 1 mM AEBSF). Further lysis was carried out by rotating samples 20 minutes at 4ºC. Lysed nuclei were spun using a Beckman Optima MAX ultracentrifuge, rotor TLS-55, 55'000 rpm for one hour at 4ºC maximum acceleration, maximum deceleration. The supernatant was dialysed against 200 ml of NE buffer (15 mM Hepes pH 8, 200 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8, 5% glycerol, 0.5 mM DTT) with a MWCO = 6-8 kDa, for 1.5 hours at 4ºC in agitation. Debris was pelleted at maximum speed for 5 minutes on a table top centrifuge at 4ºC. In parallel, the chromatin pellet from the ultracentrifugation was resuspended in 8M Urea buffer 0.05 mM DTT, sonicated in a Bioruptor (Diagenode) (16 cycles: 30 sec ON, 30 sec OFF) and collected after centrifugation at maximum speed for 5 minutes on a table top centrifuge. For loading onto an SDS-PAGE gel for analysis, LDS-400 mM DTT sample buffer was added to a 1x concentration and dilutions were made using 1x sample buffer, and heated to 70ºC for 10 minutes before loading.

2.5.2 Histone acid extraction

The brains, and the wing, haltere and third leg imaginal discs of 50 larvae were dissected and kept on ice in PBS and then transferred to ice cold PBS and 0.1% NP40. Discs and brains were quickly spun down and resuspended in 80µl of buffer N (15mM

Tris pH 8, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 3 MM EDTA pH 7.9, 0.1 mM EGTA pH 7.9, 0.5 mM spermidine, 0.2% NP-40, 10 mM NaF, 350 mM sucrose, 1 mM DTT, 1 mM AEBSF, 1x complete protein inhibitors). The mix was then transferred to a 100µl glass homogeniser and mixed by 30-50 strokes on ice. The dounce was washed twice with 100 µl of buffer N. The nuclei were then pelleted (4,000 rpm for 5 minutes at 4ºC) and washed with 200 µl of buffer N. The nuclei were then resuspended in 3 volumes of extraction buffer (0.4 M HCl, 10% glycerol) and incubated on ice for 30 minutes. The insoluble material was pelleted (13,000 rpm for 3 minutes at 4ºC) and the supernatant was transferred to a fresh tube and 10 volumes of cold acetone were added and incubated at -20ºC over night to precipitate histones. The precipitated histones were pelleted (7 minutes at 13,000 rpm at 4ºC) and resuspended in 50 µl of unfolding buffer (8 M urea, 20 mM Tris pH 8, 10 mM DTT). Then glycerol was added to 25% final concentration, and the histones were snap frozen and kept at -80ºC. For loading onto an SDS-PAGE gel for analysis, 4xLDS- 400 mM DTT sample buffer was added to a 1x concentration and dilutions were made using 1x sample buffer and heated to 70ºC for 10 minutes before loading.

2.5.3 SDS-Polyacrylamide gel electrophoresis of Proteins (SDS-PAGE)

Soluble nuclear extracts were ran either on denaturing precast NuPAGE Novex 4-12% Bis-Tris gradient gels (from Invitrogen), or on self-casted 16% polyacrylamide SDS gels (in the case of histone and chromatin extracts) or 8% polyacrylamide SDS gels (in the case of soluble nuclear extracts).

2.5.4 Western blotting

A nitro-cellulose Amersham Hybond ECL membrane (the pore size of the membrane was 0.22 µm for histones, otherwise, 0.44 µm) of the appropriate size was first equilibrated in 1X transfer buffer (20% methanol, 25 mM Tris, 192 mM Glycine, 0.05% SDS) and after preparation of a “sandwich” containing respectively, 1 thick sponge, 3 Whatman papers the PAGE gel, the membrane, 3 Whatman papers and 1 thick sponge. The transfer tank was filled with 1 X transfer buffer and the sandwich was placed in the appropriate direction so that the gel is on the side of the cathode and the membrane on the side of the anode towards which the reduced proteins migrate. Proteins were then transferred at 4ºC under mild agitation for 10 minutes at 90 V and 30 minutes at 60 V for histones and otherwise: 90 minutes at 90 V. The membrane was

stained with Ponceau S, rinsed with ddH_2O and blocked with either 5% milk TBS (PBS, 0.2% Tween 20) for 1 hour at room temperature. The membrane was incubated with the appropriate primary antibody diluted in either 4% BSA TBS or 5% milk TBS, over night at 4ºC. Then the membrane was washed 5 times for 5 minutes with TBS and incubated with the appropriate HRP labelled secondary antibody for 1 hour at room temperature under mild agitation. The membrane was washed 7 times with TBS and "developed" using a one to one mixture of detection reagent A and B (ECL plus, prime or Select, Amersham).

2.6 Antibodies used in this study

"antigen"	Host Species	Source	Concentration	
			WB	IF OR IC
PcG proteins				
Pc	Rabbit	Papp and Müller, 2006	1 in 25,000	1 in 100
Sce	Rabbit	Sdix	1 in 5,000	-
Calypso	Rabbit	Scheuermann et al., 2010	-	1 in 300
Psc	Mouse	DSHB (6E8)	1 in 700	-
Scm	Rabbit	A. Gaytan and N. Ly-Hartig	1 in 3,000	-
Ph	Rabbit	Gambetta et al., 2009	1 in 10,000	-
OGT	Rabbit	H-300, Santa Cruz Biotechnology	1 in 5,000	-
Pho	Rabbit	Papp and Müller, 2006	1 in 3,000	-
Nurf55	Rabbit	gift from A. Nowak	1 in 15,000	-
E(z)	Rabbit	M. Nekrasov	1 in 5,000	-
PcG target gene products				
Abd-B	Mouse	DSHB (1A2E9)	-	1 in 300
Ubx	Mouse	DSHB (FP3.38)	-	1 in 30
Scr	Mouse	DSHB (6H4.1)	-	1 in 200
En	Mouse	DSHB (4D9)	-	1 in 4
Salm	Rabbit	gift from R. Schuh	-	1 in 50
Prd	Rabbit	gift from M. Noll	-	1 in 1000
Elb	Rat	gift from S. Cohen	-	1 in 200
Wg	Mouse	gift from S. Cohen	-	1 in 15
Dll	Mouse	gift from S. Cohen	-	1 in10

Table 11 (i): Antibodies used in this study

"antigen"	Host Species	Source	Concentration	
			WB	IF OR IC
Histones or Histone PTMs				
H1	Rabbit	T. Kadonaga	-	1 in 100
H2A-K119ubi1	Rabbit	Cell Signalling (D27C4)	1 in 2000	-
H2AvD	Rabbit	R. Glaser	1 in 5000	1 in 1000
H3	Rabbit	Abcam (1791)	1 in 5000	-
H3	Mouse	Active Motif (MABI 0301)	-	1 in 500
H3-K27Ac	Rabbit	Abcam (4729)	-	1 in 500
H3-K27me3	Rabbit	T. Jenuwein		1 in 250
Secondary antibodies				
anti-mouse IgG Cy3	Goat	Jackson immuno-research	-	1 in 500
anti-rabbit IgG Cy3	Goat	Jackson immuno-research	-	1 in 500
anti-rat IgG Cy3	Goat	Jackson immuno-research	-	1 in 500
anti-mouse IgG Cy5	Goat	Jackson immuno-research	-	1 in 500
anti-rabbit IgG Cy5	Goat	Jackson immuno-research	-	1 in 500
anti-rabbit HRP	Donkey	Amersham Biosciences (NA934)	1 in 5000	-
anti-mouse HRP	Sheep	Amersham Biosciences (NA931V)	1 in 5000	-
anti-mouse Biotin	Horse	Vector laboratories (BA 2000)	-	1 in 100
anti-rabbit Biotin	Horse	Vector laboratories (BA 1100)	-	1 in 100

Table 11 (ii): Antibodies used in this study (continued)

3 Results

3.1 A genetic system to analyse the function of histone post-translational modifications

In the following section I will introduce the system employed to generate cells mutant for the histone residues modified by the PcG of transcriptional repressors: H3-K27 and the residues of the H2A C-terminal tail.

This genetic system is based on the strategy established by (Günesdogan et al., 2010) (**Figure 5**). In this setup, transgenes encoding twelve copies of the wild-type histone gene units (*HisGUs*), rescue animals that are homozygous for a deletion that removes the histone cluster (Δ*HisC)* with the endogenous 23 copies of the *HisGUs* (**Figure 2**). These rescued animals develop into viable adults (Günesdogan et al., 2010). However, the rescued females are semi-sterile and these animals cannot be maintained as a stock.

Animals homozygous for the histone cluster deletion, produced by heterozygous parents, complete cell cycle 14 rescued by the maternal contribution in histone mRNA and protein, but are unable to complete cell cycle 15. Since there are few cell cycles between this stage and the end of embryogenesis, wild-type, maternally contributed histones most likely persist until the end of embryogenesis also in embryos that are rescued by transgene-encoded histones. Therefore, I decided to focus on imaginal disc clones as a model system. Imaginal discs are sets of small primordia that are set aside at the end of embryogenesis, and rapidly proliferate during the larval stages to become mature tissues, for example the mature wing imaginal disc has approximately 30,000 to 50,000 cells (Martin et al., 2009; Garcia-Bellido et al., 1971). I induced the clones at the first instar larval stage (24 to 48 hours after egg laying), and analysed the phenotype of the clones at the third instar larval stage (96 hours after egg laying) unless stated otherwise. This allowed the clones to go through enough rounds of cell divisions to ensure the dilution of persisting wild-type, maternally contributed histones (**Figure 5**).

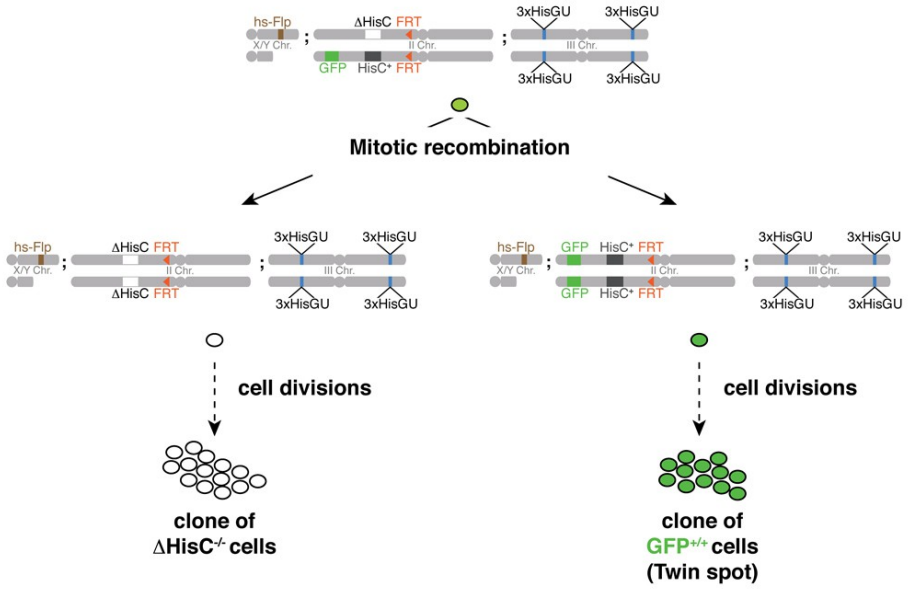

Figure 5. Experimental strategy for generating histone mutant clones in imaginal discs and adults

Top: Genotype of animals in which mutant clones were analysed. Chromosomes *X/Y*, *II* and *III* are depicted with the following genetic elements. *HisC*$^{+}$ (black box): wild-type allele of the histone gene cluster; Δ*HisC*: deletion allele lacking the entire histone gene cluster; *GFP*: *hsp70-nGFP* transgene; *hs-Flp: hsp70-Flp* transgene expressing *Flp* recombinase under control of the *hsp70,* heat-shock promoter to induce recombination at *FRT* elements (orange triangles); *3xHisGU*: cassette containing 3 histone gene units, inserted at two specific integration sites on chromosome arm *3L* and *3R* to generate a total of *12xHisGU*, as described (Günesdogan et al., 2010). Below: cell of the above genotype (green oval). After Flp-mediated recombination, cells with the indicated chromosomes are generated; a Δ*HisC* homozygous cell, that lacks the *GFP* marker gene (empty oval) that will proliferate into a clone, and the reciprocal cell that carries two copies of the GFP marker gene (dark green oval), that will proliferate into a clone of *GFP*$^{+/+}$ cells, also known as twin spot. For generation of *yellow* marked clones in adults (**Figs**. 10 and 13), a *yellow+* (*y+*) marker gene was used instead of *GFP* and clones were induced in a *y* mutant background.(Adapted from Pengelly et al., 2013)

In this setup, clones lacking histone rescue transgenes do not proliferate (**Figure 6**) and clones containing twelve copies of the wild-type histone gene units (12xHisGU) (**Figure 2**) proliferate normally and their size is identical to their corresponding, wild-type twin spots (**Figure 6**).

Given that *Drosophila* has histone variants that share a high degree of similarity with the canonical histones (section **1.1.1.2**), I first tested whether these histone variants could take over the function of canonical core histones. To achieve this, I generated clones lacking each individual core histone, by rescuing Δ*HisC* homozygous clones with twelve copies of histone transgenes lacking each core histone: *12xHisGU$^{\Delta H2A}$,12xHisGU$^{\Delta H2B}$, 12xHisGU$^{\Delta H3}$ or 12xHisGU$^{\Delta H4}$.* Strikingly, in all cases, clones marked by the absence of GFP did not proliferate (**Figure 6**). This indicates that each individual core histone is essential for proliferation in this system, and that histone variants cannot take over the proliferative function of each canonical core histone. This result is consistent with canonical histone production, being tightly coupled to DNA replication and therefore proliferation (section **1.1.1.1d**).

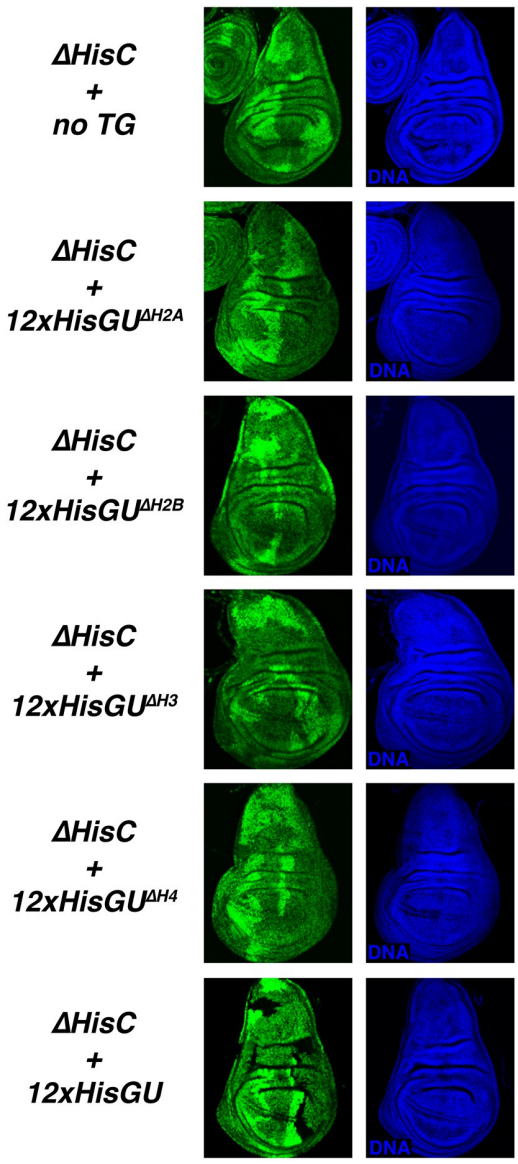

Figure 6. Histone variants cannot take over the function of canonical histones
Wing imaginal discs with clones of Δ*HisC* homozygous cells, with the indicated rescue transgenes. Δ*HisC* homozygous cells are marked by the absence of GFP (**Figure 5**) and clones were induced 96 hours prior to analysis in all cases. The blue channel represents DNA staining of each corresponding wing disc. In the case of Δ*HisC* homozygous cells in animals without transgene, or with transgenes lacking the individual core histones, non GFP clones were not detectable, but twin spots of GFP, marked by bright green, were present, confirming that the mitotic recombination reaction took place.

3.2 Functional analysis of the histone residue modified by PRC2 complexes

PRC2 type complexes mono-, di and trimethylate lysine 27 on histone H3. The methyltransferase activity of PRC2 is critically required for Polycomb repression and the upstream, promoter and coding region of Polycomb target genes display high levels of the H3-K27 trimethyl mark in their transcriptionally repressed state (Papp and Müller, 2006; Schwartz et al., 2006; Modencode project).

However, PRC2 has also been described to methylate Gata4 in murine foetal hearts and histone H1b in human cell lines (He et al., 2012; Kuzmichev et al., 2004). I therefore set up to directly investigate the role of histone H3-K27 methylation by PRC2 type complexes in Polycomb repression in *Drosophila*, using the histone rescue system described previously (section **3.1**; **Figure 5**; and Günesdogan et al., 2010).

3.2.1 H3-K27 methylation is required for Polycomb repression

3.2.1.1 *H3^{K27R}* mutant embryos show misexpression of *Abd-B* similarly to *E(z)* mutant embryos

Of note, animals heterozygous for the Histone cluster deficiency (Δ*HisC)* and containing twelve copies of *HisGU-H3^{K27R}* transgenes were viable and fertile and presented no visible phenotype. For simplicity, Δ*HisC* homozygous embryos or clones containing *12xHisGU-H3^{K27R}* will be referred to as *H3^{K27}* embryos or clones.

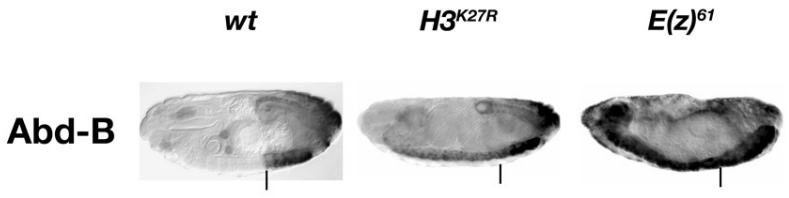

Figure 7. *H3^{K27R}* embryos show misexpression of the *HOX* gene *Abd-B*
Side views of stage 16 embryos, *wild-type* (*wt*) (left), homozygous for Δ*HisC* carrying twelve copies of the *HisGU-H3^{K27R}* transgene (*H3^{K27R}*) (middle), or homozygous for the *E(z)^{61}* allele (right) stained with an antibody against the Abd-B protein. The vertical line marks the anterior margin (Parasegment 10) of the wild-type *Abd-B* expression domain. *E(z)^{61}* homozygotes show widespread misexpression of *Abd-B* in the most anterior parasegments outside its normal expression domain. *H3^{K27R}* embryos show less widespread misexpression of *Abd-B* in the anterior parasegments.

