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INHERITANCE OF THE H3K27ME3 MODIFICATION IN THE ABSENCE OF A PRE

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“WHAT YOU THINK TO BE A PEAK IS NOTHING BUT A STEP.”

LUCIUS ANNAEUS SENECA

TO B., L., J., AND E.

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Summary

This study aimed to decipher the key mechanisms by which epigenetic information is inherited using the example of the central, repressive histone modification in *Drosophila*: histone 3 lysine 27 trimethylation (H3K27me3). This question is of special concern, since it allows for fundamental insights into the regulation of cell identities and thereby into developmental processes in general. Cell identities are defined by a very characteristic gene expression profile that is associated with the specific cell type. It is indispensable that these gene expression profiles remain constant throughout the life span of a cell and therewith maintain its identity – even after cell divisions. Whether specific genes are turned on or off is determined by different factors – the presence of histone modifications seems to play a major role. To ensure proper maintenance of gene expression profiles in each cell, histone modifications need to be faithfully propagated from one cell generation to the next.

Of special interest in this study was the question, which genetic and molecular mechanisms of inheritance preserve the maintenance of H3K27me3 in *Drosophila*. From earlier studies it was known already that in *Drosophila* the neighboring DNA sequences, the so-called Polycomb Response Elements (PREs), are required to maintain the repressive gene expression state. However, whether loss of repression is associated with loss of H3K27me3 and also, if for propagation of repression the presence of the modification itself or rather the presence of the PRE is essential were open questions. To answer these questions, levels of H3K27me3 were analyzed in a trangene locus in *Drosophila* larvae, from which a PRE was excised at different time points during development. In *Drosophila* Polycomb group (PcG) proteins bind at these defined DNA sequences, in order to modify the chromatin in the proximity, for example by trimethylation of H3K27. It was shown before that both PREs and H3K27me3, set by the Polycomb repressive complex 2 (PRC2), are decisive for maintenance of PcG-dependent repression. Most PcG complexes possess enzymatic activities; with their abilities to modify chromatin and set repressive chromatin marks they ensure correct gene expression states during the development and the propagation of epigenetic information.

In this study, I could show that H3K27me3 is completely lost within a few cell generations after removal of a transgenic PRE. This reduction of H3K27me3 could be abolished in the presence of the cell cycle inhibitor Aphidicolin, indicating that the loss of the H3K27me3 mark is caused by dilution during DNA replication of the cell cycle. This suggests that maintenance of H3K27me3 is not self-sustaining in the absence of a PRE.

Zusammenfassung auf Deutsch

Ziel dieser Studie war es, entscheidende Mechanismen der Vererbung epigenetischer Informationen zu entschlüsseln, am Beispiel einer zentralen, reprimierendenden Histon-Modifikation in *Drosophila*: Histon 3 Lysin 27 Trimethylierung (H3K27me3). Diese Frage ist von großem Interesse, da sie grundlegende Einblicke in die Regulation von Zellidentitäten und somit in Entwicklungsprozesse im Allgemeinen ermöglicht. Die Zellidentität wird durch ihr charakteristisches Genexpressionsprofil definiert, welches eng mit dem spezifischen Zelltyp in Zusammenhang steht. Es ist unabdinglich, dass diese Genexpressionsprofile durch den Lebenszyklus einer Zelle hindurch konstant bleiben und somit die Zellidentität erhalten wird, auch über Zellteilungen hinweg. Ob bestimmte Gene an- oder ausgeschaltet sind, wird durch verschiedene Faktoren bestimmt – dabei kommt Histon-Modifikationen eine zentrale Rolle zu. Um die korrekte Aufrechterhaltung von Genexpressionsprofilen in jeder Zelle zu gewährleisten, müssen Histon-Modifikationen von einer Zellgeneration zur nächsten zuverlässig und fehlerlos weitergegeben werden.

Von besonderem Interesse in dieser Studie war, welche genetischen und molekularen Vererbungsmechanismen die Aufrechterhaltung von H3K27me3 in *Drosophila* sicherstellen. Aus früheren Studien ist bereits bekannt, dass in *Drosophila* die benachbarten DNA-Sequenzen, die Polycomb Response Elemente (PREs), für die Aufrechterhaltung eines repressiven Genexpressionsstatus benötigt werden. Ungeklärt war bisher jedoch, ob dies im direkten Zusammenhang mit H3K27me3 steht und auch, ob für die Aufrechterhaltung der Reprimierung die Präsenz der Modifikation selbst oder die Anwesenheit des PREs entscheidend ist. Um diese Frage zu beantworten wurde die Präsenz von H3K27me3 an einem Transgen-Locus in *Drosophila* Larven untersucht, aus dem zu verschiedenen Zeitpunkten während der Entwicklung ein PRE ausgeschnitten wurde. An diese definierten DNA-Sequenzen binden in *Drosophila* Proteine der Polycomb Gruppe (PcG), um das umliegende Chromatin zu modifizieren, beispielsweise indem H3K27 trimethyliert wird. Es wurde bereits zuvor gezeigt, dass sowohl PREs, als auch

das vom Polycomb repressive complex 2 (PRC2) gesetzte H3K27me3, entscheidend für den Erhalt PcG-vermittelter Reprimierung sind. Die meisten PcG Komplexe sind enzymatisch aktiv; mit ihrer Fähigkeit Chromatin zu modifizieren und reprimierende Modifikationen auf dem Chromatin zu setzen, gewährleisten sie die ordnungsgemäße Genexpression während der Entwicklung und die Weitergabe epigenetischer Informationen.