I first directly examined the phenotype of embryos homozygous for a deletion of the endogenous histone gene cluster (Δ*HisC*), rescued by twelve histone gene units in which lysine 27 on H3 was mutated into an arginine (*12xHisGU-H3^{K27R}*). These embryos survive until the end of embryogenesis, but strikingly, they show a slight misexpression of the *HOX* gene *Abd-B* outside its normal expression pattern. In comparison, embryos homozygous for the temperature sensitive allele *E(z)^{61}*, reared at the restrictive temperature, and therefore lacking H3-K27 methylation (Ketel et al., 2005; Cao et al., 2002) presented widespread misexpression of *Abd-B* (**Figure 7**). A likely explanation for this striking difference might be that the *H3^{K27R}* embryos, come from heterozygous mothers for Δ*HisC* and therefore still contain a mixture of both maternally supplied wild-type histone H3 proteins and zygotic mutant H3-K27R proteins. For the reasons discussed above, it is possible that the wild-type maternally supplied histone proteins would persist until this embryonic stage and would interfere with the proper phenotype of the histone mutants at the embryonic stage.

3.2.1.2 *H3^{K27R}* imaginal disc clones show misexpression of Polycomb target genes

Since wing imaginal discs, are small primordia set aside at the end of embryogenesis and undergo multiple cell divisions during larval development, I thought that this would be the ideal system to perform the study of histone point mutants. I therefore generated clones of cells homozygous for the Δ*HisC* allele and carrying the *12xHisGU-H3^{K27R}* transgenes in imaginal wing discs of animals heterozygous for the Δ*HisC* allele. I induced the clones by mitotic recombination during the first instar larval stage and performed the analysis 96 hours after at the 3rd instar larval stage, ensuring that enough cell divisions had taken place to fully dilute the wild-type histones from the original heterozygous cell where the mitotic recombination took place (**Figure 5**).

Cells homozygous for the Δ*HisC* allele without the rescue cassette stop proliferating, as shown in (section **3.1**). However, clones of cells homozygous for the Δ*HisC* allele containing twelve copies of the wild-type cassette (*12xHisGU*) or *12xHisGU-H3^{K27R}*, proliferate normally (**Figure 8**). Furthermore H3-K27me3 signal is undetectable in *H3^{K27R}* clones, which suggests that wild-type H3 is diluted out from these cells (**Figure 8**). This confirms that imaginal disc clones are indeed a suitable system to study the phenotype of histone residue mutant cells.

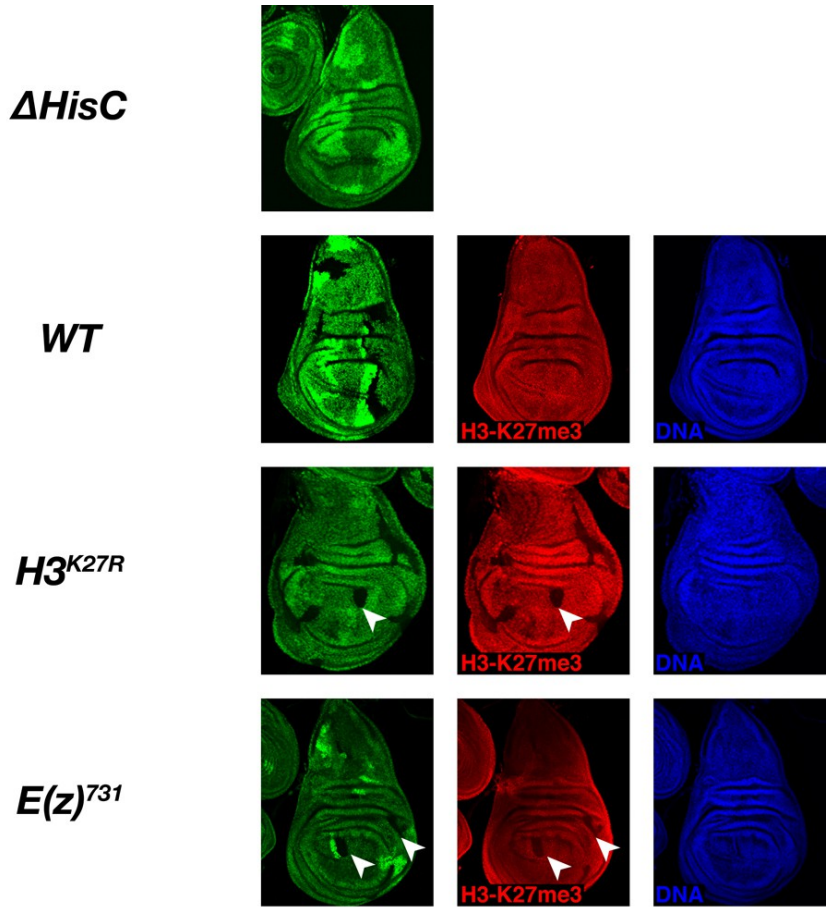

Figure 8. *H3^{K27R}* cells proliferate and lack H3-K27 methylation
Wing imaginal discs with clones of Δ*HisC* homozygous cells in animals without transgenes *(ΔHisC)* or with *12×His-GU* transgenes *(WT), or 12xHisGU-H3^{K27R}* transgenes *(H3^{K27R})*, homozygous for *E(z)^{731} (E(z)^{731}),* a null allele of *E(z)*, stained with H3-K27me3 antibody and Hoechst (DNA). Δ*HisC* and *E(z)^{731}* homozygous cells are marked by the absence of GFP (**Figure 5**) and clones were induced 96 hours before analysis in all cases. Note the reduction of H3-K27me3 signal in *E(z)^{731}* and *H3^{K27R}* homozygous cells (full arrowheads).

I next asked if repression of PRC2 target genes was affected in *H3^{K27R}* clones. Strikingly, in the same way as cells lacking the PRC2 catalytic subunit E(z), *H3^{K27R}* cells show misexpression of *Abd-B*, *Ubx, en* and *Scr* outside their normal expression patterns (**Figure 9**). There is a striking identity between the misexpression levels and the location of the misexpressing clones of *E(z)^{731}* (a null allele of *E(z);* Müller et al., 2002*)* and *H3^{K27R}* clones (**Figure 9**). In the case of *Abd-B* and *Ubx*, there is a strong misexpression in all clones except in the case of the clones in the wing notum, that do not show misexpression of *Ubx* both in *E(z)^{731}* and *H3^{K27R}* clones. In contrast, *en* and *Scr* are

misexpressed at low levels in clones of both genotypes.

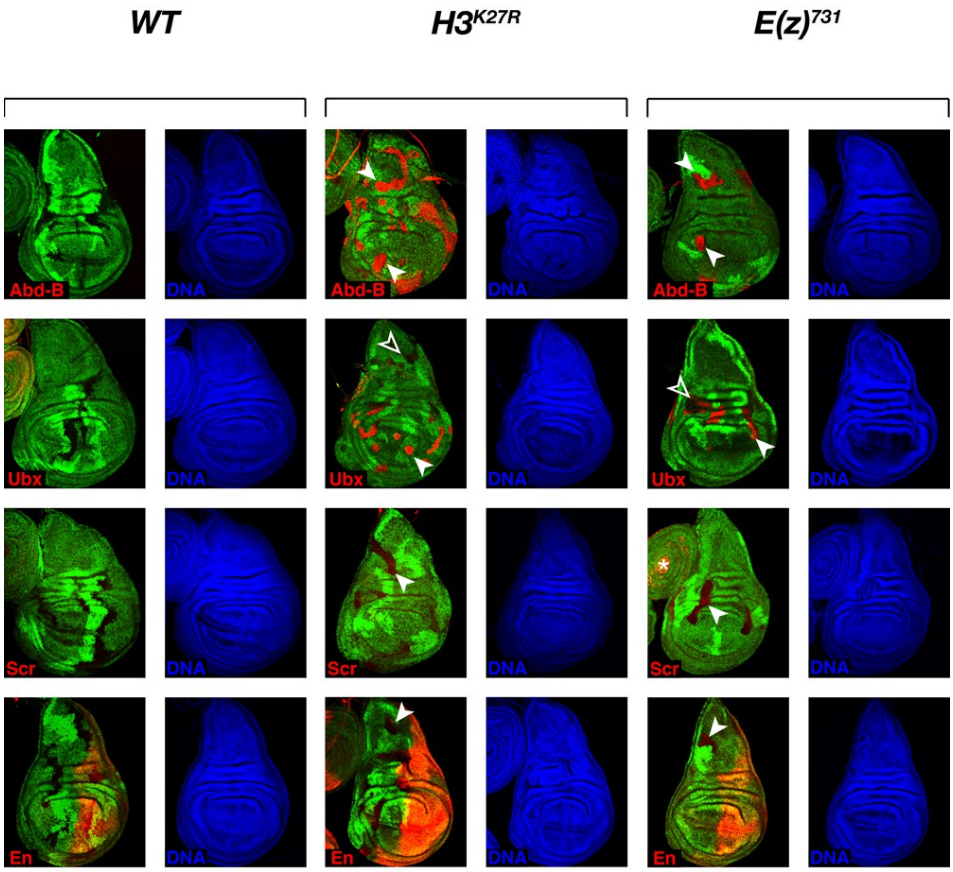

Figure 9. *H3*^{K27R} cells show misexpression of Polycomb target genes like *PRC2* mutant cells

Wing imaginal discs with clones of Δ*HisC* homozygous cells in animals carrying *12×His-GU* (first column, *WT*) or *12xHisGU-H3*^{K27R} transgenes (second column, *H3*^{K27R}), or homozygous for the *E(z)*^{731} null allele (third column, *E(z)*^{731}) stained with antibodies against the HOX proteins Abd-B (first row), Ubx (second row), Scr (third row), and the segmentation protein en (fourth row). Homozygous mutant clones are marked as in **Figure 6**. The blue channel shows the DNA staining. *Abd-B* and *Ubx* are misexpressed at high levels and *Scr* and *en* at low levels, in both *H3*^{K27R} cells and *E(z)*^{731} cells (full arrowheads). In the case of Ubx, clones present in the notum do not show misexpression (empty arrowheads). Asterisk marks Scr staining in the peripodial membrane cells of the third leg imaginal disc.

3.2.1.3 *H3^{K27R}* cells differentiate into adult structures and show homeotic transformations

Next I asked if *H3^{K27R}* cells could differentiate into adult structures. To answer this question, I generated clones in the larval histoblasts using the *yellow* gene as a genetic marker, and examined if these histoblats differentiated into adult bristles. Both *E(z)^{731}* and *H3^{K27R}* cells differentiate into epidermal adult structures (**Figure 10**). In addition, in both cases, the mutant bristles present in the anterior abdominal compartments (A2 for example), resemble the bristles that are normally only present in the most posterior abdominal compartment, the anal plate. This suggests that both *E(z)^{731}* and *H3^{K27R}* show homeotic transformations, which is consistent with the misexpression of *HOX* genes in imaginal disc clones. Remarkably, the differentiated bristles which are formed by both *E(z)^{731}* and *H3^{K27R}* mutant cells show no other obvious patterning defects.

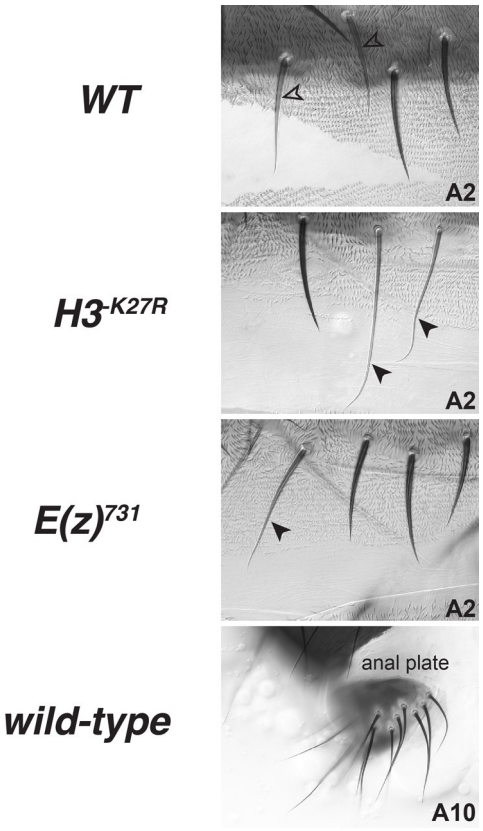

Figure 10. *H3^{K27R}* differentiated adult epidermal structures show homeotic transformations

Top three panels show abdominal segment A2 fragments of adult flies with clones of Δ*HisC* homozygous cells in *12×His-GU (WT*) or *HisGU-H3^{K27R} (H3^{K27R}*) transgenics or with clones of *E(z)^{731}* homozygous cells (*E(z)^{731}*); in all cases, mutant cells are genetically marked with a yellow mutation (light pigmentation). *H3^{K27R}* mutant clones show homeotic transformations into more posterior body segments; the mutant sensory bristles (arrowheads) are finer and more tapered than the neighbouring wild-type bristles in A2 and resemble characteristic abdominal segment A10 bristles in wild-type animals (bottom row). In the case of *E(z)^{731}*, mutant bristles (arrow-heads) are less extensively transformed, which may reflect the perdurance of E(z) protein in the mutant cells. Δ*HisC, 12×His-GU* bristles (open arrowheads), shown as controls, are indistinguishable from neighbouring wild-type bristles.

3.2.2 H3-K27Ac and H3.3-K27Ac are not required for ectopic expression of *HOX* genes in imaginal disc clones.

Since H3-K27 acetylation is strongly correlated with enhancer activity (Rada-Iglesias et al., 2011) and transcriptional activation, I next asked whether H3-K27 acetylation was affected in $H3^{K27R}$ cells. To answer this question I stained imaginal disc clones with an anti-H3-K27 acetyl antibody. Clones lacking E(z), show an increase of H3-K27Ac (**Figure 11A**). This is consistent with the two chromatin marks, H3-K27Ac and H3-K27 trimethylation being mutually exclusive because it is chemically not possible to have both acetylation and methylation at lysine 27 at the same time(Tie at al., 2009; Pasini et al., 2010a).

In contrast, H3-K27 acetylation is reduced in $H3^{K27R}$ cells, but some residual staining persists in the clones. As the two histone H3 variants in *Drosophila* H3.3A and H3.3B are nearly identical to the canonical H3 except for four amino acid residues, I reasoned that the residual level of bulk H3-K27Ac in $H3^{K27R}$ cells might be due to the presence of the H3.3-K27Ac. In addition, it has also been proposed that H3-K27Ac antagonises H3-K27 methylation and contributes to the activation of Polycomb target genes (Tie at al., 2009, Pasini et al., 2010a). However, *HOX* genes are misexpressed in $H3^{K27R}$ clones outside their normal expression patterns,which is not consistent with the idea that H3-K27Ac would be required for the transcriptional activation of *HOX* genes.To investigate whether acetylation of the histone variant H3.3 on lysine 27, might be compensating for the lack of acetylatable canonical H3, I generated $H3^{K27R}$ clones in imaginal wing discs of animals lacking any zygotic histone H3.3A or H3.3B (Figure 11**B**). This was achieved by specifically selecting male larvae by examination of the genital imaginal disc. Strikingly, these clones still show misexpression of the *HOX* gene *Ubx* in the wing imaginal disc where it normally is not expressed. This result demonstrates that neither H3 nor H3.3 acetylation on lysine 27, are required for transcriptional activation of *HOX* genes in the absence of H3-K27 methylation in larval imaginal discs.

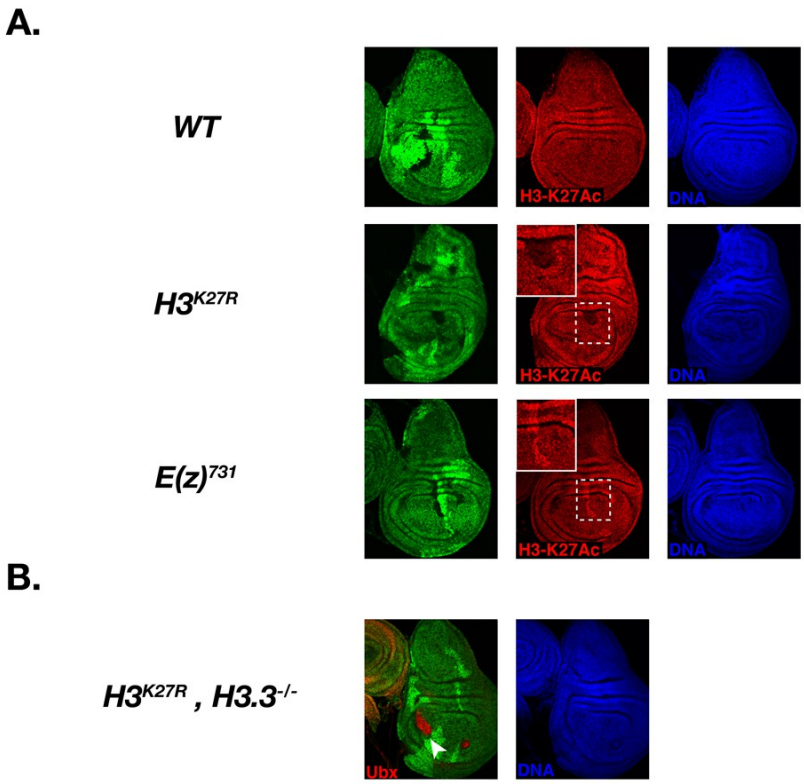

Figure 11. Neither H3-K27 nor H3.3-K27 acetylation play a major role in the ectopic expression of *HOX* genes in the absence of H3-K27me3

A. Wing imaginal discs with clones of Δ*HisC* homozygous cells in animals bearing *12×His-GU* or *12xHisGU-H3^{K27R}* transgenes (*WT* and *H3^{K27R}* respectively), or with clones of *E(z)^{731}* homozygous cells (*E(z)^{731}*), stained for H3-K27Ac in red, and DNA in blue. Clones are marked by the absence of GFP and induced as in **Figure 6**. In *E(z)^{731}* cells, bulk H3-K27Ac levels are increased (see inset), and in *H3^{K27R}* cells, H3-K27Ac levels are reduced, but a residual signal persists (see inset), likely due to H3.3-K27Ac. **B.** Wing imaginal discs with clones of Δ*HisC* homozygous cells in animals homozygous for *H3.3A^{KO}* and *H3.3B^{KO}*, null alleles for *H3.3A* and *H3.3A* respectively, with *12xHisGU-H3^{K27R}* transgenes (*H3^{K27R}, H3.3 $^{-/-}$*), stained for the Ubx product. Clones show misexpression of *Ubx* in the wing imaginal disc pouch (full arrowhead).

3.3 Functional analysis of H2A C-terminal residues that are modified by PRC1-type complexes

PRC1-type complexes are required for transcriptional repression of PcG target genes. These complexes have been shown to have chromatin compaction abilities (Shao et al., 1999). Additionally, PRC1-type complexes are E3 ubiquitin ligases that monoubiquitylate lysine 118 of the C-terminus of H2A (119 in mammals). The function of H2A monoubiquitylation is not clear. Studies in *Drosophila* and mammalian ES cells have yielded somewhat contradictory results (Gutiérrez et al., 2012; Eskeland et al., 2010, Endoh et al., 2012). On one hand, in *Drosophila,* the E3 ubiquitin ligase Sce/Ring is required for *HOX* gene repression (Gutiérrez et al., 2012). On the other hand a study in mouse ES cells suggested that H2A ubiquitylation is dispensable for *HOX* gene compaction (Eskeland et al., 2010). However, this report presents limitations since the authors only study the effects of mutating one of the two Ring paralogs in murine ES cells. This resulted in considerable residual levels of H2Aub in these mutant ES cells. Therefore these data do not provide sufficient evidence to rule-out H2Aub having a function in PcG repression of HOX genes. Finally, a study in mouse ES cells, in which both murine Ring paralogs were deplete, reported that although H2A ubiquitylation does not seem to be critical for *HOX* gene repression, it is important for the repression of other target genes (Endoh et al., 2012). In order to better understand the function of H2Aub, I set about to directly analyse the function of H2A residues that are monoubiquitylated by PRC1-type complexes in *Drosophila*.

3.3.1 Functional analysis of H2A ubiquitylation in the maintenance of Polycomb repression

I first analysed the function of H2A ubiquitylation during larval stages of development, during which PcG complexes maintain the repression of their target genes. In my analysis of histone H2A point mutants, it is important to note that animals heterozygous for the Δ*HisC* allele, containing twelve copies of the *H2A* mutated transgenes histone transgenes are viable and fertile. They were maintained as a stock and do not present any visible phenotype.

3.3.1.1 Larval cells lacking ubiquitylatable H2A proliferate normally, show wild-type expression of *HOX* genes and differentiate to form wild-type epidermal structures.

H2A-K119 is the main substrate of PRC1-type complexes in mammals, (H2A-K118 in *Drosophila*) (Nickel and Davie, 1989; Wang et al., 2004b). However, there is another lysine in position 118 in mammals (position 117 in *Drosophila*). Structural studies on a minimal PRC1 complex bound to the nucleosome showed that structurally both of these lysines can be ubiquitylated (McGinty et al., 2014). Interestingly, *Drosophila* H2A possesses two additional lysine residues at position 121 and 122, which are not present in mammals. Instead mammalian H2A exhibits lysine residues at positions 125, 127 and 129 (**Figure 12**)**.**

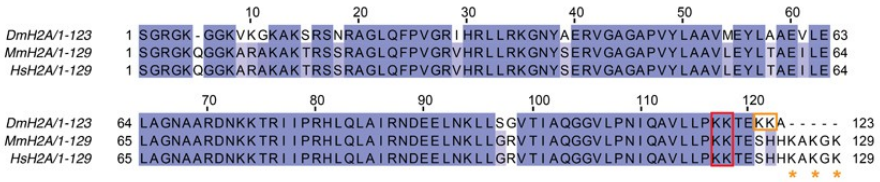

Figure 12. Alignment of *Drosophila*, Mouse and Human H2A protein sequences
Alignment generated with Clustal W2 http://www.ebi.ac.uk/Tools/msa/clustalw2/ of *Homo sapiens* H2A (Uniprot: P04908), *Mus musculus* H2A (Uniprot: P22752) and *Drosophila melanogaster* H2A (Uniprot: P84051). The two lysines at positions 117-118 in *Drosophila* and 118-119 in *Human* and *Mouse* are boxed in red. The highlight purple colour represents percentage of identity: the darker the colour the higher the percentage of identity. The *Drosophila* H2A C-terminal tail is 8 amino acids shorter than the human and mouse tail. In particular, the two lysines at positions 121-122 (boxed in orange) in *Drosophila* H2A are not conserved in human or mouse H2A, which instead contain three lysines separated by one residue each at positions 125, 127 and 129 (orange asterisks). The scale depicted above the alignment corresponds to the *Drosophila* H2A amino acid residue numbering.

In order to dissect the function of H2A ubiquitylation in PcG repression, I first analysed the phenotype of clones mutant for the H2A residues monoubiquitylated by PRC1-type complexes.

In an *in vitro,* E3 ubiquitin ligase assay, using a minimal recombinant PRC1 complex on *Drosophila* mononucleosomes, showed that H2A lysines 121 and 122 can be monoubiquitylated when lysines 117 and 118 are mutated into arginines (Reinhard Kalb, personal communication).

Therefore I generated Δ*HisC* homozygous clones in animals containing twelve copies of the histone gene units (12xHisGU) as in (section **3.1**), in which the H2A C-terminal lysines at position 117, 118, 121 and 122, were mutated into arginines: *12xHisGU-H2A*$^{4K->4R}$.

For simplicity I will refer to Δ*HisC* homozygous embryos or clones containing *12xHisGU-H2A*$^{4K->4R}$ transgenes, as *H2A*$^{4K->4R}$ embryos or clones.

H2A$^{4K->4R}$ embryos die at the end of embryogenesis, but do not show misexpression of a classical Polycomb target gene such as the *HOX* gene, *Abd-B* (data not shown). In agreement with this result, cuticles from *H2A*$^{4K->4R}$ embryos show a wild-type denticle belt pattern (**Figure 13A**). However, as discussed above, the chromatin of these embryos contains a mixture of wild-type maternally supplied H2A and mutant H2A$^{4K->4R}$ histones, because of the maternally supplied H2A product (Günesdogan et al., 2010). I therefore performed an imaginal disc clone analysis of H2A C-terminal mutants employing the same system as for H3-K27.

Intriguingly, I found that *H2A*$^{4K->4R}$ imaginal disc clones proliferate normally and show no misexpression of the *HOX* genes *Ubx* and *Abd-B* (**Figure 13B**). Furthermore, *H2A*$^{4K->4R}$ adult clones differentiate into wild-type epidermal structures (**Figure 13B,** lower panel). This strongly contrasts to homozygous *Sce*^{KO} imaginal disc clones which misexpress both *Ubx* and *Abd-B* and *Sce*^{KO} adult clones which show homeotic transformations (**Figure 13**).

To explain these surprising results I hypothesised that in cells where H2A can no longer be ubiquitylated, another protein might be ubiquitylated and compensate to bring about PcG repression.

H2AvD is the only essential H2A histone variant in *Drosophila* (van Daal and Elgin, 1992), it also shares a high degree of homology with the canonical H2A (**Figure 14A**). Moreover, it contains two lysines at positions 120 and 121, which have been reported to be ubiquitylated by PRC1-type complexes in murine cells (**Figure 14A**) (Sarcinella et al., 2007; Ku et al., 2012). I therefore set about to test whether H2Av is either the biologically relevant target of PRC1-type complexes, or compensating for the lack of ubiquitylatable H2A residues in *H2A*$^{4K->4R}$ clones. For this purpose, I first undertook to generate clones without any ubiquitylatable H2Av residues.

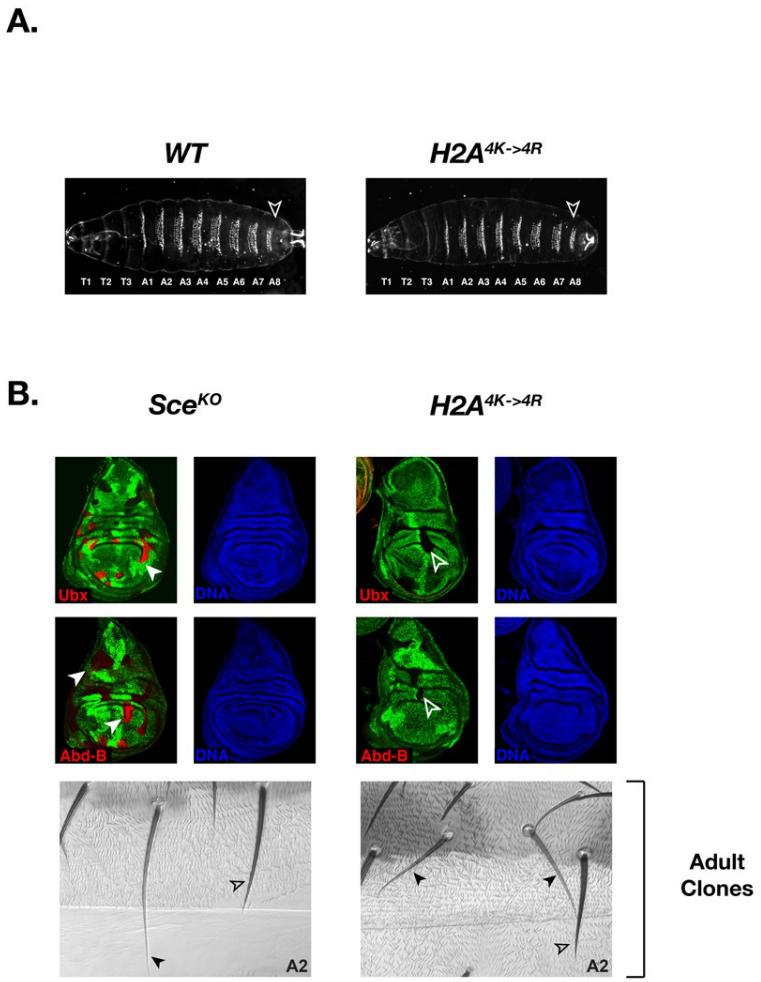

Figure 13. H2A residues are not required for *HOX* gene repression
A. Cuticles of Δ*HisC* homozygous embryos containing either*12×His-GU (WT)* or *12xHisGU-H2A*$^{4K->4R}$ (*H2A*$^{4K->4R}$) transgenes. Empty arrowheads mark the A8 denticle belt. *H2A*$^{4K->4R}$ embryos show a wild type denticle belt pattern. Δ*HisC*, *12×His-GU* cuticles are shown as a wild-type control. **B.** Wing imaginal discs with clones homozygous for the *Sce*^{KO} null allele (Gutiérrez et al., 2010), or Δ*HisC* homozygous clones in animals bearing *12xHisGU-H2A*$^{4K->4R}$ transgenes (*H2A*$^{4K->4R}$), stained for the Ubx (upper panel) and the Abd-B (middle panel) products. Homozygous mutant clones are marked as in **Figure 6.** Lower panel shows abdominal segment A2 fragments of *Drosophila* adults with clones of *Sce*^{KO} homozygous cells (*Sce*^{KO}) or Δ*HisC* homozygous cells in *12×His-GU-H2A*$^{4K->4R}$ transgenics (*H2A*$^{4K->4R}$); mutant cells are genetically marked with a yellow mutation (light pigmentation, marked by full arrowheads) as in **Figure 10**. *Sce*^{KO} mutant clones show slight homeotic transformations into more posterior body segments; the mutant sensory bristles are somewhat finer and more tapered than the neighbouring wild-type bristles (empty arrowheads) in A2 and resemble characteristic abdominal segment A10 bristles in wild-type animals (**Figure 10**, bottom panel).

3.3.1.2 H2Av C-terminal ubiquitylation is not required for PcG target gene repression, viability or fertility

In the following section I first characterised the *H2Av*^{810} allele, I then generated an *H2Av*^{KO} allele by homologous recombination in order to analyse the phenotype of cells lacking any ubiquitylatable residues on H2Av alone or on H2Av and H2A.

a *H2Av*^{810} is not an *H2Av* null allele

In order to produce the stocks to generate clones lacking H2Av ubiquitylatable residues, I first turned to the only available *H2Av* allele: *H2Av*^{810} described in (van Daal and Elgin, 1992). This allele is a deletion of exon 2 resulting in an in frame fusion of the first methionine, (exon 1) to the phenylalanine at position 27 (**Figure 14A** and **B**). In consequence, although the allele was described as null, and not producing any H2Av protein product, I probed acid extracted histones from *H2Av*^{810} homozygous larvae and wild-type larvae for H2AvD product by Western blot. H2Av^{810} homozygous larvae lack wild type H2Av product but express a shorter product detected by the anti-H2Av antibody, of approximately 12 kDa that can be visualised in all three serial dilutions (**Figure 14B** lanes 4 to 6); this shorter product is not detectable in wild-type larval extracts (**Figure 14B** lanes 1 to 3). The size of this short peptide corresponds approximately to the size of H2Av devoid of the 26 amino acid residues corresponding to Exon 2 (predicted Molecular weight (MW): 12.53 kDa). These observations suggest that *H2Av*^{810} is a hypomorphic allele and is not a null allele of *H2Av* as previously described.

Since I intended to use an *H2Av* null allele to generate cells containing a non-ubiquitylatable version of H2Av, I decided to generate an *H2Av* deletion allele.

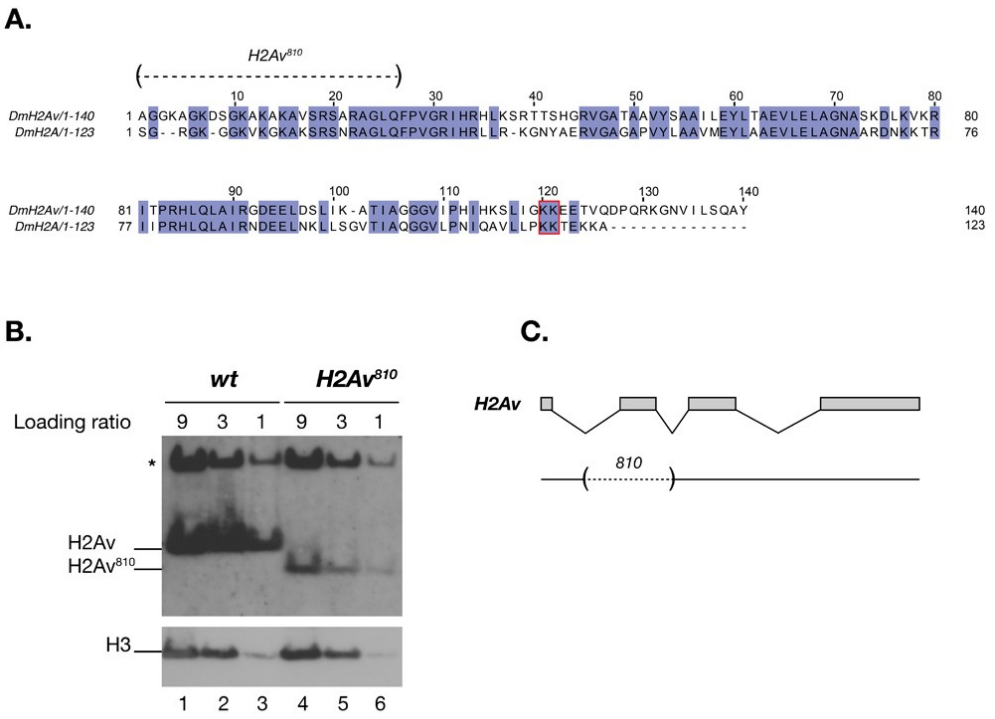

Figure 14. *H2Av*^{810} is not an *H2Av* null allele

A. Alignment generated with Clustal W2 http://www.ebi.ac.uk/Tools/msa/clustalw2/ *Drosophila melanogaster* H2Av (Uniprot:P08985) and H2A (Uniprot:P84051). The two lysines at positions 117-118 in H2A are conserved at positions 120 and 121 of H2Av (red box). Colouring according to percentage of identity: the darker the colour the higher the percentage of identity. The scale above the alignment corresponds to the H2Av amino acid residue numbering. The dashed line in parenthesis above the alignment represents the region deleted in the *H2Av*^{810} allele. **B.** Western blot on serial dilutions (9:3:1) of acid extracted histones from *wild-type* (lanes 1 to 3) or *H2Av*^{810} (lanes 4 to 6) homozygous larval central nervous systems and imaginal discs probed for H2Av. The histone H3 signal was used as a loading control. Note the presence of a shorter protein product (~ 12 kDa) recognised by the anti-H2Av antibody only in the H2Av810 and not in the wild-type extracts. Asterisk marks a cross reacting band .**C.** Schematic representation of the *H2Av* gene (boxes filled in grey represent exons and black lines represent introns). The H2Av gene is spliced and contains four exons. The *H2Av*^{810} allele is a deletion of exon 2 that results in an in-frame fusion of exon1 (the first methionine) and flanking intronic sequences upstream of exon 2. The predicted molecular weight of the putative H2Av810 product is 12 kDa, which corresponds to the size band observed in A.

b An *H2Av^{KO}* deletion allele

In a next step, I generated a new *H2Av* null allele by homologous recombination (Gong and Golic, 2003). I deleted the whole *H2Av* coding region by replacing it with the *white^{+}* (*w^{+}*) gene (**Figure 15A**) and isolated several independent targeting events that all failed to complement the lethality of the *H2Av^{810}* allele. Furthermore, I confirmed these lines as genuine replacements with the *white^{+}* gene by PCR (**Figure 15B**). After verification by sequencing of the whole upstream, *white^{+}* gene and downstream genomic regions of the different lines, I chose one line (number 6) as *H2Av^{KO}.*

H2Av^{KO} homozygous animals die at the end of the 3rd instar larval stage, in the same way as *H2Av^{810}* homozygous larvae. However, *H2Av^{KO}* homozygous larvae have nearly undetectable imaginal wing, 3rd leg and haltere discs. In contrast to *H2Av^{810}* homozygous discs that are of normal size. The lack of analysable discs prevented me from successfully probing for H2Av protein levels in diploid larval tissues. I did however make larval brain histone acid extracts from *H2Av^{KO}* homozygous animals and could still detect a faint band corresponding to the maternally contributed H2Av product (data not shown). I assume that this represents the maternal contribution in H2Av since larval brains contain many cells that are of embryonic origin (Truman, 1990). Additionally, somatic *H2Av^{KO}* homozygous clones induced 48 hours prior to analysis in imaginal wing discs show no detectable H2Av product by immunofluorescence (**Figure 15C**). It was not possible to probe for the H2Av product in *H2Av^{KO}* homozygous 96 hour clones, since these clones are no longer detectable, presumably because they are eliminated by cell death (**Figure 16A**). Interestingly, this is not the case of *H2Av^{810}* homozygous clones (**Figure 16A**) which are detectable and proliferate, most likely rescued by the H2Av^{810} truncated product.