In dieser Studie konnte ich zeigen, dass H3K27me3 innerhalb weniger Zellgenerationen nach Entfernen eines transgenen PREs vollständig verschwindet. Diese Reduzierung von H3K27me3 konnte in Gegenwart des Zellzyklusinhibitors Aphidicolin aufgehoben werden, was darauf hinweist, dass der Verlust von H3K27me3 durch eine Verdünnung während der DNA-Replikation ausgelöst wird. Dies wiederum suggeriert, dass die Aufrechterhaltung von H3K27me3 in Abwesenheit einer entsprechenden PRE-Sequenz nicht selbsterhaltend ist.

I. Introduction

I. 1. Developmental regulation of gene expression

In multicellular organisms stringent regulation of gene expression is required for the correct execution of developmental programs. Although nearly all cells of an individual organism contain the very same DNA sequence, the cells undergo vastly different developmental programs in order to give rise to the entirety of specified cell types.

Cell type-specific gene expression profiles are controlled by transcription factors that regulate not only which genes are expressed in a specific cell, but also in which developmental stage genes become differentially expressed. Early during development, cells have to take the decision to turn specific genes on or off and therewith commit to a specific cell fate. Once committed to a distinct cell fate, cells need to reliably express transcription factors for many cell generations in order to maintain their differentiated cell type and remember their cellular identity throughout their lifespan. Remarkably, only very few genes control these cell fate decision and play a role in developmental programs. Once a differentiated cell identity is established, it is very unlikely to switch into other, unrelated cell fates (Kato *et al.*, 1993).

Differentiated cell types contain a so-called epigenetic signature, highly specific for their gene expression state. The current definition of *epigenetics* defines an: epigenetic trait (as) stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (Cold Spring Harbor Meeting, 2008).

It is generally believed that the cellular identity is transmitted from one cell generation to the next by epigenetic marks, such as marks on the chromatin – potentially by histone modifications. The ability to transmit a gene expression state from one cell generation to the next is referred to as epigenetic memory.

I. 2. Epigenetic memory

The term epigenetic memory does not comprise memory of DNA sequences. It rather provides memory of transcriptional states and gene expression profiles, which will be outlined in this paragraph.

Epigenetic memory retains differential gene expression states throughout cell generations, even without the initial trigger (Bird *et al.,* 2002; Bird *et al.,* 2007; Ng and Gurdon, 2014). It provides therewith a robust feature of gene regulatory networks to ensure long-term stability of differentiated cell states, including potential positive feedback loops, regulated by transcription factors.

The underlying mechanisms are not well understood and may differ not only between organisms, but also from histone modification to histone modification. To better understand potential mechanisms that could be causal for maintenance of epigenetic memory, we first have to take a closer look at the molecular structures that actually provide information about gene expression states – the nucleosomes.

I. 2. 1. Nucleosome composition and their inheritance

In chromatin, DNA is tightly packed and wrapped around nucleosomes, which consist of octamers of the four core histones. In the nucleosome structure, 147 bp of DNA are required to fully enclose the octamer of histones, comprising two histones of each of the canonical core histones H2A, H2B, H3, and H4 (*see Fig. I.1*).

In order to form the nucleosome octamer, histone dimers are assembled through a sequence of steps. During the assembly, DNA is first wrapped around a tetramer of histones, comprising H3-H4, before two dimers of H2A-H2B are incorporated (Worcel *et al.*, 1978; Smith *et al.*, 1991).

Newly synthesized, as well as recycled nucleosomes are assembled with the DNA by chaperones shortly after DNA itself is synthesized. Most of the new histones that are incorporated into the chromatin are synthesized during S-phase of the cell cycle (Osley*,* 1991). Newly synthesized histones carry a

specific histone modification pattern that is distinct from the histones found within mature chromatin. In *Drosophila melanogaster,* for instance, these marks of newly synthesized histones include acetylation of lysine 14 and lysine 23 in histone H3 (Loyola *et al.,* 2006).