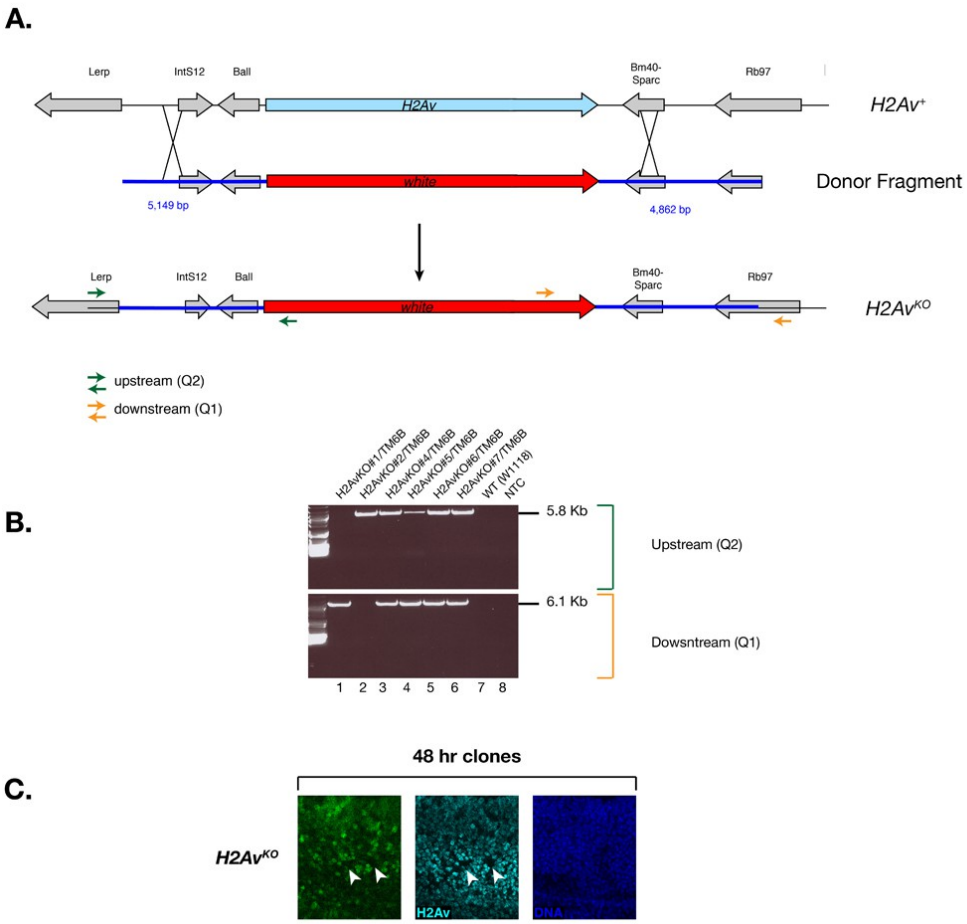

Figure 15. Generation of an *H2Av*^{KO} null allele

A. Diagram depicting the general strategy employed to generate the *H2Av*^{KO} allele. Genomic region containing the *H2Av* gene in *wild-type* animals (*H2Av*$^{+}$); the targeting construct containing the indicated genomic region in which the whole *H2Av* coding region was replaced by the *white* (*w*$^{+}$) marker gene (donor fragment); and the deletion allele, resulting from the ends out gene replacement by homologous recombination with the donor fragment, in which the complete *H2Av* coding region is replaced by *w*$^{+}$ (*H2Av*^{KO} allele). The blue lines represent the homologous regions upstream and downstream of the *H2Av* gene .**B.** Diagnostic Polymerase Chain Reaction (PCR) of the different *H2Av*^{KO} lines obtained form the ends out gene replacement strategy. The primers used for the diagnosis are mapped in A. While most lines are positive for both PCRs, I chose *H2Av*$^{KO\#6}$ as the *H2Av*^{KO} allele. This allele was further confirmed by sequencing the whole upstream, *w*$^{+}$ gene and downstream regions of *H2Av*. **C**. Imaginal disc clones of cells homozygous for the *H2Av*^{KO} allele, stained with antibodies against H2Av (Cyan) and Hoechst (DNA, blue channel). *H2Av*^{KO} homozygous cells are marked by the absence of GFP**,** and clones were induced 48 hours before analysis. The H2Av staining shows that H2Av protein is not detectable in the *H2Av*^{KO} clones (full arrowheads).

c Monoubiquitylation of H2Av is not required for PcG repression, viability or fertility

I next asked whether H2Av ubiquitylation itself was required for Polycomb repression of PRC1 target genes. To answer this question I constructed *H2Av* coding transgenes bearing either the wild-type *H2Av* genomic sequence or a version in which both lysines at position 120 and 121 were mutated into arginines *($H2Av^{KK->RR}$)* (**Figure 14A**). I then carried out a somatic clone analysis in imaginal discs of $H2Av^{KO}$ homozygous clones, rescued either by the $H2Av^{WT}$ or the $H2Av^{KK->RR}$ constructs. In both cases the clones proliferate normally, in comparison to their corresponding $GPF^{+/+}$ twin spot clones. Moreover, $H2Av^{KK->RR}$ maintain repression of the Polycomb group target genes *Ubx* or *Abd-B* in wing imaginal discs outside their normal expression patterns (**Figure 16A** and data not shown). Furthermore, $H2Av^{KO}$ homozygous animals are rescued into viable and fertile adults with no detectable phenotype, by both the $H2Av^{WT}$ and the $H2Av^{KK->RR}$ transgenes. Western blot analysis of H2Av ubiquitylation in acid extracted histones from embryos further confirmed the absence of H2Av ubiquitylation in $H2Av^{KK->RR}$ rescued animals (**Figure 16B,** lanes 4 to 6 compared to lanes 1 to 3). In addition, western blot analysis also revealed that in embryos lacking the maternal and zygotic catalytic activity of Sce/Ring ($Sce^{I48A\ m-;\ z-}$*),* described in (section **3.3.2**), H2Av ubiquitylation is depleted (**Figure 16B,** lanes 7 to 9 compared to lanes 1 to 3). This indicates that Sce/Ring is the main E3 ubiquitin ligase for H2Av in *Drosophila*.

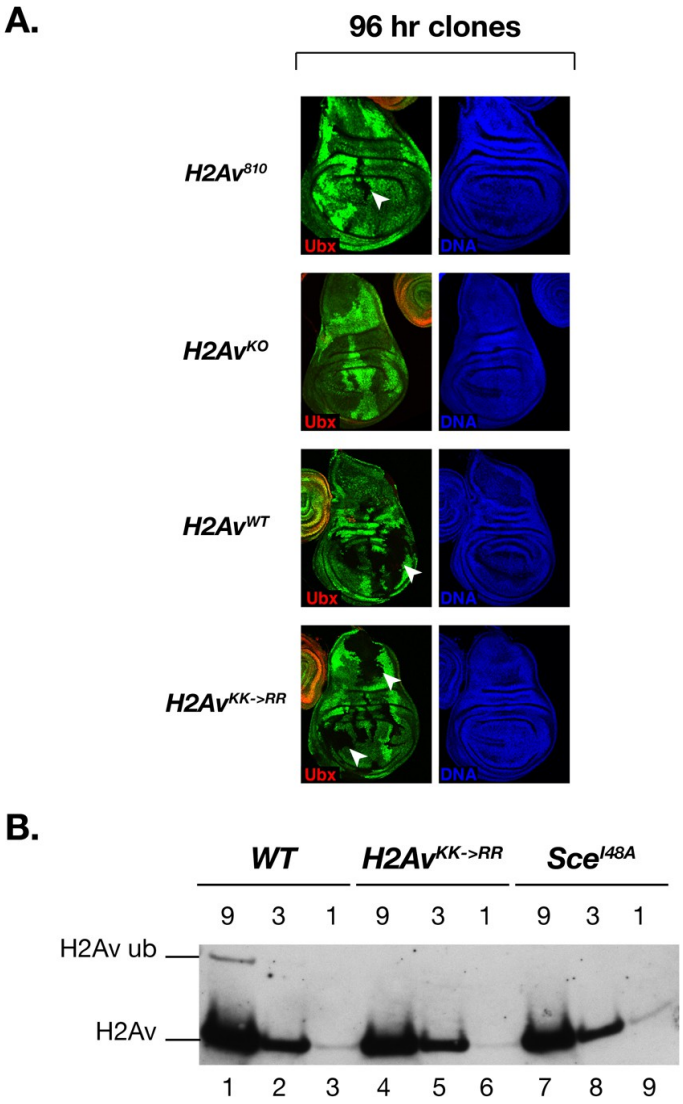

Figure 16. Monoubiquitylation of H2Av is not required for Polycomb repression, viability or fertility

A. Imaginal disc clones of cells homozygous for the *H2Av*^{810} allele or the *H2Av*^{KO} alleles alone (first and second row respectively), or homozygous for the *H2Av*^{KO} allele containing one copy of either the genomic *H2Av* wild-type (*H2Av*^{WT}) or *H2Av*$^{KK->RR}$ (*H2Av*$^{KK->RR}$) transgene, stained with antibodies against the Ubx product (red) and Hoechst (DNA, blue channel). Homozygous mutant cells are marked by the absence of GFP, and clones were induced 96 hours before analysis in all cases. *H2Av*^{810} 96 hour clones proliferate to a certain extent (full arrowhead) unlike *H2Av*^{KO} clones that either do not proliferate or enter in apoptosis. Lack of proliferation is fully rescued in *H2Av*^{KO} clones in the presence of one copy of either the *H2Av*^{WT} or the *H2Av*$^{KK->RR}$ transgene. **B.** Western blot analysis of serial dilutions (9:3:1) from acid extracted histones from 7 to 24 hour old embryos of wild type (lane 1 to 3), *H2Av*^{KO} homozygous animals rescued by the *H2Av*$^{KK->RR}$ transgene (lane 4 to 6) or *Sce*^{KO} (lanes 7 to 9) animals lacking both zygotic and maternal Sce rescued by a transgene encoding a catalytically inactive version of *Sce* lacking the E3 ubiquitin ligase activity of Sce: *Sce*^{I48A}. *H2Av*$^{KK->RR}$ and *Sce*$^{I48A\ m-;\ z-}$ embryos show no detectable H2Av ubiquitylation.

In summary, these data suggest that H2Av ubiquitylation is not required for the maintenance of Polycomb group target gene repression, viability or fertility in *Drosophila melanogaster*. These data are in opposition to the hypothesis that in mammals H2A.Z ubiquitylation might be required for transcriptional silencing of PcG target genes (Sarcinella et al., 2007; Creyghton et al., 2008). Nevertheless, this finding might indicate differences between H2A.Z function in mammals and in *Drosophila*.

3.3.1.3 Larval cells lacking H2A and H2Av ubiquitylation maintain repression of Polycomb group target genes

a Imaginal disc clones lacking ubiquitylatable H2A and H2Av residues maintain Polycomb repression

I next asked if in cells lacking wild-type ubiquitylatable H2A, H2Av is able to maintain repression of PcG target genes. It was genetically not possible to generate embryos containing only $H2Av^{KK->RR}$ and $H2A^{4K->4R}$. I thus employed the imaginal disc clone strategy in larvae to generate cells lacking wild-type H2A and H2Av, and containing only non-ubiquitylatable H2A and H2Av. These cells proliferate in a similar way as wild-type cells, although they are slightly smaller than the corresponding $GFP^{+/+}$ twin spots present in the same disc (**Figure 17,** GFP channel). Surprisingly, $H2A^{4k->4R}$, $H2Av^{KK->RR}$ clones do not show misexpression of *Ubx* or *Abd-B* (**Figure 17** first and second row). This prompted me to reconsider my hypothesis and investigate the expression of other putative PRC1 target genes in my setup. Given that the murine ortholog of *Paired (Prd)* was recently found to be misexpressed in mouse ES cells lacking the E3 ubiquitin ligase activity of PRC1-type complexes (Endoh et al., 2012), and that the *Prd* gene is bound by PRC1 subunits in *Drosophila* (Gambetta et al. 2009), I chose to examine *Prd* expression in $H2A^{4K->4R}$, $H2Av^{KK->RR}$ imaginal disc clones. In order to ensure that anti-Prd antibody was suitable for immunostaining, I also stained larval brains that presented Prd signal (data not shown).In contrast, *Prd* expression was undetectable in $H2A^{4K->4R}$, $H2Av^{KK->RR}$ mutant clones (**Figure 17** third row).

These results suggest that neither H2A nor H2Av ubiquitylation are essential for repression of the Polycomb target genes *Ubx* and *Abd-B* or the *Prd* gene. In contrast, the E3 ubiquitin ligase Sce/Ring itself is required for *HOX* gene repression in imaginal wing discs (Gutiérrez et al., 2012; **Figure 13**); and in mammals, the catalytic activity of Ring1B is required for normal expression levels of the *Prd* ortholog *Pax3* (Endoh et al., 2012).

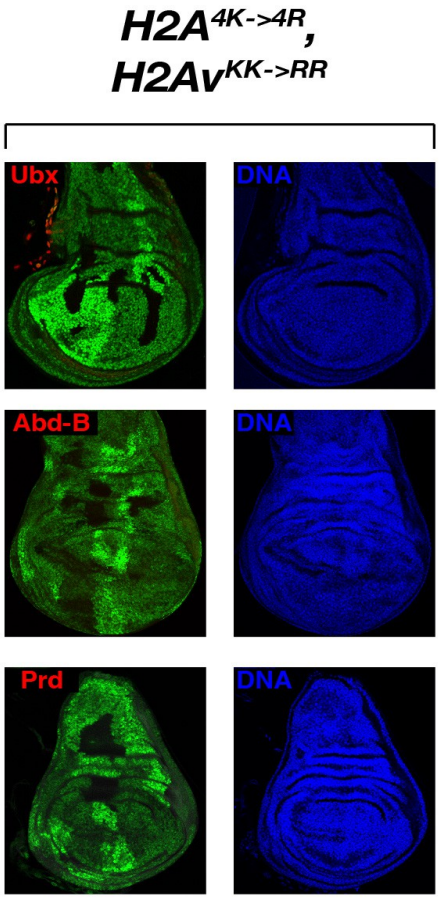

Figure 17. Imaginal disc clones lacking ubiquitylatable H2A and H2Av residues maintain Polycomb repression of *HOX* genes and *Prd*.
Wing imaginal discs with clones of Δ*HisC* homozygous cells in animals bearing 1*2xHisGU-H2A*$^{4K->4R}$ transgenes, also homozygous for the *H2Av*^{KO} allele, containing one copy of the *H2Av*$^{KK->RR}$ transgene (*H2A*$^{4K->4R}$, *H2Av*$^{KK->RR}$) stained with antibodies against the HOX proteins Ubx (first row), Abd-B (second row), the segmentation protein Prd (third row) and Hoechst (DNA). Clones are marked by the absence of GFP signal and were induced as in **Figure 6**.

b *Sce^{I48A}* is a catalytically inactive mutant of Sce/Ring

To complement these observations, I generated constructs containing a version of Sce/Ring in which a critical residue on the interaction surface of Sce/Ring and the E2 ubiquitin conjugating enzyme, UbcH5c, is mutated (Sce$^{\mathbf{I48A}}$) (Buchwald et al., 2006). Notably, the genomic sequence of *Sce* under its own promoter and regulatory sequences was used in this transgene (section **2.1.6**)**.** *In vitro*, this mutation highly reduces the E3 ligase activity for H2A monoubiquitylation (Buchwald et al., 2006). Additionally, *in vivo,* in mouse ES cells this mutation also highly reduces bulk ubiquitylated H2A (Endoh et al., 2012). Since *Sce^{KO}* animals die at the end of embryogenesis, and in contrast *Sce $^{I48A\ m+;\ z-}$* animals develop into pharate adults without any striking defects, I set out to to test whether in *Drosophila, Sce$^{I48A\ m+;\ z-}$* animals also lack H2A ubiquitylation. It is important to note that the *Sce^{WT}* wild-type version of the transgene rescues *Sce^{KO}* homozygotes into viable and fertile adults. In addition, bulk H3-K27me3 levels are similar in *Sce^{WT}* and *Sce$^{I48A\ m+;\ z-}$* animals (**Figure 18**). Western blot analysis on acid extracted histones from larvae rescued either with the wild-type *Sce (Sce^{WT})* transgene or with the *Sce^{I48A}* transgene, confirmed that *Sce$^{I48A\ m+;\ z-}$* larvae have no detectable bulk ubiquitylated H2A as compared to *Sce^{WT}* animals (**Figure 18** lanes 5 to 8 compared to lanes 1 to 4). This suggests that Sce^{I48A} is indeed a catalytically inactive version of Sce in *Drosophila.*

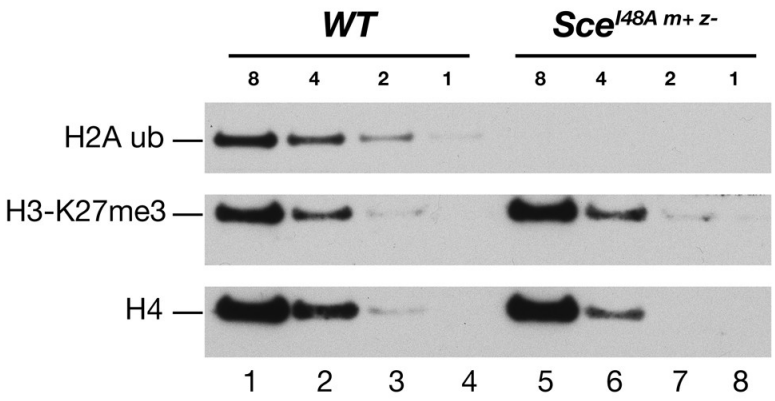

Figure 18. *Sce*^{I48A} mutants lack H2Aub
Western blot on serial dilutions (8:4:2:1) of acid extracted histones from *wild-type* (lanes 1 to 4) or *Sce*$^{I48A\ m+;\ z-}$ (lanes 5 to 8) homozygous larval central nervous systems and imaginal discs probed for ubiquitylated H2A and H3-K27me3. The histone H4 signal was used as a loading control. ubiquitylated H2A (first row) is reduced beyond detection levels in *Sce*$^{I48A\ m+;\ z-}$ extracts. In contrast, H3-K27me3 levels are not significantly different in the mutant extracts.

c *Sce*$^{I48A\ m+;\ z-}$ animals show no misexpression of Polycomb target genes

I then asked if *Sce*^{I48A} animals show misexpression of Polycomb target genes. To address this question, I examined the expression of several PRC1 bound genes: the *HOX* gene *Ubx*, the developmental regulator zinc finger transcription factors *Distalless (Dll), elbow B (elB)*, *wingless (wg)* and *spalt major (salm)* (Oktaba et al., 2008; Gutiérrez et al., 2010) in the wing imaginal discs of *Sce*$^{I48A\ m+;\ z-}$ animals. Of these genes, only *Ubx* and *elB* have been shown to require *Sce* for repression in imaginal discs. However, I found that all of these genes remained stably repressed outside their normal expression domains in *Sce*$^{I48A\ m+;\ z-}$ wing discs (**Figure 19**). This result complements the data from the H2A and H2Av point mutant analysis in (sections **3.3.1.1** and **3.3.1.2**) indicating that H2Aub is not required for the repression of *HOX* genes or of the tested PRC1-bound genes in *Drosophila* larvae.