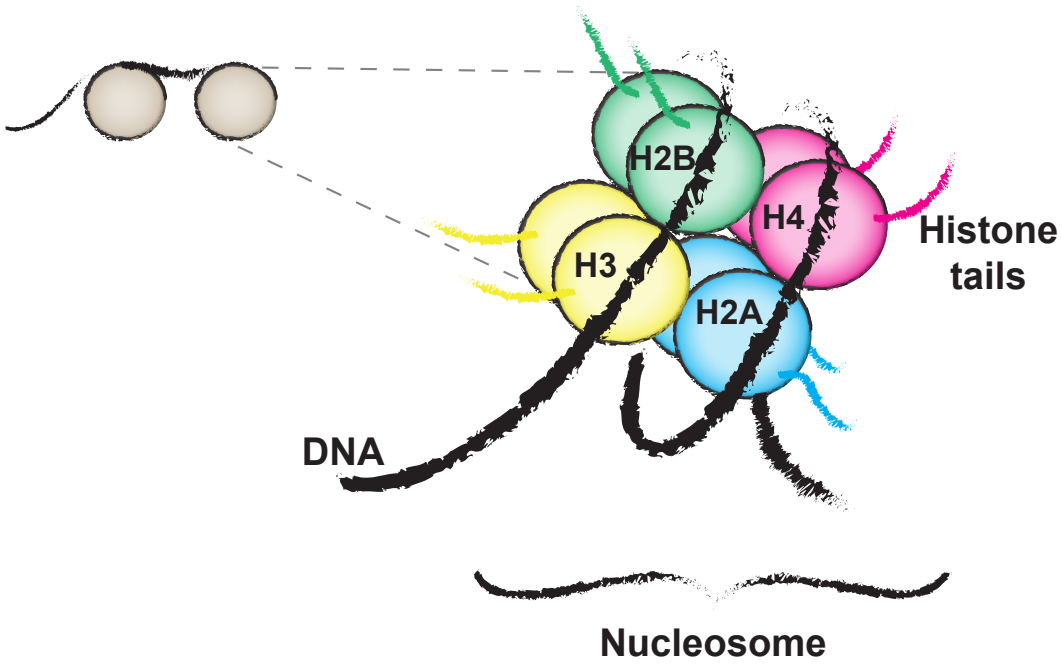

***Fig.* I.1: Nucleosome composition**: DNA is wrapped around two histones of each of the canonical histones H2A, H2B, H3, and H4 to comprise a nucleosome. The histone tails extrude out from the nucleosome.

For successful cell division, first, DNA must be replicated. During replication, the progressing DNA replication machinery disrupts nucleosomes in front of the replication fork. The nucleosomes are then reassembled behind the fork onto newly synthesized DNA strands in a random manner (Jackson and Chalkley, 1985; Gruss *et al.*, 1993). This semi-conservative DNA replication ensures the faithful duplication of genetic information during cell divisions.

H3-H4 tetramers were shown to be deposited as dimers (Tagami *et al.,* 2004; English *et al.,* 2005; Benson *et al.,* 2006) and their tetramer structure was shown to be disrupted by chaperones (Natsume *et al.,* 2007). Interestingly, dependent on the histone dimer species, during replication histones remain either associated or dissociate from the octamer structure. For instance, only

some of the H3-H4 dimers remain bound to replicating chromatin, whereas others dissociate and need to be reestablished (Xu *et al.,* 2010).

Inevitably, duplication of chromatin requires double the amount of histones, around which the double strand of DNA can be wrapped. This implies that only half of the histones can be comprised of the parental histones – the rest has to be supplied from the pool of newly synthesized histones. In this context, tetramer splitting of H3-H4 allows for maintenance of specific histone dimers, which could potentially carry epigenetic information and remain bound to specific regions during replication (Xu *et al.,* 2010).

I. 3. Epigenetic mechanisms

During mitosis cells divide to give rise to two genetically and mostly epigenetically identical daughter cells (Maton *et al.*, 1997). The process of mitosis thus separates two cell generations from one another.

This process requires a variety of cellular players that have the potential to convey epigenetic information from one cell generation to the next. Among these, DNA methylation (1), replacement of histones with histone variants (2), PcG proteins, as well as trithorax group (trxG) proteins (3), and modifications of histone tails (4) potentially play crucial roles as the carrier of epigenetic information (Ng and Gurdon, 2014).

In the following, the prospective players will be discussed in the context of epigenetic memory.

I. 3. 1. DNA methylation

Methylation of DNA, in particular methylation of cytosines, is generally associated with gene silencing (Bird and Wolffe, 1999). In replicating cells, methylated DNA is retained on one of the two newly synthesized daughter strands of DNA, resulting in two hemi-methylated DNA strands. The fully methylated pattern will be restored through action of DNA methyltransferases,

such as Dnmt1, which specifically bind to these hemi-methylated DNA strands (Pradhan and Esteve, 2003).

While omnipresent in mammals and vertebrates, lysine DNA methylation seems to be absent in some lower eukaryotes, such as *Caenorhabditis elegans* and *Drosophila*[1]. *Drosophila* does not contain any of the canonical DNA methyltransferases (neither Dnmt1 nor Dnmt3). Furthermore, no functional homologs of known 5-methylcytosine reader proteins are found in *Drosophila*. The only known protein of the Dnmt class is Dnmt2, which is involved in processes such as telomere integrity and retrotransposon silencing (Phalke *et al.*, 2009).

Overall, any functional role of DNA methylation in *Drosophila* is controversial and remains to be elucidated. Therefore details will not be further addressed in this study. For critical analysis please refer to (Krauss and Reuter, 2011).

I. 3. 2. Histone variants

Another possible mechanism of epigenetic inheritance is provided by the existence of histone variants. Besides the canonical histones (H2A, H2B H3, and H4), various types of H1, H2A, and H3 proteins exist that differ in their functions as well as their cellular properties. These variants differ from the core histones by a few changes in their amino acid sequence and represent only a small fraction of histone proteins. They are thought to be incorporated into chromatin in order to mark regions of chromatin for specialized function.

While synthesis and deposition of canonical histones is linked to S-phase of the cell cycle, most histone variants are deposited into chromatin by replication-independent mechanisms (for review on S-phase-linked histone deposition see Elgin and Weintraub, 1975; Osley, 1991; Wu and Bonner, 1981; Tagami *et al.*, 2004).

[1] Abbreviation *Drosophila* connotes *Drosophila melanogaster*

First hints on the role of histone variants in transmission of gene expression states came from transplantation experiments in *Xenopus*, where epigenetic patterns from the donor cell type were maintained, instead of the ones from acceptor cells. These patterns refer to a preservation of histone variant H3.3, predominantly at active gene regulatory regions (Ng and Gurdon, 2008).

In addition to histone proteins themselves, two other classes of proteins are commonly found in the context of epigenetic memory – the PcG and trxG protein family, which will be discussed in the next paragraphs.

I. 3. 3. Polycomb and trithorax group proteins

DNA methylation and histone variants are thought to act globally on all sorts of different genes. In contrast, the PcG and trxG proteins have been identified as regulators that primarily control the expression of developmental regulator genes. They act at the level of chromatin.

The PcG and trxG proteins represent classical examples of *epigenetic* activator and repressor complexes. The term *epigenetic* in this context is currently under dispute, since until today it has not been shown whether PcG or trxG proteins as well as their associated marks act as cause or consequence of activation or silencing.

The two protein families regulate gene expression in an antagonistic manner and are highly conserved throughout different kingdoms of life and evolution (Sawarkar and Paro, 2010).

Homeotic genes (also known as Hox genes) are the main target genes of PcG and trxG proteins. They play a crucial role in specification of the cellular identity along the anteroposterior (A-P) body axis in *Drosophila* and all other bilaterally symmetrical animals (Lewis, 1978; Prince *et al.*, 2002; Duboule, 2007). Hox genes compose a family of transcription factors that is tightly controlled during development. During early embryo development in *Drosophila*, segmentation genes define the localized expression patterns of Hox gene expression along the A-P embryo body axis (Maeda and Karch, 2006). The

expression domains established by this regulatory network have to remain constant throughout the development of the fly and during its adult life, even in the absence of the initial trigger. When the segmentation genes cease to be expressed early during development (Struhl, 1981), the PcG and trxG proteins take over and faithfully maintain the right level of Hox gene expression in each parasegment of the fly.

I. 3. 3. 1. Polycomb group proteins

Most PcG proteins were identified in *Drosophila* mutants in the 1980s, owing to their specific Polycomb phenotype, and were classified accordingly. Loss-of-function mutations in the PcG genes result in misexpression of Hox genes and often lead to their activation in cells or developmental stages in which they should not be active. Therefore, it is generally accepted that PcG proteins act as gene repressors.

PcG proteins usually assemble into multi-protein complexes. To date, five of these complexes are known: Polycomb repressive complex 1 (PRC1) (Shao *et al.,* 1999), PRC2 (Muller *et al.,* 2002; Cao *et al.,* 2002; Czermin *et al.,* 2002; Kuzmichev *et al.,* 2002), dRing-associated factor complex (dRAF) (Lagarou *et al.,* 2008), Polycomb repressive deubiquitinase complex (PR-DUB) (Scheuermann *et al.,* 2010), and Pho-repressive complex (PhoRC) (Klymenko *et al.*, 2006). The four main *Drosophila* complexes with their minimal subunits are depicted in Figure I.2.

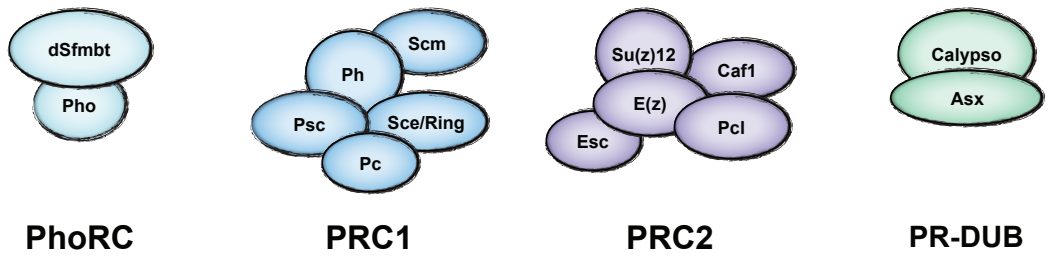

Fig.* I.2: PcG complexes with their components in *Drosophila. PhoRC, PRC1, PRC2, and PR-DUB.

Additionally, the enzyme O-glycosyltransferase (Ogt) was identified as PcG protein that does not require a multi-protein complex environment for its action (Gambetta *et al.,* 2009; Sinclair *et al.,* 2009). Ogt glycosylates the PcG protein polyhomeotic (Ph) to prevent its aggregation (Gambetta and Muller, 2014).

PcG complexes interact with specific DNA elements, the PREs (Schwartz *et al.,* 2006; Chan *et al.,* 1994). The structure and function of these elements will be discussed in detail in I. 5. 2.

Different aspects of PcG function were shown to be involved in gene repression. These functions range from induction of changes in the chromatin architecture – e.g. preventing transcription through a closed conformation – to addition of specific histone modifications to histone-amino tails, which could play a repressive role (Margueron and Reinberg, 2010).

Only one PcG protein is known to directly interact with DNA, specifically the PREs: pleiohomeotic (Pho) (Mohd-Sarip *et al.,* 2002) as part of the PhoRC (Klymenko *et al.,* 2006) (*Fig. I.3*). Pho is therefore thought to tether the entity of PcG complexes to the PREs and therewith towards their sites of action. A number of studies have proposed interaction between Pho and various PcG complexes (Franke *et al.,* 1992; Poux *et al.,* 2001; Mohd-Sarip *et al.,* 2002; Kahn, *et al.,* 2014); however, the molecular basis remains largely elusive. A current study by Felice Frey in our lab underlines – on a molecular level – the hypothesis that PhoRC and PRC1 interact (Frey, unpublished data).