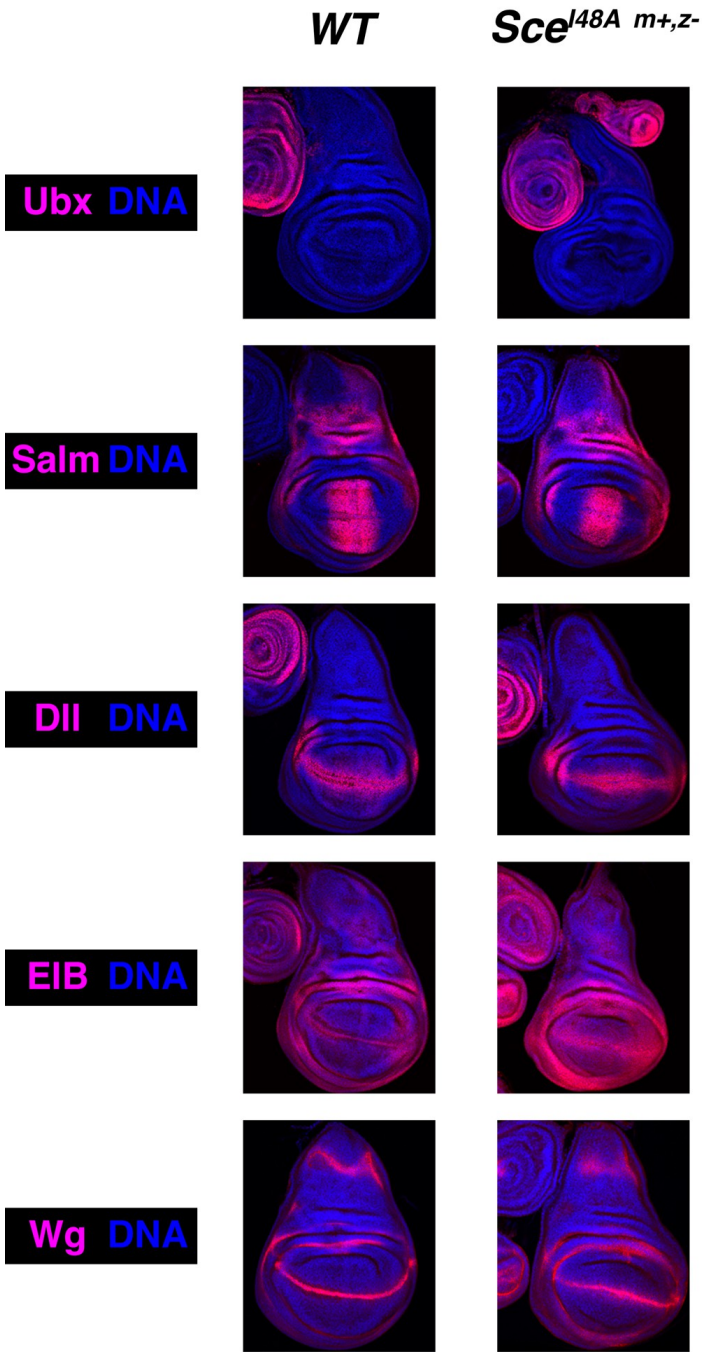

Figure 19. *Sce*^{I48A} mutants show no misexpression of *HOX* genes
Imaginal wing discs of *Sce*$^{0\ m+;\ z-}$ homozygous third instar larvae with two copies of either the genomic wild-type (*WT*) or the catalytically inactive point mutant *Sce*^{I48A} (*Sce*$^{I48A\ m+;\ z-}$), Sce transgenes, stained with antibodies against *Ubx, Spalt major (Salm), Distal-less (Dll), Elbow B (EIB)*, or *Wingless (Wg)*, in magenta. DNA is shown in the blue channel. All genes are expressed in their wild type expression domains in *Sce*$^{I48A\ m+;\ z-}$ imaginal discs.

3.3.1.4 Pharate adults lacking H2A ubiquitylation present sensory organ abnormalities.

However, whilst *Sce*^{WT} animals develop into viable and fertile adults, *Sce*$^{I48A\ m+;\ z-}$ animals die as pharate adults. Additionally, the *Sce*$^{I48A\ m+;\ z-}$ differentiated adult cuticles, show defects in the bristle sensory organs (**Figure 20**)**.** The adult *Drosophila* bristles are part of the peripheral nervous system (PNS). They are mechanosensory organs that play an important role in the sensation of external mechanical stimuli. There are two distinct types of bristles: the macrochaete that are long and the microchaete that are short. *Sce*$^{I48A\ m+;\ z-}$ pharate adults show a reduction of both macro and microchaetae and present an altered bristle morphology compared to the *Sce*^{WT} control (**Figure 20**). In addition, the pattern of both kinds of bristles is not properly organised in *Sce*$^{I48A\ m+;\ z-}$ adults. The *Sce*$^{I48A\ m+;\ z-}$ bristles are also not orientated in the same direction as *Sce*^{WT} bristles. These abnormalities in *Sce*$^{I48A\ m+;\ z-}$ mechanosensory organs might reflect the misexpression of uncharacterised PcG target genes. Intriguingly, adult *H2A*$^{4K->4R}$ clones in the abdomen do not show any bristle morphological defects (**Figure 13B**)**.** This could be due to the fact that the *H2A*$^{4K->4R}$ clones were visualised in the abdomen and not in the thorax, and that different pathways might regulate the proper development of bristles in the adult abdomen versus the thorax.

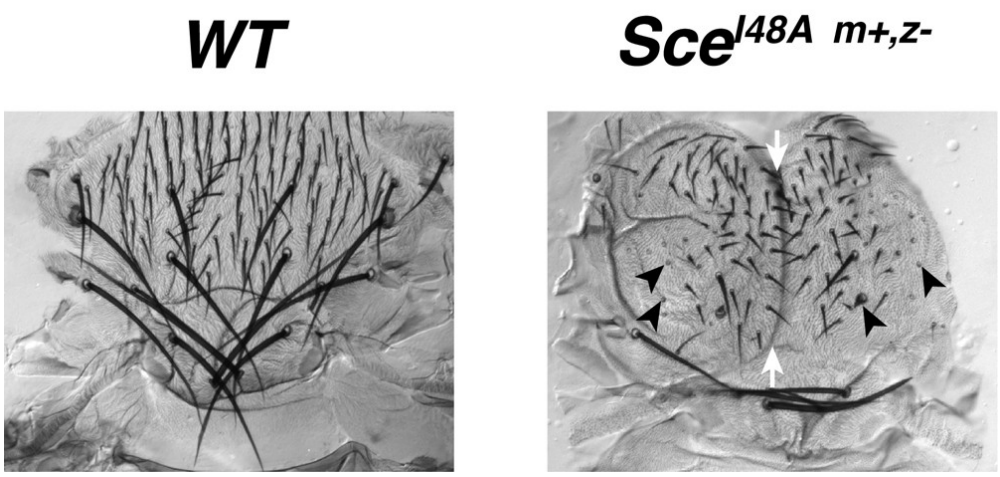

Figure 20. *Sce*^{I48A} animals develop into pharate adults and show sensory organ defects

Portions of thoraxes from pharate adults, homozygous for the *Sce*^{KO} *r*escued either by the wild-type *Sce* transgene *(WT)* or by the *Sce*^{I48A} transgene (*Sce*$^{I48A\ m+;\ z-}$). The sensory organs of *Sce*$^{I48A\ m+;\ z-}$ animals are smaller and thicker than the wild-type control organs and in some variable regions, bristles are lacking in the *Sce*$^{I48A\ m+;\ z-}$ thorax (full arrowhead). Additionally, *Sce*$^{I48A\ m+;\ z-}$ bristles do not follow the same overall pattern or orientation as the wild-type bristles. Moreover, the *Sce*$^{I48A\ m+;\ z-}$ animals do not fully complete dorsal closure as evidenced by the split thorax (white arrows).

Together, these results suggest that the E3 ubiquitin ligase activity of Sce/Ring or H2A-H2Av ubiquitylation itself are not required for the maintenance of repression of classical, characterised PRC1 target genes or the tested PRC1 bound genes in larval tissues. However, H2Aub is required for normal bristle formation and for viability at an organism level.

3.3.2 Functional analysis of H2A ubiquitylation in embryonic development

On the one hand, because *HOX* genes are the best characterised PcG target genes in *Drosophila*, and they are critically required for proper anterior-posterior body patterning, I asked whether ubiquitylated H2A is required to establish PcG repression at *HOX* genes, rather than for the maintenance of *HOX* gene repression.

On the other hand, the unexpected observation that ubiquitylation of H2A is not required for the maintenance of Polycomb group repression of PcG target genes, is contrary to what has been previously described in imaginal wing disc cells either lacking or depleted for *Sce/Ring* (**Figure 13**, Gutiérrez et al., 2010). Furthermore *Sce*$^{KO\ m+;\ z-}$ embryos die at the end of embryogenesis (Gutiérrez et al., 2010) unlike *Sce*$^{I48A\ m+;\ z-}$

animals that die as pharate adults. Thus, I hypothesised that these apparent discrepancies might be due to Sce/Ring having another function independent of its E3 ubiquitin ligase function. Perhaps, Sce/Ring has an architectural function that is required for the structure and physical stability of PRC1-type complexes. PRC1-type complexes are perhaps destabilised in the absence of Sce/Ring. This architectural function might be essential for PRC1-type complexes to achieve transcriptional repression of target genes. This would be in line with studies in mouse ES cells, which demonstrate that several subunits of the PRC1 complexes are degraded in cells lacking the Sce ortholog Ring1B likely reflecting the destabilisation of PRC1 complexes (Eskeland et al., 2010). However, in imaginal disc tissue depleted for Sce by RNAi (RNA interference), the protein levels of the PRC1 subunits Psc and Ph do not seem to be affected (Gutiérrez et al., 2012). The reason for this might be that, in *Drosophila,* the complex is destabilised by the absence of Sce but the individual proteins do not get degraded. Alternatively, other members of the PRC1 complex, that were not examined in (Gutiérrez et al., 2012) like Pc or Scm, might be degraded in *Sce* depleted tissue.

To address both of these issues, I generated embryos either completely lacking Sce protein, both zygotically and maternally or containing exclusively the Sce^{I48A} catalytic inactive product and no maternal or zygotic wild-type Sce protein.

3.3.2.1 H2A ubiquitylation is required for viability but not for *HOX* gene repression

a Strategy to generate embryos lacking maternally and zygotically *Sce* and containing only mutant Sce^{I48A} product.

To examine the phenotype of animals completely lacking Sce protein or only containing Sce^{I48A} product, I employed the FLAP (Flippable LoxP AttB containing Plasmid) technique adapted from (Gambetta and Müller, 2014) (**Figure 21**). The efficiency of this strategy was calculated by scoring the number of GFP positive embryos from parents lacking Sce protein in their germ line. The GFP positive embryos do not excise the *Sce-FLAP* transgene (**Figure 21A**). The efficiency was approximately 98%. In all cases the GPF positive embryos were sorted out. It is important to note that the genomic *Sce* sequence of the *Sce-FLAP* vector was identical to the *Sce* wild-type rescue transgene employed in (section **3.3.1.3b**).

b Animals lacking both maternal and zygotic Sce/Ring catalytic activity die at the end of embryogenesis and show wild-type expression of *HOX* genes.

Strikingly, *Sce*^{0} embryonic cuticles show severe homeotic transformations of all thoracic and abdominal denticle belts into the most posterior denticle belt A8, in agreement with (Gutiérrez et al., 2010). In addition, these embryos present widespread misexpression of both *Ubx* and *Abd-B* along the anterior segments of the central nervous system (CNS) and the epidermis outside their normal expression patterns (**Figure 22**) and die before the end of embryogenesis. In contrast, *Sce*$^{I48A\ m-;\ z-}$ embryos did not display any homeotic transformations and did not misexpress either *Ubx* or *Abd-B* (**Figure 22**). However, the vast majority of *Sce*$^{I48A\ m-;\ z-}$ embryos die as pharate first instar larvae, although some of them do hatch into first instar larvae.

These results suggest that the E3 ubiquitin ligase activity of PRC1-type complexes is not required for *HOX* gene repression or proper body patterning in *Drosophila* embryos. Nevertheless, the PRC1 E3 ubiquitin ligase activity is essential for viability at the organism level.

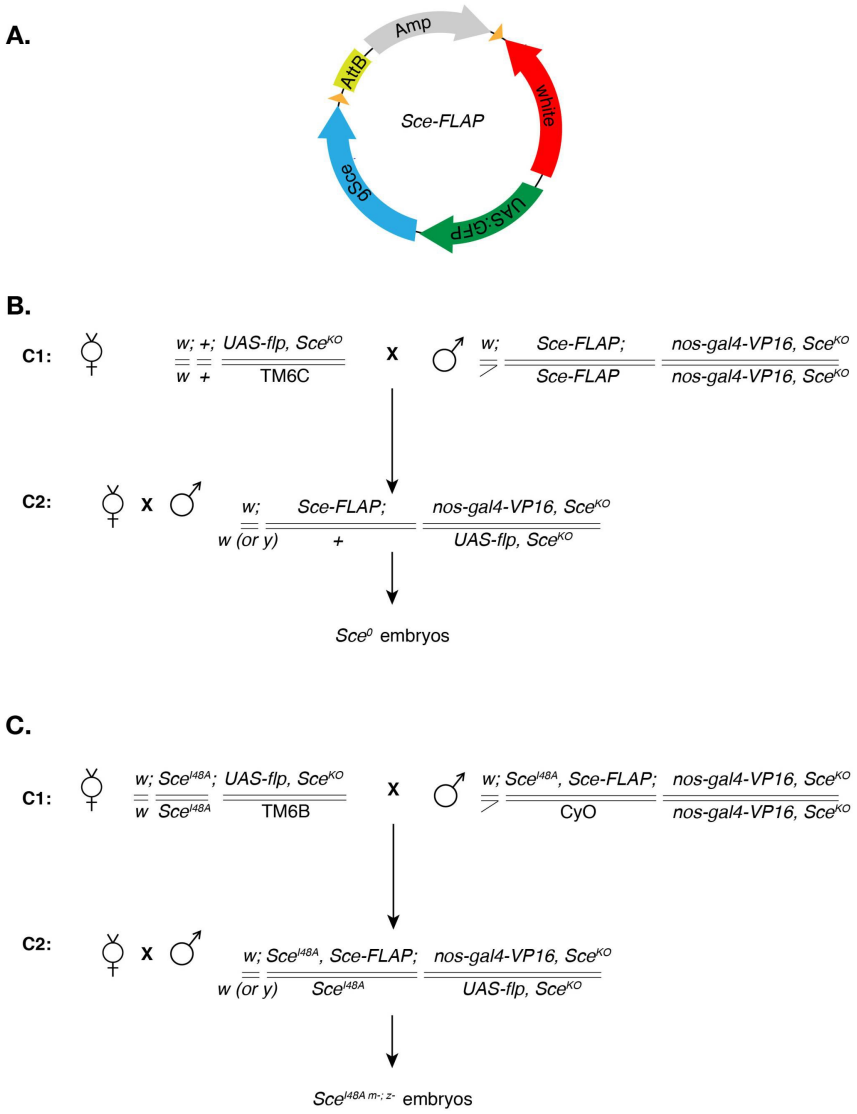

Figure 21. Strategy employed to generate *Sce*$^{I48A\ m-;\ z-}$ embryos
A. Diagram depicting the *Sce-FLAP* vector. It contains a *white* maker gene, a *UAS:GFP* sequence and the genomic *Sce* gene region (*gSce*) flanked by FRT sites (orange triangles); an *AttB* site (light green) that can be recombined with an *AttP* sequence on a specific landing site using germline specific phiC31 integrases (Bischof et al., 2007). It also contains a bacterial Ampicillin resistance gene (*Amp).* **B**. Crosses performed to flip-out the *Sce* genomic region from the *Sce-FLAP* integrated construct. In brief, virgins containing the flipase under the control of the UAS regulatory sequence (*UAS-flp*) and bearing the *Sce*^{KO} allele were crossed to males containing the *Sce-FLAP* construct, and the Gal4-VP16 fusion protein under the control of the *nanos* promoter (*nos-gal4-VP16*). Virgins and males heterozygous for the *Sce-FLAP* and trans-heterozygous for *nos-gal4-VP16* and *UAS-flp,* and therefore expressing the *flipase* in their germline*;* and homozygous for the *Sce*^{KO} allele were selected from the progeny of cross 1 (c1) and crossed together (c2). The progeny from c2 was then collected and the embryos that did not "flip out" the *Sce* genomic region and therefore also not the *UAS-GFP* were removed by detection of GFP signal. **C**. As in B but in this case all stocks additionally contain a genomic *Sce*^{I48A} rescue transgene on the second chromosome.

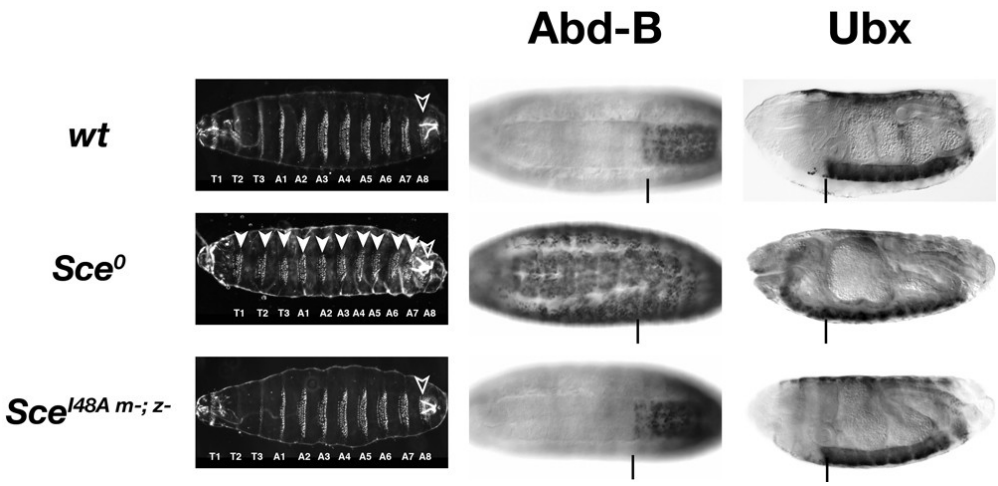

Figure 22. *Sce^{0}* embryos show homeotic transformations and misexpression of *HOX* genes unlike *Sce$^{I48A\ m-;\ z-}$* embryos

(Left panel) Ventral views of *Drosophila* embryonic cuticles. The empty arrowhead marks the wild-type denticle belt of the the 8th abdominal segment (A8). In *Sce^{0}* cuticles, all the more anterior denticle belts are transformed to A8 (full arrowheads), unlike in *Sce$^{I48A\ m-;\ z-}$* cuticles, that are indistinguishable form wild-type cuticles. (Middle panel) Ventral views of stage 16 embryos stained for the HOX protein Abd-B. In wild-type embryos, *Abd-B* is expressed in the last 5 most posterior parasegments. *Sce^{0}* embryos show *Abd-B* misexpression from head to tail, unlike *Sce$^{I48A\ m-;\ z-}$* embryos that show wild-type expression. (Right panel) Lateral views of stage 15 to 16 embryos stained for the HOX protein Ubx. In wild-type embryos *Ubx* is expressed from parasegment 5 to parasegment 12. In *Sce^{0}* embryos, *Ubx* is misexpressed in the more anterior parasegments, though the misexpression is only mild, possibly owing to the misexpression of *Abd-B* in this domain. *Sce$^{I48A\ m-;\ z-}$* embryos show wild-type *Ubx* expression. The vertical line marks the anterior boundaries of wild-type *Abd-B* and *Ubx* expression (parasegment 10 and 5 respectively).

c Polycomb protein levels are reduced in animals lacking Sce/Ring protein

In order to explain the striking differences between the *Sce^{0}* and *Sce$^{I48A\ m-;\ z-}$* embryos, I decided to first check if the protein levels of all other subunits of the canonical PRC1 complex were affected in embryonic soluble nuclear extracts from both genotypes by western blot. In both *Sce^{0}* and *Sce$^{I48A\ m-;\ z-}$* embryos, PRC1 subunits Ph and Psc showed slightly increased levels compared to wild-type (**Figure 23,** lanes 4 to 6 and 7 to 9 compared to lanes 1 to 3). This is not surprising, since *Ph* and *Psc* transcription is repressed by PcG complexes, via their PREs. Strikingly however, Pc protein levels were significantly reduced in *Sce^{0}* extracts (**Figure 23,** lanes 4 to 7) as compared to wild-type and *Sce$^{I48A\ m-;\ z-}$* extracts (**Figure 23,** lanes 1 to 3 and 7 to 9 respectively). To rule out the possibility that the lack of Polycomb phenotype in *Sce$^{I48A\ m-;\ z-}$* embryos was due to residual ubiquitin ligase activity of the Sce^{I48A} protein, I probed chromatin pellet extracts of the same material used for the soluble nuclear extraction, with H2Aub anti-

bodies (**Figure 24**). As expected (section **3.3.1.3b**), ubiquitylation of H2A was strongly reduced in both *Sce*^{0} and in *Sce*$^{I48A\ m-;\ z-}$ extracts.