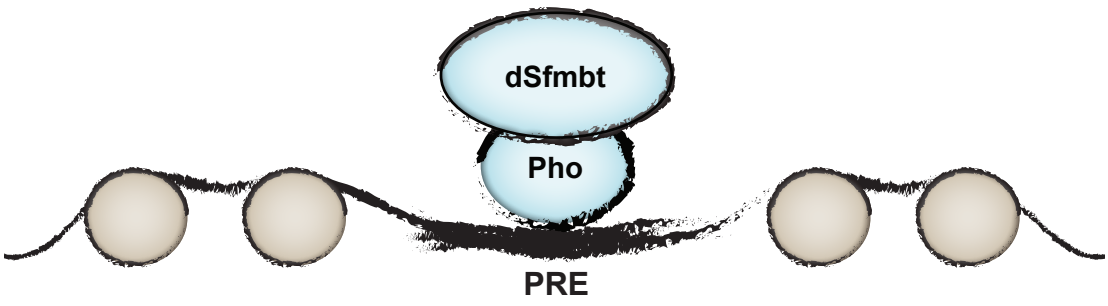

***Fig.* I.3**: **Graphic illustration of Pho-RC interacting with a PRE.** As illustrated the PRE is a nucleosome-poor stretch of chromatin that functions as binding platform for PcG complexes through Pho-RC.

The PRC1 complex exerts a histone ubiquitilase activity on H2A. This E3 ligase activity (i.e., to form H2A mono-ubiquitination (H2Aub)) is provided by sex combs extra (Sce) in *Drosophila* and Ring1a/b in vertebrates, respectively (de Napoles *et al.,* 2004; Wang *et al.,* 2004).

The PRC2 complex on the other hand represents the *Drosophila* histone methyltransferase (HMTase) that specifically methylates histone 3 lysine 27 (H3K27) on nucleosomes. This methylation is performed by its catalytic subunit enhancer of zeste (E(z)). Additionally, the PRC2 subunit embryonic ectoderm development (EED) is known to recognize H3K27me3 and allosterically facilitate the enzymatic activation of PRC2 (Margueron *et al.,* 2009).

By today, different compositions of PRC2 complexes with additional subunits (compared to the core PRC2 complex) are known. One of them is a complex, called Pcl-PRC2, which additionally features Polycomb-like (Pcl) (O'Connel *et al.,* 2001; Tie *et al.,* 2003; Nekrasov *et al.*, 2007). Pcl plays a critical role in generating high levels of repressive H3K27me3 at PcG target genes (Nekrasov *et al.*, 2007).

The PRC1 subunit Polycomb (Pc) interacts with H3K27me3 (Cao and Zhang, 2004). Therefore, it was suggested that H3K27me3 is sufficient to recruit PRC1 in a sequential manner (Wang *et al.,* 2004). Meanwhile, a number of studies have disproven this idea of sequential binding of the complexes – most notably the finding that there are H3K27me3 domains that lack binding of PRC1 (Ku *et al.,* 2008).

Importantly, components of the PRC2 complex (and other PcG proteins) bind their target genes in a highly localized manner at PREs. In contrast to that, the histone modification that is added onto the chromatin by PRC2, H3K27me3, widely spreads across the promoter as well as the coding region of the target genes (Papp and Muller, 2006; Schwartz, *et al.,* 2006). How this histone modification and the repressed state are maintained over time is not well understood.

I. 3. 3. 2. Trithorax group proteins

The trxG proteins represent the antagonists of the PcG proteins. Similar to their counterparts they interact with trithorax response elements (TREs) and

play an important role in keeping active genes active throughout the development (Tie *et al.,* 2014). TrxG proteins provide a more heterogenous group of proteins with many different functions. Interestingly, one protein of this family turned out to be the only demethylase of H3K27me3 in *Drosophila* (Agger *et al.,* 2007; de Santa *et al.,* 2007; Hong *et al.*, 2007; Swigut and Wysocka, 2007).

Details will not be further discussed here (for review, please refer to Ringrose and Paro, 2004; Schuettengruber *et al.,* 2007).

I. 3. 3. 3. Inherent differences between transcriptional on and off states

Strikingly, the difference between cells in which expression of a specific developmental regulatory gene is turned on versus those where its expression is turned off is not the occupancy with PcG or trxG proteins (Papp and Muller, 2006; Kwong *et al.,* 2008). In fact, the inherent difference between the on and off state was found to be the presence or the absence of the histone modification H3K27me3. H3K27me3 is exclusively bound to gene regulatory regions in cells where the associated gene is repressed (Papp and Muller, 2006) (*Fig. I.4*). This finding raises the question of how H3K27me3 can be specifically maintained at repressed chromatin, although PcG and trxG proteins are bound, independently of the transcriptional state.

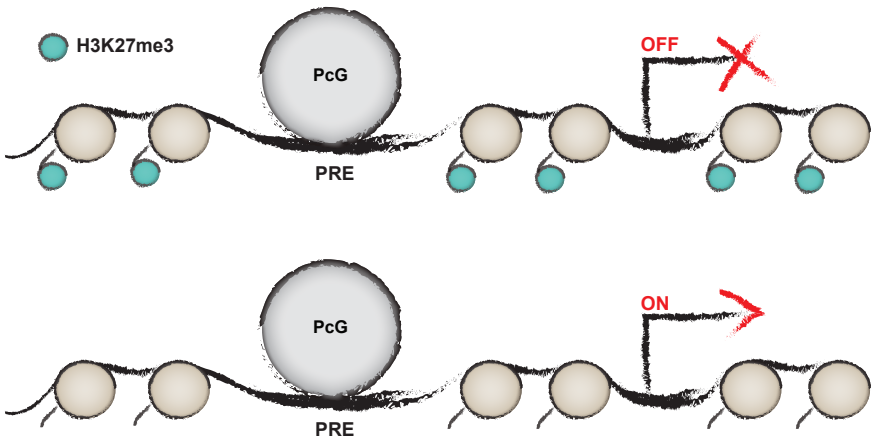

***Fig.* I.4: PcG proteins bind to PREs irrespective of the transcriptional state of the associated gene.** On the contrary, the repressive H3K27me3 mark (green circle) is only found when gene expression is turned off (upper panel). In cells where gene expression is turned on, H3K27me3 is absent (lower panel).

I. 3. 4. Histone modification states during replication

Histone modifications are considered to be causal for the maintenance of gene expression states and cell identity (Jenuwein and Allis, 2001). They are set by histone modifying enzymes, which are capable of adding a variety of chemical residues to the histone tails, such as for example acetyl-, methyl-, or ubiquityl-residues. Many histone modifiers fall into the category of PcG proteins, as described above in I. 3. 3. 1.

The chromatin modifications they set, have different functions on chromatin, which can be attributed in part to their general sterical and chemical properties, but also to their localization on the histone tails (for review, please refer to Kouzarides, 2007).

If histone modifications convey epigenetics information, duplication of chromatin in S-phase of the cell cycle is critical to copy this epigenetic information onto newly incorporated nucleosomes. Importantly, the question of how histone modifications or their modifiers know where they have to be reset remains to be answered.

An essential requirement for a mark capable to carry epigenetic information is its stable transmission through mitotic cell divisions. Comparing the half-lives of distinct histone modifications allows first conclusions about the eligibility of different histone modifications to carry epigenetic information.

The half-life of histone acetylation, for instance, is estimated to be in the range of minutes (Jackson *et al.*, 1975), already indicating that this mark might not be stable enough to confer information throughout cell generations (which are separated from one another by many hours). Additionally, the high turnover rate of acetyl groups argues against a role in cellular memory (Chestier and Yaniv, 1979).

This is different when it comes to methylation of lysine residues. Although dynamics of methylation of different lysine residues differ greatly, Zee *et al.* were able to show in mass spectrometry studies that lysine methylation, specifically H3K27me3, can be stable for more than three days (Zee *et al.,* 2010).

The specific localization of H3K27me3 argues for the modification, rather than the PcG proteins themselves – which are bound to PREs independently of the transcriptional state – being the carrier of epigenetic information. Apart from H3K27me3, a second histone lysine methylation state, histone 3 lysine 9 dimethylation (H3K9me2), is involved in another classical epigenetic phenomenon, which is known as position variegation effect (Rea *et al.,* 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001). H3K9me2 was also shown to be stable for a long time period (Zee *et al.,* 2010). In contrast to stability of H3K9me2 and H3K27me3, histone 3 lysine 9 monomethylation (H3K9me1) has among the shortest half-lifes of methylation states (Zee *et al.,* 2010). Thus, both, H3K27me3 and H3K9me2 are comparably stable modifications and therefore appear to qualify well as epigenetic marks. They will be discussed in detail below.

I. 3. 4. 1. H3K27me3

For the reason mentioned above, the stable H3K27me3 mark complies with the requirements to potentially carry information about gene expression states from one cell generation to the next.

The H3K27 residue itself is required for maintenance of repression, proving for the first time that a histone modification is indeed the crucial physiological substrate that is required for PcG repression (Pengelly *et al.,* 2013). Pengelly *et al.* showed that mutation of H3K27 to a non-methylatable residue resulted in loss of PcG-mediated repression, as had been seen in E(z) mutants.

Interestingly, this does not hold true for H2Aub, the repressive mark that is added onto the chromatin by PRC1, since mutation of the ubiquitylatable residues of H2A does not lead to misexpression of Hox genes (Pengelly *et al.,* 2015).

The PRC2 complex with its enzymatic subunit E(z) is exclusively responsible for mono-, di-, and trimethylation of H3K27 in the fly (Kuzmichev *et al.,* 2002; Czermin *et al.*, 2002; Muller *et al.,* 2002; Cao and Zhang, 2004). Furthermore, domains of H3K27me3 are often found to cover chromatin

stretches that exceed 10 kb in length (Schwartz, *et al.,* 2006; Tolhuis *et al.,* 2006) and are linked to repression of the associated genes in *Drosophila*.

It has been suggested that trimethylation of H3K27 is sufficient – once set - to recruit PcG proteins to the chromatin and transmit the repressive H3K27me3 mark from one cell generation to the next in mammalian cells (Hansen *et al.,* 2008). Moreover, several studies indicated that bulk levels of H3K27me3 are reduced nearly by 50% after mitosis, followed by a slow reset with completion of the cell cycle (Aoto *et al.,* 2008; Alabert *et al.,* 2015). These data allow to speculate that the H3K27me3 mark accounts for a potential memory element of the cell, that stably carries information throughout mitosis and mirrors the formation of two DNA strands that in part contain newly synthesized histones. Furthermore, the finding that all high methylation states of H3 (H3K9me2/3, H3K27me2/3, and histone 3 lysine 36 di- and trimethylation (H3K36me2/3)) show similar decay rates indicates that their reduction is more likely to be coupled to a general event, such as mitosis, than to a modification-specific event (Xu *et al.,* 2012).

The hypothesis that H3K27me3 is a stable mark was further corroborated by the fact that the H3K27me3 peptide was shown to stimulate PRC2, providing a positive feedback loop of propagation (Margueron *et al.,* 2009).