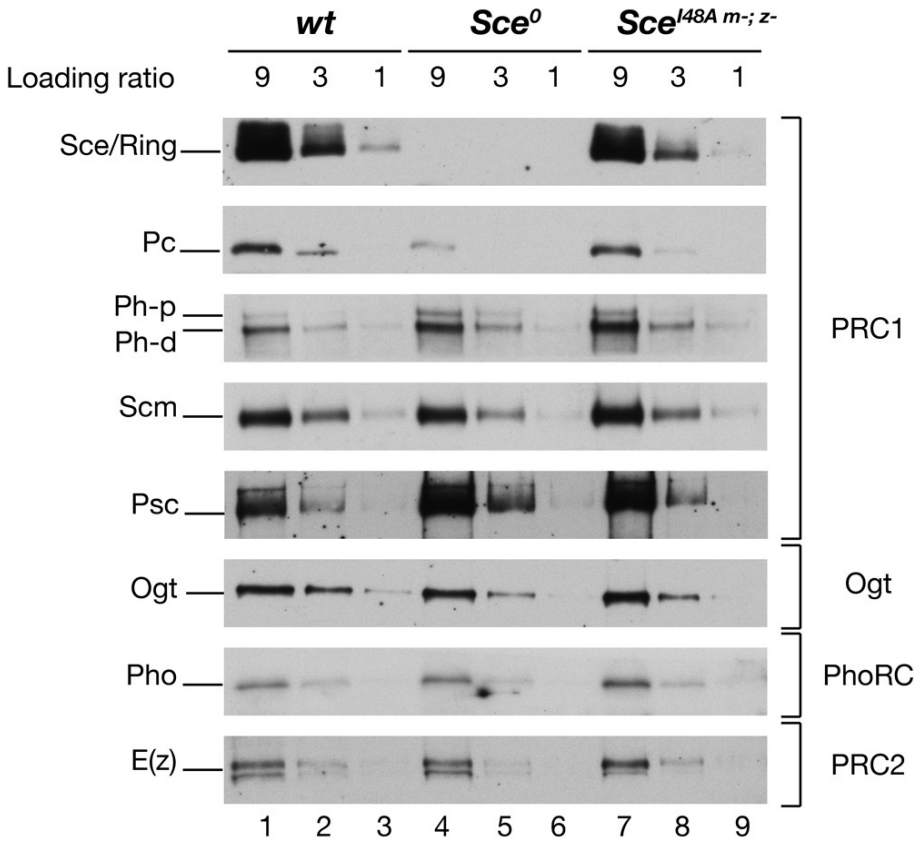

Figure 23. *Sce*^{0} embryos show reduced levels of Polycomb protein unlike *Sce*$^{I48A\ m-;\ z-}$ embryos

Western blot analysis on serial dilutions (9:3:1) of soluble nuclear extracts from embryos of *wild-type* (*wt*), *Sce*^{0} and *Sce*$^{I48A\ m-;\ z-}$ embryos. Analysis of the PRC1 subunits show that Sce/Ring protein (first row) is not detectable in *Sce*^{0} embryos and *Sce*$^{I48A\ m-;\ z-}$ mutants show wild-type levels of Sce/Ring. Interestingly, Pc protein levels (second row) are reduced in *Sce*^{0} embryos compared to wild-type and *Sce*$^{I48A\ m-;\ z-}$ embryos (3rd row, lane 4 compared to lane 1 and 7). Scm levels (4th row) are not affected in both *Sce*^{0} and *Sce*$^{I48A\ m-;\ z-}$ embryos compared to wild-type. However, the Ph homologs: Ph proximal (Ph-p) and Ph distal (Ph-d) and Psc levels are slightly increased in *Sce*^{0} extracts and to a lesser extent in *Sce*$^{I48A\ m-;\ z-}$ embryos. The levels of Ogt, Pho and E(z) in both *Sce*^{0} and *Sce*$^{I48A\ m-;\ z-}$ embryos are indistinguishable form levels in wild-type animals.

Together, these results strongly suggest that ***Sce*^{0}** embryos fail to repress *HOX* genes and show homeotic transformations due to the lack of Sce protein and the

reduced levels of Polycomb protein. It is likely that the architecture of PRC1 complexes is destabilised in the absence of Sce protein, perhaps affecting the link between Psc and Pc.

d H3-K27 methylation levels are reduced in embryos lacking Sce/Ring protein and in embryos lacking the catalytic activity of Sce/Ring

Several independent studies indicated that variant PRC2 histone methyltransferase activity is stimulated on H2A ubiquitylated nucleosome arrays (Kalb et al., 2014), and that H2A ubiquitylation is sufficient to bring about H3-K27me3 at ectopic sites in the genome of mouse ES cells (Blackledge et al., 2014, Cooper et al., 2014). Therefore, I asked whether H3-K27me3 levels were changed in $Sce^{I48A\ m-;\ z-}$ embryos. I compared H3-K27me3 levels in chromatin extracts from $Sce^{I48A\ m-;\ -}$ and Sce^{0} animals by western blot. Strikingly, both $Sce^{I48A\ m-;\ z-}$ and Sce^{0} embryo chromatin extracts show reduced bulk H3-K27me3 levels (**Figure 24**). This finding suggests that H2A ubiquitylation is required for wild-type levels of bulk H3-K27me3 during the early stages of *Drosophila* development. This result contrasts the finding that bulk H3-K27me3 levels are not drastically affected in $Sce^{I48A\ m+;\ z-}$ larval tissues (**Figure 18**).

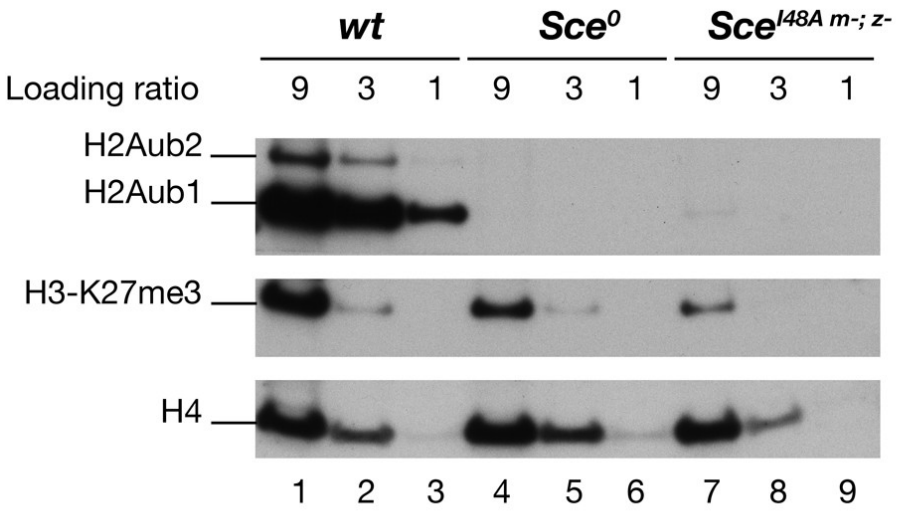

Figure 24. Sce^{0} and $Sce^{I48A\ m-;\ z-}$ embryos show reduced levels of bulk H3-K27me3

Western blot of serial dilutions (9:3:1) of chromatin extracts from *wild-type* (*wt*), Sce^{0} and $Sce^{I48A\ m-;\ z-}$ embryos probed with antibodies against ubiquitylated H2A and trimethylated H3-K27. ubiquitylated H2A (first row) is not detectable in Sce^{0} extracts (lanes 4 to 6), and reduced to less than 5% in $Sce^{I48A\ m-;\ z-}$ extracts (compare lanes 1 to 3 and 7 to 9). Trimethylated H3-K27 is slightly reduced in embryos from both Sce^{0} and $Sce^{I48A\ m-;\ z-}$ embryos (compare lane1, lane 4 and 7).

3.3.3 *Sce^{I48A}* acts as a suppressor of the PR-DUB phenotype in larval cells

Having found that H2A ubiquitylation is not required either for the establishment or for the maintenance of PcG repression of *HOX* genes, I turned to the potential interaction between the deubiquitylating activity of PR-DUB and the E3 ubiquitin ligase activity of PRC1-type complexes in *HOX* gene repression.

The PR-DUB complex is required for repression of PcG target genes, in particular *Ubx.* Intriguingly, PR-DUB has the ability to remove ubiquitin from H2A ubiquitylated nucleosomes *in vitro,* and mutants lacking PR-DUB subunits show an increase in bulk levels of H2Aub *in vivo* (Scheuermann et al., 2010). Therefore, deubiquitylation of H2Aub seems to be critical for PcG repression. This posed an apparent conundrum since the E3 ubiquitin ligase subunit of PRC1-type complexes is also critically required for repression of *HOX* genes (Gutiérrez et al., 2012; Scheuermann et al., 2012). However, my data suggests that H2A ubiquitylation itself does not have a function in repression of *HOX* genes. This appears to explain the H2A ubiquitylation puzzle: H2A in fact must not be ubiquitylated at *HOX* gene chromatin in cells where *HOX* genes should be repressed. This would imply that when H2A is not ubiquitylated at *HOX* genes, the presence of wild-type PR-DUB may become irrelevant for PcG repression. In other words, in cells lacking the catalytic activity of PRC1, removal of PR-DUB would not result in misexpression of *HOX* genes. To test this hypothesis, I set about to generate clones of cells lacking the PR-DUB ubiquitin carboxy-terminal hydrolase *Calypso* in imaginal discs of *Sce$^{I48A\ m+;\ z-}$* larvae. Strikingly, unlike clones lacking *Calypso* only, which misexpress *Ubx* in imaginal wing discs, clones lacking the E3 ubiquitin ligase activity of Sce/Ring and *Calypso* no longer show misexpression of *Ubx* (**Figure 25**). These results indicate that *Sce^{I48A}* acts as a suppressor of the *PR-DUB* phenotype. This is in line with the idea that the function of PR-DUB in the repression of *Ubx* is perhaps to deubiquitylate ubiquitylated H2A specifically on the *Ubx* chromatin. Alternatively, suppression of *Ubx* misexpression in the *Calypso* homozygous clones in *Sce^{I48A}* larvae might be an indirect effect of the depletion of H2A ubiquitylation.

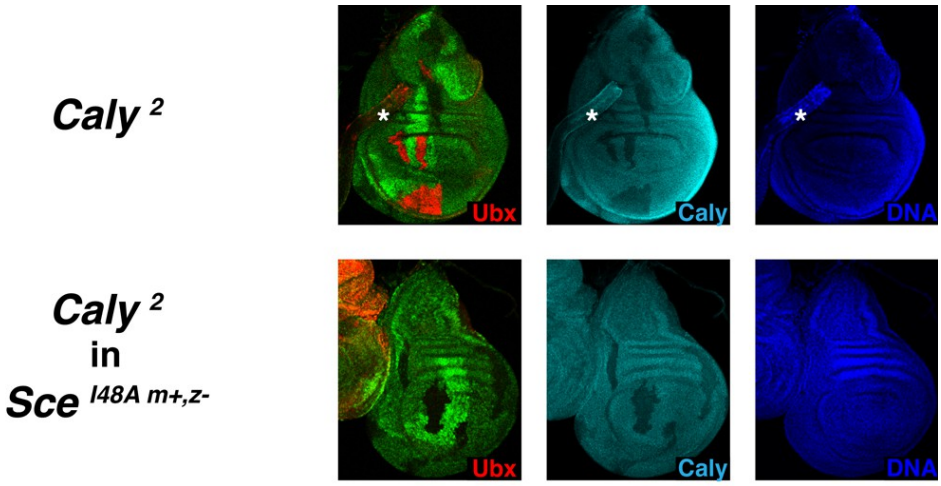

Figure 25. *Sce*^{I48A} acts as a suppressor of the *PR-DUB* phenotype in imaginal discs**.**

Wing imaginal discs with clones of *Caly*^{2} (a calypso null allele) homozygous clones, in a wild-type background (*Caly*^{2}*,* upper panel) or *Caly*^{2} homozygous clones in animals homozygous for the *Sce*^{0} allele bearing one copy of the *Sce*^{I48A} transgene (*Caly*^{2} *in Sce*$^{I48A\ m+;\ z-}$, lower panel), stained with antibodies against Ubx (in red), the Calypso protein (in cyan), and the DNA signal (in blue). Clones are marked by the absence of GPF and were analysed 96 hours after induction. The white asterisk marks a fragment of larval trachea.

4 Discussion

Previous to the work described in this thesis, the function of histone post-translational modifications carried out by the PcG of transcriptional repressors was not clear. In this thesis I demonstrate that H3-K27 methylation is critically required for PcG repression, and that H3-K27 is the physiologically relevant substrate of PRC2 complexes. Furthermore, I present strong evidence showing that H2A and H2Av ubiquitylation are not required for the repression of *HOX* genes or for anterior-posterior body patterning in *Drosophila.* I also find that H2A ubiquitylation is however essential for viability at an organism level.

4.1 Functional analysis of H3-K27

4.1.1 H3-K27 methylation is critically required for Polycomb repression.

Taken together, my results from the H3-K27 functional analysis provide, for the first time, direct functional evidence that H3-K27 is the physiologically relevant substrate of PRC2 complexes, and that H3-K27 methylation is critically required for PcG repression of *HOX* genes and the *engrailed* gene, a homeobox gene responsible for the proper anterior-posterior patterning of each compartment of the body (Morata and Lawrence, 1975).

It has been widely accepted that H3-K27 trimethylation is important for PcG repression, based on the phenotype of mutants lacking the catalytic activity of PRC2 (Müller et al. 2002). However, other histone and non-histone substrates of PRC2 have been identified. The linker histone H1 was found to be methylated at lysine 26 by a PRC2 variant complex *in vitro* and in human cell lines. Based on luciferase reporter assays, the authors of this study propose that H1-K26 methylation is required for Polycomb repression (Kuzmichev et al., 2004). In addition, the Gata4 transcription factor was shown to be methylated by PRC2 in mouse foetal hearts, and the authors of this work suggest that this methylation modulates Gata4 transcription factor activity (He et al., 2012). Both of these putative substrates are conserved in *Drosophila,* thus it is possible that H1 and the orthologs of Gata4: Pannier (Pnr) and grain (grain), are also

substrates of PRC2 in the *Drosophila* system. However, here I demonstrate not only that there is a perfect correlation between the genes misexpressed in *H3^{K27R}* cells and the genes misexpressed in cells mutant for PRC2 subunits, but that these genes are misexpressed at identical levels in both *H3^{K27R}* clones and clones mutant for PRC2. In addition, the homeotic transformation morphology of *H3^{K27R}* adult bristles is identical to the morphology of bristles mutant for PRC2. Therefore, H3-K27 is the direct, physiologically relevant target of PRC2, in *Drosophila,* and given the strong conservation of the PcG system and H3-K27 methylation, this result also stands for mammals and metazoans in general. These findings directly argue against H1 and Gata4 methylation by PRC2 complexes being important in PcG repression.

As stated in the introduction (section **1.2.1.2c**), the core PRC2 complex mono-, di- or trimethylates H3-K27 (Müller et al., 2002). However, it has been shown that H3-K27me1 and H3-K27me2 are present genome wide (Ebert et al., 2004; Modencode project), and that only H3-K27me3 specifically decorates nucleosomes on PcG target genes (Papp et al., 2006; Schwartz et al., 2006; Nekrasov et al., 2007; Kahn et al., 2006). In addition, H3-K27me3 is present at target genes only in their repressed state (Papp et al., 2006). Furthermore, the PRC2 subunit, Pcl, which is required for the catalysis of high levels of H3-K27me3 *in vitro* and *in vivo,* is critically required for PcG repression (Nekrasov et al., 2007). Therefore, it is reasonable to assume that the misexpression of PcG target genes in *H3^{K27R}* cells is due to the lack of H3-K27 trimethylation and not to the lack of the mono- and dimethylated versions of H3-K27 that are present genome wide.

4.1.2 H3-K27 acetylation

H3-K27 is also acetylated by the CREB-binding protein (CBP) histone acetyltransferases (Nejire in *Drosophila*), and acetylation has been shown to be mutually exclusive with H3-K27 methylation (Tie et al., 2009). The acetylated form of H3-K27 is associated with transcriptional activation, and is present at enhancer elements (Rada-Iglesias et al., 2011). *H3^{K27R}* cells not only lack H3-K27 methylation, but also H3-K27 acetylation (**Figure 11**). However, *H3^{K27R}* cells ectopically express *HOX* genes, which suggests that H3-K27 acetylation is not required for the ectopic expression of *HOX* genes. I then hypothesised that, given that the histone variant H3.3 has a high degree of homology with H3, perhaps in *H3^{K27R}* cells, H3.3-K27 might be acetylated and this would be sufficient for transcriptional activation of PcG target genes. Nonetheless, I found that *H3^{K27R}*

clones in animals lacking any H3.3, still misexpress *HOX* genes (**Figure 11**). This provides strong evidence that neither H3-K27 acetylation nor H3.3-K27 acetylation are required for ectopic and likely normal expression of *HOX* genes in the absence of H3-K27 methylation. It is tempting to speculate and extrapolate this to general transcription. This would imply that H3-K27 acetylation is either only helping transcriptional activation, or that H3-K27Ac is not a cause but a consequence of transcription or, finally, that it is a by-product of the acetylation of other substrates. It might also be that H3-K27 acetylation is only required to antagonise or block methylation of H3-K27 by PRC2. This would be in accordance with recent findings that H3-K27 acetylation by CBP counteracts Polycomb repression (Tie et al., 2012).

Interestingly, recent studies in mouse ES cells show that H3.3 is required for high levels of H3-K27 trimethylation by PRC2 at bivalent and developmentally regulated genes, by generating a dynamic environment that would facilitate PRC2 in methylating H3-K27 (Banaszynski et al., 2013). In the system employed in my study, I did not observe a more severe phenotype in cells lacking H3.3 (**Figure 11**). This might be because once there is misexpression of PcG target genes it would be difficult to detect a "higher" misexpression, in my system. The more severe phenotype that could be expected is the lack of proliferation of the*, $H3^{K27R}$, $\Delta H3.3$* clones, which I do not observe. Furthermore this function of H3.3 might be a mammalian specific function. Alternatively, since it is known that H3.3 is not essential for *Drosophila* viability but required for fertility, it might be that H3.3 plays a role in facilitating H3-K27 methylation by PRC2 in the *Drosophila* germ line (Hödl and Basler, 2009).

4.1.3 $H3^{K27M}$ and $H3.3^{K27M}$ mutants

Histones H3 and H3.3 in which lysine 27 is mutated into methionine ($H3^{K27M}$ and $H3.3^{K27M}$) are associated with a specific type of paediatric glioblastoma (Wu et al., 2012; Schwartzentruber et al., 2012). Further studies demonstrated that expression of $H3^{K27M}$ and $H3.3^{K27M}$ in a wild-type background significantly reduced the bulk levels of H3-K27me3. The same study suggested that replacement of the lysine 27 with a methionine on H3 and H3.3 acts as a dominant negative mutation by inhibiting the histone methyltransferase activity of PRC2 complexes (Lewis et al., 2013). The authors also express $H3^{K27R}$ in mammalian cell lines as a control. Indeed, in a wild-type background, expression of $H3^{K27R}$ does not seem to affect the levels of H3-K27 trimethylation. This is in line with my fly stocks containing twelve copies of $H3^{K27R}$ transgenes versus twenty-

three endogenous copies of the wild-type histone H3, presenting a normal physiology. This suggests that the *H3^{K27R}* mutation does not behave like the *H3^{K27M}* mutation, which seems to specifically inhibit the enzymatic activity of PRC2.

4.1.4 How does H3-K27 methylation bring about PcG repression of target genes?

How H3-K27me3 brings about transcriptional repression of *HOX* genes is still an open question. One hypothesis would be that H3-K27me3 provides an anchor for a transcriptional repressor. Knowing that the Pc subunit of PRC1 binds to H3-K27me3 via its chromodomain, it is tempting to speculate that H3-K27me3 facilitates the binding of Pc and thereby facilitates the recruitment of PRC1. Which in turn could execute chromatin compaction on target genes. However, to date there is no experimental evidence that Pc's chromodomain is required for repression. Systematic mutation of Pc 's chromodomain *in vivo* might help shed light on this problem. Although, the high dissociation constant of the interaction between Pc and H3-K27me3 indicates that this interaction is probably not strong enough to be the only force involved in recruitment of PRC1, but it might in any case facilitate the binding of PRC1. Another hypothesis to be considered is that H3-K27 methylation contributes to the spreading of a repressive environment, by for example facilitating "looping" between the PRC1 complex bound at PREs and the body of target genes (Kahn et al., 2006). The looping might be facilitated by Pc binding to H3-K27me3 via its chromodomain, and by the DNA binding ability of other PcG proteins (Pasini et al., 2010b). This looping might facilitate the spreading of repression. It would still be unclear how exactly PcG complexes would bring about transcriptional repression. Would recruitment of PRC1 be sufficient? Indeed, PRC1 has been shown to interact directly with TFIID, raising the possibility that this interaction might block the transcriptional machinery (Saurin et al., 2001). In addition, PRC1-type complexes could also directly promote chromatin compaction to bring about transcriptional repression (Shao et al., 1999; Francis et al., 2004).

It is likely that PcG repression requires several different modes of recruitment and spreading to repress target genes properly. It seems intuitive that all PcG target genes are not regulated in the same manner this will be discussed in (section **4.2.5**). Some target genes might for example require Ph to be properly repressed but not H2A ubiquitylation, for example. This is illustrated by the finding that Pc target genes can be sorted into two classes, the ones that require *Pc* and *Sce/Ring*, *Ph* and *Psc-Su(z)2* for

repression and the ones that don't require *Pc* or *Sce* but only *Ph* and *Psc-Su(z)2* (Gutiérrez et al. 2012). In support for the hypothesis that the function of H3-K27me3 in PcG repression is to provide an anchor site for the Pc subunit, in somatic cells, *Pc* mutants just like *H3^{K27R}* mutants and *PRC2* mutants, misexpress *HOX* genes and *en.* This indicates that they have compatible phenotypes and are likely to be involved in the same pathway.

4.1.5 Outlook

Cells from the *H3^{K27R}* clones might be a suitable model to study how H3-K27 methylation affects transcription of target genes at the molecular level. Despite it being complicated to acquire a sufficient amount of cells to perform molecular assays it could yield interesting results. For example, one could think about performing ChIP analysis on these cells to see how the lack of H3-K27 methylation affects the binding of PcG complexes and transcriptional activators. In a similar way, genome wide studies like RNA sequencing might provide information about how transcription is affected in the absence of H3-K27 methylation. However one must take into account that *H3^{K27R}* cells not only lack trimethylation of H3-K27, but they also lack mono- and dimethylation of H3-K27 and H3-K27 acetylation. This might make the results difficult to interpret although in the case of the misexpression phenotype in the imaginal disc clones and the embryos, it is clear that the *H3^{K27R}* phenotype mimics the phenotype of cells lacking E(z), the histone methyltransferase subunit of PRC2 (section **3.2**).

Having demonstrated that H3-K27 trimethylation is critically required for Polycomb repression, it will now be crucial to dissect the mechanisms of epigenetic inheritance of the H3-K27me3 mark through generations of cells during development. Indeed, several studies have addressed the question of transmission of histone post-translational modifications through the cell cycle (Hansen et al., 2008; Margueron et al., 2009; Angel et al., 2011). Interestingly a model for the transmission of H3-K27me3 has been proposed in which, during S phase, PRC2 binds to established H3-K27me3 and methylates the neighbouring nucleosomes and thereby maintains H3-K27me3 through the cell cycle (Hansen et al., 2008). However, this study was performed in transformed cell lines, and not in a developing animal. Moreover, the authors don't provide direct *in vivo* evidence for the stable binding of PRC2 to H3-K27me3 during S phase at target genes, they show H3-K27me3 peptide pull downs on soluble nuclear extracts of human cell lines, although they do not exclude the possibility that perhaps PRC2 subunits associate with

H3-K27me3 peptides indirectly via other protein components. Additionally the authors proposed that establishment of transcriptional silencing is independent of the catalytic activity of EZH2, but they also state that H3-K27me3 is required for the maintenance of transcriptional repression.

Another study reported that the murine ortholog of Esc, Eed, binds to the H3-K27me3 nucleosomes and that this binding stimulates the methyltransferase activity of PRC2 complexes (Margueron et al., 2009). The authors propose that this mechanism contributes to the transmission of the H3-K27me3 mark through the cell cycle and thereby to the inheritance of H3-K27me3 from mother to daughter cells. However the authors do not confirm this hypothesis *in vivo.*

Therefore the question of the epigenetic inheritance of H3-K27me3 through the cell cycle still remains open. A more thorough *in vivo* analysis of the inheritance of H3-K27me3 through replication will be required to draw clear conclusions about this matter.

4.2 Functional analysis of H2A ubiquitylation

In summary, my results on the analysis of H2A ubiquitylation by PcG repressors provide compelling evidence that: (1) H2A ubiquitylation is not required for PcG repression of *HOX* genes but is required for viability at the organism level; (2) H2A ubiquitylation is required early in embryonic development; (3) H2A ubiquitylation is required for normal levels of bulk H3-K27me3 in embryos but not in larval tissues; (4) H2Av ubiquitylation by PRC1-type complexes is not required for PcG repression, viability or fertility; (5) In the absence of Sce/Ring protein, the Pc subunit of PRC1 is destabilised which suggests the PRC1 complex is architecturally destabilised; (6) PR-DUB, the enzyme that removes H2A ubiquitylation, is only required for Polycomb repression of *Ubx* when H2A is ubiquitylated. It is important to note that, my histone point mutant analysis of cells lacking the histone residue substrates of PRC1-type complexes, and the analysis of cells lacking the catalytic activity of PRC1-type complexes show complementary results that lead to the same conclusions.

My findings set the stage for a new vision of how PcG transcriptional repression is achieved.

4.2.1 Promiscuity of H2A ubiquitylation by PRC1-type complexes

In this thesis, I perform a mutagenesis on the H2A C-terminal tail, in order to remove ubiquitylatable residues by PRC1 E3 ligases. To ensure that all putative ubiquitylation sites were removed, I mutated all four lysines on the C-terminus of H2A (**Figure 12**) under the hypothesis that PRC1 could potentially ubiquitylate all four lysines. An *in vitro* E3 ubiquitin ligase assay using a minimal recombinant PRC1 complex on recombinant *Drosophila* mononucleosomes, showed that H2A ubiquitylation was only completely abolished when all four lysines were mutated (Reinhard Kalb, personal communication).

4.2.2 H2A ubiquitylation is not required for PcG repression of *HOX* genes.

Previous findings in mouse ES cells demonstrate that in cells KO for one of the mammalian orthologs of Sce/Ring, Ring1B, most of the PRC1 subunits are destabilised and degraded (Eskeland et al., 2010). Interestingly, the authors of this study suggest that when Ring1B KO cells are rescued by a catalytically inactive version of Ring1B, that can no longer ubiquitylate H2A, chromatin compaction of *HOX* genes is maintained. Thus, the authors propose that chromatin compaction can be achieved in the absence of H2A ubiquitylation. However, in these Ring1B catalytically inactive mutant cells, the Ring1A paralog of Ring1B is still present, and so are substantial residual levels of H2A ubiquitylation. It is therefore difficult to come to the conclusion, from this data, that H2A ubiquitylation is not required for chromatin compaction of *HOX* genes. In addition, another report in murine ES cells, in which both Sce/Ring orthologs were removed and rescued with a catalytically inactive version of Ring1B, showed that in fact there is a mild misexpression of previously uncharacterised PcG bound genes, in particular the *Pax3* gene (Endoh et al., 2012). Therefore, in mammals, the precise role of H2A ubiquitylation in PcG repression also remains unresolved.

In this thesis, by analysing the phenotype of cells lacking H2A and H2Av ubiquitylatable residues, I provide, for the first time direct conclusive evidence that H2A ubiquitylation is not required for PcG repression of *HOX* genes.

My histone rescue experiments with mutant H2A versions that can no longer be ubiquitylated, complemented with the study of animals lacking the E3 ubiquitin ligase activity of PRC1-type complexes, provide direct *in vivo* evidence that H2A ubiquitylation

is not required for Polycomb repression of *HOX* genes. This finding was unexpected, since recent functional analysis of the E3 ligase Sce/Ring had shown that Sce/Ring itself was required for repression of PcG target genes (Gutiérrez et al., 2012). However in this previous study, the catalytic activity of Sce/Ring was not analysed, only the complete depletion of Sce/Ring1B. Instead, I not only analyse the catalytic activity of Sce/Ring and its impact on transcriptional repression of target genes, but I also directly study the requirement of the substrate residues of Sce/Ring on the C-terminal tail of H2A and H2Av for normal expression of PcG target genes (**Figure 13** and **Figure 19**)**.** Furthermore, I show that in embryos lacking the Sce/Ring protein, the protein levels of the Pc subunit of PRC1 are highly reduced (**Figure 23**). This indicates that the PRC1 complex might be destabilised without Sce/Ring. It also suggests that the reason why Sce/Ring is required for PcG repression is to architecturally stabilize the PRC1 complex, and not because of its E3 ubiquitin ligase activity for H2A or H2Av.

4.2.3 H2A ubiquitylation is required for viability

I observe a striking difference between the lifespans of catalytically inactive mutants for *Sce* (*Sce*^{I48A}) that still have the maternal contribution in wild-type Sce product (*Sce*$^{I48A\ m+;\ z-}$) and the ones that no longer receive the maternally contributed wild-type Sce product (*Sce*$^{I48A\ m-;\ z-}$) (**Figure 20** and **Figure 22**). In particular, *Sce*$^{I48A\ m+;\ z-}$ animals develop into third instar larvae, and some develop into pharate adults (**Figure 20**), whereas the vast majority of *Sce*$^{I48A\ m-;\ z-}$ animals do not complete embryogenesis, and only very few develop into first instar larvae. Interestingly, my analysis of embryos lacking zygotically expressed endogenous wild-type H2A histone proteins but containing mutant H2A histones (H2A$^{4K->\ 4R}$) that cannot be ubiquitylated shows that these animals develop into normal looking embryos that however arrest development before hatching into larvae (section **3.3.2.1b**). In *H2A*$^{4K->\ 4R}$ embryos, histones need to be readily available to package the replicating DNA at every rapid S-phase during *Drosophila* embryogenesis and therefore the maternal load of wild-type histones is rapidly diluted. In contrast, in *Sce*$^{I48Am+;\ z-}$ embryos containing the maternal contribution of wild-type Sce/Ring, it is unlikely that the wild-type Sce/Ring protein is diluted out so rapidly. It is instead conceivable that the maternal wild-type Sce/Ring protein persists until later stages than the maternally contributed wild-type histones. One could speculate that wild-type Sce and ubiquitylated H2A persist until later stages in embryogenesis, thereby enabling *Sce*$^{I48A\ m+;\ z-}$ embryos to survive until the pharate adult stage. Therefore, these

findings are both consistent with H2Aub having a critical role in the early stages of embryogenesis. Another conclusion that can be drawn from this observation is that when supplied maternally, at the beginning of embryogenesis, H2A ubiquitylation is not required for development into relatively normal pharate adults. However, the question of why these animals die still remains open. The answer to this question lays perhaps in the relatively subtle abnormalities of the PNS sensory organs (bristles) these animals present.

4.2.4 H2A ubiquitylation is required for wild-type levels of bulk H3-K27me3 in embryos but not in larval tissues

I find that *Sce*$^{I48A\ m-;\ z-}$ embryos exhibit a decrease in the bulk levels of H3-K27me3 (**Figure 24**). This is consistent with very recent reports that show that a variant PRC2 complex binds to nucleosomes monoubiquitylated on H2A and that this increases the H3-K27me3 histone methyltransferase activity of the variant PRC2 complex (Kalb et al., 2014). Two additional studies show that tethering of a minimal ubiquitin ligase PRC1 module to ectopic sites on the genome of mouse ES cells is sufficient to bring about high levels of H3-K27me3 (Blackledge et al., 2014; Cooper et al., 2014). My data suggest that H2A ubiquitylation is required for normal bulk levels of H3-K27me3 but not for repression of *HOX* genes. This would imply, that at least at *HOX* genes, H2A monoubiquitylation is not required for the necessary levels of H3-K27me3 for PcG repression. The question then is, is there a threshold on the levels of H3-K27me3 that decides if the mark is repressive or not, and if so, is this threshold the same at all PcG target genes? If the answer to this question is positive, then the reduction of the bulk levels of H3-K27me3 is not causative of high-level misexpression of any (*HOX* or uncharacterised) PcG target genes. This would imply that the increase of PRC2 histone methyltransferase activity on H2Aub nucleosomes is some sort of backup mechanism that facilitates the establishment of a certain level of H3-K27me3 at target genes, but that this is not critically required for repressive levels of H3-K27me3. In support of this hypothesis, it has been shown that in mouse ES cells lacking the Sce/Ring orthologs Ring1A and Ring1B, there is a global genome wide reduction of H3-K27me3 (Blackledge et al., 2014).

Alternatively, the reduction of H3-K27me3 might be differential for distinct sets of target genes. This would imply that H2Aub is required for proper levels of H3-K27me3 and transcriptional repression at only a subset of target genes. This problem could be

addressed by performing genome wide analysis of H3-K27me3 coupled to H2Aub levels in *Sce*$^{I48A\ m-;\ z-}$ embryos.

Intriguingly, this reduction in H3-K27me3 observed in *Sce*$^{I48A\ m-;\ z-}$ embryos is not detectable in *Sce*$^{I48A\ m+;\ z-}$ larvae (**Figure 24** versus **Figure 18**). This leads to the following hypothesis. The reduction of H3-K2me3 in *Sce*$^{I48A\ m-;\ z-}$ embryos is established very early in embryogenesis, probably before the start of zygotic transcription. In contrast, *Sce*$^{I48A\ m+;\ z-}$ embryos are rescued by the maternal contribution in wild-type Sce, which provides sufficient H2A ubiquitylation at genes that require H2A ubiquitylation for establishment of H3-K27me3 during embryogenesis. This would be in line with the idea that the bulk reduction in H3-K27me3 observed, together with other unknown effects, is causal of the phenotype observed in the *Sce*$^{I48A\ m-;\ z-}$ embryos. However, if this bulk level reduction is the cause of the arrest of *Sce*$^{I48A\ m-;\ z-}$ embryos or not remains unclear. A combined approach of RNA sequencing and ChIP sequencing on these embryos will perhaps provide useful insights into the answer to this question as proposed in (section **4.2.6**)**.**

4.2.5 PcG repression of target genes: one-size-does-not-fit-all.

My results suggest that H2A ubiquitylation is not required for *HOX* gene repression (section **3.3**). However, due to the lack of adequate antibodies for ChIP, whether H2A ubiquitylation decorates the nucleosomes of *HOX* gene chromatin or not is not known in *Drosophila* embryos or imaginal discs. Nevertheless, a report in which ChIP microarray was performed in the *Drosophila* BG3 central nervous system cell line, suggests that H2A ubiquitylation is absent from *HOX* genes in these cells (Schaaf et al, 2012). Given the dynamic nature of H2Aub, it is tempting to speculate that the fact that no H2Aub is observed on *HOX* genes might be due to H2Aub nucleosomes being present only very transiently at *HOX* genes, rapidly deubiquitylated by PR-DUB. The alternative hypothesis would be that H2Aub does not occur at *HOX* genes, but this would be difficult to explain given that PRC1 localises to *HOX* genes; unless one imagines that a repressor of the E3 ubiquitin ligase activity of PRC1-type complexes is also present at *HOX* genes, or that a variant PRC1 complex in which the E3 ubiquitin ligase activity is blocked or reduced is present at these genes. In any case, although one cannot extrapolate these findings from one cell type specific cell line to embryos or imaginal discs, it is conceivable that perhaps H2A ubiquitylated nucleosomes are only present transiently at *HOX* genes in embryos and imaginal discs. However, this does

not exclude the possibility that H2A ubiquitylation at other, yet uncharacterised PcG target genes is more persistent.