However, there are two observations that argue against the hypothesis that in *Drosophila* H3K27me3 itself is sufficient to transmit epigenetic information required for repression.

First, *in vivo* excision of the PRE as PcG binding platform, from genes that had been under control of PcG repression before, results in loss of PcG-mediated repression in proximity of PREs of the associated genes (Sengupta *et al.*, 2004). Second, nucleosomes in vicinity of PREs that are modified by E(z) turn over faster than the time period required for the cell cycle to pass (Deal *et al.*, 2010).

These discrepancies between H3K27me3 behaving like a self-sustainable mark in mammals (Hansen *et al.,* 2008) and repression being lost after removal of a PRE in *Drosophila* (Sengupta *et al.*, 2004), may be due to general differences in PcG recruitment between distinct species, but might also be a result of differences in the experimental setups.

I. 3. 4. 2. H3K9 methylation

H3K9 methylation is predominantly found at constitutive heterochromatin domains and is mainly catalyzed by the SET-domain HMTase Su(var)3-9 (Suv39h in *human*) and its homolog Clr4 in fission yeast. More specifically, Su(var)3-9 dimethylates H3K9 in the bulk of pericentromeric heterochromatin, but not at telomeric or euchromatic sites (Schotta *et al.*, 2002; Ebert *et al.*, 2004).

In yeast, experiments using reporter genes inserted into heterochromatin domains show epigenetic inheritance properties of these H3K9 domains in mitotic and meiotic cell divisions (Allshire *et al.*, 1994; Grewal *et al.*, 1996; Nakayama *et al.*, 2000). Furthermore, it was demonstrated recently that H3K9me2/3 could indeed be inherited throughout cell divisions after removal of the sequence-specific initiator – at least for a few cell generations (Audergon *et al.*, 2015; Ragunathan *et al.,* 2015). The decay seen in these experiments was shown to be dependent on the H3K9 demethylase Epe1 – in its absence H3K9me2/3 levels stayed high for many cell generations.

These findings identify H3K9 methylation as heritable epigenetic mark in prokaryotes; its demethylase Epe1 prevents unauthorized inheritance of constitutive heterochromatin domains (Audergon *et al.,* 2015; Ragunathan *et al.,* 2015). Whether this holds true for *Drosophila,* for other histone modifications or even for other chromatin environments remains to be elucidated.

In order to understand the possible mechanisms underlying the potential propagation of a histone mark throughout mitotic cell divisions and replication, the mark of interest has to be considered in the context of chromatin.

I. 4. Challenges for carriers of epigenetic information

The above listing of possible carriers of epigenetic information demonstrates the potential and the features of the prospective players. The requirements for a molecule to convey information throughout cell generations

are high – the system has to cope with challenges such as DNA replication and mitosis. Another challenge, specific for histone modifications or histone variants, is borne by cell division-uncoupled histone turnover. This turnover requires resetting histone marks or histone variants outside of the periodic processes of cell division. In the next paragraphs potential challenges for epigenetic information are discussed, focusing on the ones for PcG proteins and histone modifications.

I. 4. 1. Histone turnover

As mentioned earlier, histones are mainly incorporated into chromatin in an S-phase-dependent manner (Osley, 1991). However, assembly of histones on newly synthesized DNA is not that well understood; neither is the process of their establishment on newly incorporated histones (Henikoff *et al.*, 2004; Tagami *et al.*, 2004). To shed light on the kinetics of how histone modifications are established on newly incorporated histones after cell division, several studies were performed in the past decade, using mass spectrometry analysis of isotopically labeled HeLa cells (Loyola *et al.,* 2006; Scharf *et al.,* 2009). While acetylation as well as monomethylation levels of newly incorporated histone are adjusted within a few hours after release into S-phase – to equalize modification patterns between old and new histones – di- and trimethylation of prominent lysine residues are relatively slow processes (Alabert *et al.,* 2015). Long-winded conversion of monomethylation to di- and trimethylation was observed for H3K27, as well as H3K36.

Importantly, another histone mark, histone 4 lysine 20 trimethylation (H4K20me3), was identified as slowly maturing mark, leading to the current understanding that the presence of H4K20me3 marks the fully assembled, mature nucleosome (Scharf *et al.,* 2009).

These findings allow better interpretations of the mechanisms underlying dynamics of histone modifications.

I. 4. 2. DNA replication

Replication constitutes a major challenge for chromatin integrity, including dissociation of bound proteins – such as PcG proteins – to allow for passage of the replication machinery (Probst *et al.,* 2009). Additionally, histone modifications have to be faithfully transferred to newly replicated chromatin in order to maintain correct gene expression states.

It was suggested that in *Drosophila* recruitment of PcG proteins to PREs, as well as H3K27 trimethylation in the vicinity of PREs, are augmented shortly before replication (Lanzuolo *et al.,* 2011). These increased levels of proteins and histone marks should compensate for dilution of both factors during replication. Similar results were found in a cell-free SV40 replication system (Lo *et al.,* 2012). In line with that, quantitative mass spectrometry experiments that examined kinetics of histone methylation mark restoration after replication in HeLa cells further demonstrated that histone marks are most likely diluted during S-phase (Scharf *et al.,* 2009; Xu *et al.,* 2012). For review of PcG protein dynamics in different organisms please refer to Steffen and Ringrose, 2014.

Obviously, this hypothesis entails that histone modifications have to be restored after replication, including *de novo* modification of newly synthesized or newly incorporated histones. How could this be achieved?