In any case this raises the possibility that we might not be aware of all PcG target genes, and that perhaps, not all PcG genes are regulated in the same manner. This observation has been made for other target genes that differentially require distinct subunits of the PRC1 complex (Gutiérrez et al., 2012). Like for example *Dachshund* and *Prospero* that require *Psc-Su(z)2* and *Ph* but not *Sce* or *Pc* for their normal expression patterns. Gutiérrez et al. propose a classification of PcG target genes based on their requirement for PRC1 subunits. It is perhaps even more complex than that and there are many different classes of target genes, and one or more categories could require H2A ubiquitylation for repression by PcG complexes, and their misexpression might be responsible for the death of $Sce^{I48A\ m-;\ z-}$ and $Sce^{I48A\ m+;\ z-}$ animals. However this remains to be confirmed.

4.2.6 Outlook: Why do embryos lacking H2A ubiquitylation die?

To answer the question of why these embryos die, RNA sequencing experiments on $Sce^{I48A\ m-;\ z-}$ embryos could be conducted to study which genes are misregulated. This approach has the caveat that one would be looking at many different cell types, in particular if the experiment is carried out in late stage embryos. This however would probably be the best approach, since by this stage, most of the radical transcriptional changes occurring early during embryogenesis will have already taken place and the normal transcriptional variation between different developmental stages will not interfere with the analysis. Nevertheless, it would be important to keep in mind that this experiment is not ideal since a cell type specific data set will not be generated. The data will represent the average of many different cell types, so if there is a subtle transcriptional change in a few cells, it will not be detectable.

In addition, with this technique, if a gene is differentially expressed in the $Sce^{I48A\ m-;\ z-}$ embryos versus the wild-type, it will be difficult to distinguish between this being a direct effect or an indirect effect. Therefore this experiment would have to go hand in hand with ChIP sequencing experiments on these embryos to determine which genes are decorated by H2Aub and H3-K27me3 and which are not, and thereby potentially identify genes that are directly affected by the lack of the modification. It would also be interesting to define which genes are affected by the reduction of bulk levels of H3-

K27me3. At the moment it is not known if this reduction in the bulk levels reflects a high level reduction of H3-K27me3 localised at certain genes, that causes up-regulation of certain genes and a 'cascade' of transcriptional events that lead to death or, if the bulk reduction in H3-K27me3 merely represents a genome wide low-level reduction of H3-K27me3 (in which case the transcriptional misregulation might not be due to the lack of H3-K27me3 but rather to other effects). The latter hypothesis would be in line with recent work in mouse ES cells showing that the lack of one of the mouse orthologs of dKdm2, Kdm2b, that is required for high levels of H3-K27me3 at target genes, has a mild effect on the transcription of these genes (Blackledge et al., 2014). H2A ubiquitylation might be required for the transcriptional regulation of a subset of genes independently of H3-K27me3. Lack of ubiquitylation might result in low-level misexpression or in up-regulation of a subset of target genes. This low misexpression might collectively be toxic and result in the death of *Sce*$^{I48A\ m-;\ z-}$ animals.

If the RNA sequencing does not provide evidence of any changes in transcriptional outputs in *Sce*$^{I48A\ m-;\ z-}$ embryos, it will be necessary to consider other hypotheses, independent of H2Aub having a function in transcriptional regulation.

It is conceivable that the levels of free ubiquitin in the cell and even in the nucleus in the absence of the E3 ubiquitin ligase activity of PRC1-type complexes, becomes too high and has a toxic effect for the embryos which leads them to death. This effect might happen only in a subset of embryonic cells not in the whole embryo. To confirm such a hypothesis it would be necessary to test the free ubiquitin levels in *Sce*$^{I48A\ m-;\ z-}$ embryos, preferably in a cell type specific way, which will be challenging.

4.3 H2A must not be ubiquitylated for *Ubx* repression

The genetic interaction analysis between PR-DUB, the enzyme that removes H2A ubiquitylation, and a catalytically inactive allele of PRC1, the E3 ligase that monoubiquitylates H2A, suggests that H2A monoubiquitylation needs to be removed for Polycomb repression of *Ubx* to be achieved (**Figure 25**). However, the question remains: at which genomic locations does H2A ubiquitylation need to be removed by PR-DUB for proper repression of target genes?

Two main hypotheses can be formulated to address this issue:

1 Extra H2Aub might drain PRC2 away from target genes. This hypothesis is based on the idea that PRC1 would ubiquitylate H2A at target genes and at ectopic sites genome wide. PR-DUB in turn would remove the H2Aub from the ectopic sites specifically. In PR-DUB mutants, hyper-H2Aub at ectopic sites would cause PRC2 (that cruises the genome generating H3-K27me1 and H3-K27me2) to dwell at these ectopic sites with H2Aub and thereby would effectively deplete PRC2 from the genes that need it, like *HOX* genes. The implication of this hypothesis would be that PR-DUB mutants would have hypo-H3-K27me3 at *HOX* genes, which would explain their misexpression in these mutants. Nonetheless, the only ChIP data available for H2Aub in *Drosophila*, from a BG3 cell line, shows that H2A ubiquitylated nucleosomes do not localise at *HOX* genes (Schaaf et al., 2013). Despite these results not being directly extrapolatable to imaginal wing discs or *Drosophila* embryos, this data would be in line with the removal of ubiquitylated H2A by PR-DUB on *HOX* gene chromatin and thus the lack of signal in ChIP for ubiquitylated H2A on *Ubx*. This leads to my second hypothesis.

2 PRC1 type complexes have a dual function. As mentioned in (section **1.2.1.2a**), PRC1 has two main functions: a non-enzymatic function which lies in the ability to compact nucleosomes; and an enzymatic function, which consists in E3 ubiquitin ligase activity for H2A. The chromatin compaction function of PRC1 type complexes relies on the compaction abilities of the Psc and Ph subunits and also on the Pc subunit to scan for the H3-K27me3 mark. Sce would be an architectural link between Psc and Pc and thus required for repression. Sce also has an E3 ubiquitin ligase activity for H2A. However H2Aub would be detrimental for chromatin compaction and an “unwanted side product” on the chromatin of *Ubx*. Therefore, H2Aub on *Ubx* chromatin would need to be removed by PR-DUB in order for PRC1 to compact and repress *Ubx* transcription.

To properly confirm or disprove any of these hypotheses it will be necessary to determine the genome wide localisation of H2Aub and H3-K27me3 in PR-DUB mutants, compared to wild-type by ChIP sequencing.

Taken together, these striking and surprising findings not only finally seem to shed light on the function of H2A deubiquitylation in PcG repression, but perhaps also on the apparent paradox of two different enzymes (PR-DUB and PRC1-type complexes), with antagonistic catalytic activities on the same substrate (the modified or unmodified H2A

C-terminal tail) being involved in the same biological process. However, it is puzzling from an evolutionary perspective: why would cells “waste energy" ubiquitylating nucleosomes that subsequently have to be deubiquitylated to bring about transcriptional repression? Different complementary hypotheses could explain this apparent conundrum. Firstly *in vitro* studies show that, at least in mammals, the efficiency of H2A monoubiquitylation varies with the composition of the PRC1-type complexes (Gao et al., 2012). One could imagine that the complexes which inefficiently monoubiquitylate H2A are present on *Ubx* chromatin and perhaps monoubiquitylating nucleosomes at a relatively low rate or not at all. PR-DUB would then ensure that all the “residual” H2Aub from these reactions is removed. Removal of H2A ubiquitylation, would in turn facilitate chromatin compaction and transcriptional repression of *Ubx,* perhaps by generating a chromatin environment more permissive to compaction.

Secondly, one might ask, how come an E3 ligase is targeted to *Ubx* if this activity is not required, and even perhaps detrimental to the proper transcriptional silencing of the gene? It is important to remember that Sce plays an important architectural role in the PRC1 complex, most likely by bridging the Psc subunit to the Pc subunit. Therefore, the complex needs this E3 ligase subunit physically for its stability and to link the chromatin compaction ability of Psc to the H3-K27me3 scanning function of Pc (**Figure 23**).

In summary, ubiquitylated H2A is most likely a very dynamic mark, that depending on the target genes might be present just transiently, it might confer a special chromatin conformation that facilitates the binding of PcG repressors, establishment or spreading of H3-K27 methylation, but that needs to be removed in order for chromatin compaction, and thus transcriptional repression to take place.

4.4 H2Av ubiquitylation by PRC1-type complexes is not required for PcG repression, viability or fertility

With the aim of analysing cells lacking all ubiquitylatable residues on H2A and H2Av at the same time (**Figure 14A**), I first tested the existing H2Av allele *H2Av^{810},* for lack of H2Av product. I find that the existing “null" allele of H2Av: *H2Av^{810}*, is in fact a hypomorphic allele that still produces a truncated version of H2Av, lacking only its second exon. However, the levels of this H2Av^{810} truncated product are very low, therefore, it is likely that this minimal product does not affect the validity of the results found in all the studies that have been published using this allele (van Daal and Elgin, 1992).

Second, I generated a proper KO allele of *H2Av* by homologous recombination (Gong and Golic, 2003), and found that although these animals still develop into 3rd instar larvae, they present wild-type cuticle and normal central nervous systems but hardly detectable imaginal discs, unlike the larvae homozygous for the *H2Av*^{810} allele. This indicates that the low levels of the H2Av^{810} truncated product are sufficient to drive imaginal disc development until the end of the larval stages. It is important to consider that this truncated version of H2Av still contains not only the C-terminal lysines that are monoubiquitylated by PRC1-type complexes, but they also contain the H2Av acidic patch, which has been shown to be essential for *Drosophila* adult development (Clarkson et al., 1999). Although in this study a rescue assay was performed with the *H2Av*^{810} allele. This implies that the expression of a rescue transgene of H2Av lacking the acidic patch actually has a dominant negative effect. This indicates that the acidic patch of H2Av plays a crucial role for viability.

Interestingly, I find that *H2Av*^{KO} clones do not proliferate (**Figure 16**), and as mentioned before, that imaginal discs from *H2Av*^{KO} homozygous animals are hardly detectable. This is somewhat intriguing, given that *H2Av*$^{KO\ m+;\ z-}$ animals, survive into the third instar larval stage. This might be explained by the maternal contribution in H2Av. Indeed, several studies have found H2Av protein in lipid droplets of maternal origin in *Drosophila* embryos (Cermelli et al., 2006; Li et al., 2012; Li et al., 2014), and it is well established that the larval CNS in *Drosophila* have embryonic origins (Truman, 1990). In addition, most larval tissues instead of dividing, become polyploid and larger in size, with the exception of imaginal discs that are larval diploid tissues, that rapidly proliferate during the larval stages, like for example the imaginal wing discs proliferate from a 20 cell primordium set aside at the end of embryogenesis to a 30,000 to 50,000 cell mature imaginal disc at the 3rd instar larval stage (Martin et al., 2009). Therefore it is conceivable that in imaginal discs, the maternal contribution of wild-type H2Av is exhausted very soon during larval development, and that in the case of the cuticle and the larval CNS, that go through considerably fewer cell divisions, the maternally contributed H2Av protein persists much longer. It is also tempting to speculate that the H2Av histone products stored in lipid droplets are somehow directed to the CNS and the cuticle.

Third, I do not observe any homeotic transformations of *H2Av*^{KO} homozygous larvae, or any other typical Polycomb phenotypes, which is in contrast to what has been proposed by Swaminathan et al., 2005. It is important to note that this is the only study proposing such a hypothesis (section 1.1.1.2).

Fourth, I demonstrate that H2Av ubiquitylation on lysine 120 and 121 (**Figure 14A**) by PRC1 complexes is not required either for PcG repression of *HOX* genes (**Figure 16**)**,** for viability or fertility. This is in contrast to models proposing that ubiquitylation of the mammalian counterpart of H2Av, H2A.Z might be critical for PcG repression (Creyghton et al., 2008). If this hypothesis is proven to be true in mammals, it suggests either that in mammals, H2A.Z ubiquitylation is required for PcG repression, or that neither in mammals nor in *Drosophila* H2Av ubiquitylation is required for PcG repression. To distinguish between these two possibilities, it will be necessary to perform H2A.Z point mutant studies in mouse ES cells and embryos, perhaps taking advantage of the CRISPR/Cas system (Yang et al., 2013).

Given that in murine ES cells H2Av seems to confer a permissive chromatin structure for the binding of PcG complexes, it is conceivable that H2Av is localised at PcG target genes. This could imply that H2Av ubiquitylation might just be a side effect of the ubiquitylation of canonical H2A by PRC1-type complexes because of its localisation at +1 nucleosomes (Hu et al., 2013).

Finally, I find that imaginal disc cells in which all ubiquitylatable residues on H2A and H2Av are mutated (*H2A$^{4K->4R}$, H2Av$^{KK->RR}$*), do not show misexpression of *HOX* genes, or of the orthologs of genes found to be up-regulated in mouse ES cells lacking H2A ubiquitylation (**Figure 17**) (Endoh et al., 2012). This is strong direct evidence, that in larval imaginal disc cells, neither H2A nor H2Av ubiquitylation are required for the maintenance of PcG repression.

4.5 Polycomb repression beyond histone modifications

In summary, my results suggest that histone modifications are differentially required for Polycomb repression. H3-K27 methylation is required for the repression of *HOX* genes and other developmental regulator target genes. In stark contrast, H2Aub is not required for *HOX* gene repression, or any other developmental regulator gene tested. This implies that these two histone modifications have very different functions, and one of them is dispensable for the repression of *HOX* genes (sections **3.2** and **3.3**). This leads to the question, if one of the histone modifications that were thought to bring about Polycomb repression does not actually have that function, then how is Polycomb repression achieved? Is solely H3-K27me3 sufficient to bring about repression? This seems unlikely. A more plausible idea would be that the chromatin compacting ability of

PRC1 complexes via their Psc and Ph subunits is the main trigger for transcriptional repression, at least at *HOX* genes (Francis et al., 2004). Additional features of PRC1 are likely to also contribute to the spreading of compaction, like for example the ability of PRC1-type complexes containing Ph to form ordered PRC1 polymers via the Ph sterile alpha motif (SAM) domain (Francis et al., 2004; King et al., 2005; Isono et al., 2013; Gambetta and Müller, 2014). This polymerisation function might cause the spreading of PRC1-type complexes and thereby the spreading of chromatin compaction. Additional spreading of the compaction is likely facilitated by the binding of the Pc subunit of PRC1 complexes to H3-K27me3.

4.6 The histone rescue system

I find that twelve copies of wild-type *Drosophila* histone gene units (HisGUs) (that contain one copy of each of the canonical core histone genes and the linker histone gene, **Figure 2**) are sufficient, to rescue the proliferation of imaginal disc clones homozygous for the deletion of the endogenous histone gene cluster (Δ*HisC*). In addition, these twelve copies of wild-type HisGUs are also sufficient to rescue Δ*HisC* homozygous animals into viable adults. This is in agreement with the study that originally established this histone rescue system (Günesdogan et al., 2010).

Interestingly, the endogenous histone cluster contains 23 haploid copies of the HisGUs, and 46 diploid copies. Strikingly, this suggests that twelve copies of the HisGUs encoded by transgenes can fully replace the 46 endogenous HisGU copies. It is interesting to ask, how come the *Drosophila* genome has 46 copies of HisGUs when it only requires twelve copies for viability?

Interestingly, even if animals homozygous for the Δ*HisC* deletion, rescued by twelve copies of wild-type HisGUs, develop into viable adults, I find that they are only semi-fertile, and that it is not possible to maintain them as a stock. This suggests that proper germ line development requires more than twelve copies of the HisGUs. Indeed, several reports have demonstrated that during stage 10 of *Drosophila* oogenesis there is a burst of histone gene expression in the nurse cells, and then these *Histone* messages are exported to the oocyte (Ruddell et al., 1985, Ambrosio et al., 1985). The authors of these studies suggest that these mRNAs are then translated and used up during the rapid nuclear divisions at the early stages of embryogenesis. This implies that perhaps, in adults rescued by only twelve copies of HisGUs this burst of histone

transcription during oogenesis might not be sufficient to supply the early embryonic nuclear divisions in histones.

From an evolutionary perspective, considering that the histone genes of *Drosophila* form tandem repeats of HisGUs in a single cluster, it is tempting to speculate that these repeats originated from several duplications of the histone gene unit in the *Drosophila* lineage. Indeed this is the manner in which histone genes are thought to have evolved initially from Archaebacteria (Eirín-López et al., 2009).

4.7 General conclusion

Taken together, my results demonstrate that H3-K27 methylation but not H2A-K118 monoubiquitylation is required for Polycomb repression of *HOX* genes.

This is, to my knowledge the first time that it is directly shown, in metazoans, that histone modifications are required for the maintenance of a transcriptional status during development. In addition, my results provide the first direct *in vivo* proof that H2A ubiquitylation is not required for *HOX* gene repression. The exact function of H2A ubiquitylation remains however a mystery but does not seem to be related to PcG repressive function. It will be important to understand the function of H2Aub beyond PcG repression.

This striking result indicates that multimeric protein complexes containing histone modifying activities do not necessarily use their enzymatic activities to exert a transcriptional readout, but instead, perhaps in an enzymatic activity independent manner and in a composition specific manner, they dictate a transcriptional readout. It is not because a complex has a histone modifying activity and it is localised on target gene chromatin, that this enzymatic activity will be necessarily the medium of repression employed by the complex.

These results open a new door into the molecular understanding of the function of PcG proteins *in vivo*. It will now be possible not only to direct studies into understanding how H3-K27me3 brings about transcriptional repression but also to investigate how this modification is interrelated to non-histone-modifying and chromatin compacting mechanisms, which are crucial for PcG transcriptional repression.

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- Supervised 3 Biochemistry Master's students from the Ludwig-Maximilians-University (LMU), Munich and from the Eberhard-Karls-University, Tübingen, during a six-week internship/placement (2011 and 2013).

Research Experience

- **Wellcome Trust Centre for Cell Biology, Edinburgh, U.K.** Six month Master's thesis, subject: "Affinity selection of intact centromeric heterochromatin on plasmids from fission yeast"; supervised by Prof. Robin Allshire. (January 2010 to July 2010).
- **Fred Hutchinson Cancer Research Center, Seattle, U.S.A.** Five months internship, subject: "The role of the miR-200 family in the hypoxia pathway"; supervised by Dr. Muneesh Tewari. (April 2009 to August 2009).
- **Karolinska Institute, Stockholm, Sweden.** Three months summer internship, subject: "Study of the Nse proteins of the SMC5/6 complex in budding yeast"; supervised by Prof. Camilla Sjogren. (June 2008 to August 2008).
- **University of Liverpool, U.K.** Two months and a half internship, subject: "Expression and characterisation of certain domains of the PfEMP 1 protein involved in severe malaria" supervised by Dr. Ed Yates. (April 2007 to June 2007).