As lined out in I. 3. 3. 1, the H3K27me3 mark in particular can be recognized by different components of the PcG system. On the one hand, PRC1 is capable of interacting with H3K27me3 through the chromodomain of Pc. On the other hand, the human homolog of the PRC2 subunit extra sex combs (Esc) – EED, can bind H3K27me3, which stimulates PRC2's HMTase activity (Margueron *et al.,* 2009).

An elegant study by Anja Groth's and Axel Imhof's lab suggested two distinct modes for propagation of different histone modifications. Histone modifications could be reestablished either within one cell generation, or, as for H3K9me3 and H3K27me3, within several cell generations (Alabert *et al.*, 2015).

I. 4. 3. Mitosis

During mitosis, chromatin is compacted up to 300-fold, accompanied by a general shutdown of transcription. Nevertheless, PcG and trxG proteins remain bound to mitotic chromatin before and during mitosis (Buchenau *et al.*, 1998; Fonseca *et al.,* 2012).

Furthermore, histone modifications are maintained on mitotic chromatin only to a small extent. Specifically, modifications that play a role in PcG-mediated repression, such as H3K27me3 or H3K36me3 can be found on mitotic chromatin (Wang and Higgins, 2013). On the other hand, different marks are strongly decreased during mitosis, such as H2Aub (Mueller *et al.*, 1985). Whether the marks that persist through mitosis are sufficient to reset pre-existing chromatin states is one of the most compelling questions in the field of epigenetics. For details on mitosis and chromatin modifiers, please refer to Steffen and Ringrose, 2014.

I. 5. Elucidating H3K27me3 dynamics

Among all the candidates that could potentially convey epigenetic information in *Drosophila* – based on the current state of knowledge – histone modifications are the most promising ones. Therefore, I aimed to test whether H3K27me3 could indeed behave like an epigenetic mark and be sufficient to propagate memory of repressed chromatin.

In order to understand whether or not H3K27me3 plays a role in inheritance of gene repression states, several approaches could help to answer this question. The most obvious way to look at H3K27me3 dynamics would be to remove the enzyme responsible for the modification, E(z), and follow the dynamics of H3K27me3 in the absence of its establisher.

I. 5. 1. Enhancer of zeste

E(z) is crucial for the development of the fly. Homozygous mutants develop into larvae before they die, but imaginal discs in these larvae are only poorly developed (Mueller *et al.,* 2002).

Depletion of the enzymatic function by removing E(z) or inhibiting its enzymatic activity *in vivo* would not only involve to completely eliminate translation of the protein, but also to inhibit the enzymatic activity of the remaining cellular proteins. Overall, depletion of the enzyme in its entirety requires several layers of interference with the endogenous system.

In order to minimize the number of intrusions and still get a better idea of H3K27me3 kinetics, I decided to take a closer look at the binding platforms – the PREs, whose removal might have similar effects as loss of E(z).

I. 5. 2. Polycomb Response Elements

In *Drosophila* the binding platforms of PcG proteins are clearly defined. As mentioned before, among the PcG proteins, solely Pho was shown to directly interact with DNA and potentially tether all other PcG proteins to their site of actions – the PREs.

I. 5. 2. 1. How are PREs defined?

Sequence analysis unraveled combinations of consensus-binding sites for PcG proteins as PREs (Ringrose *et al.*, 2003). Among them are the binding site for Pho and its paralog Pho-like, the consensus site which is bound by the GAGA factor (GAF) (Hagstrom *et al.*, 1997), as well as the consensus site for zeste, a trxG protein (Saurin *et al.*, 2001; Hur *et al.*, 2002; Ringrose *et al.*, 2003). These consensus-binding sites differ in number and order from one PRE to another (Brown and Kassis, 2013; Oktaba *et al.,* 2008). Pho binding sites are the most essential component of PREs for PcG repression *in vivo* (Brown *et al.*, 1998; Fritsch *et al.*, 1999).

Fly PREs are typically a few hundred to a few thousand base pairs in length and can be found either in close proximity of each other, such as in the bithorax complex (BX-C), or rather isolated. The sequence specificity and, therewith, the prediction of PREs is limited to *Drosophila* – in mammals the identification of PREs is more complicated and does not seem to correlate with consensus sequences. Although sequence motifs in vertebrates are yet to be identified, in the meantime a few mammalian PREs could be identified (Sing *et al.,* 2009; Woo *et al.,* 2010). For a detailed overview of differences between general mammalian and metaozoan PREs, please refer to Bauer *et al.,* 2015. A correlation between CpG islands (CGI) and PREs in mammals is currently under dispute (Mendenhall *et al.*, 2010; Farcas *et al.*, 2012; Lynch *et al.,* 2012; Klose *et al.*, 2013).

I. 5. 2. 2. PREs: specific DNA sequences for targeting chromatin-modifying complexes

The specific and highly localized binding of PcG proteins to PREs (Papp and Muller, 2006; Schwartz *et al.,* 2006) indicates the importance of these binding sites for PcG-mediated repression.

During the 1990s, various transgenic reporter assays resolved many characteristics of PREs. Their main function – as the original names ‘maintenance elements’ implies – was defined as maintaining a pattern that was given by an initiator (until late embryogenesis). This maintenance function is strictly dependent on the performance of PcG proteins (Simon *et al.*, 1993; Chan *et al.,* 1994; Christen and Bienz, 1994).

Key experiments with various combinations of enhancers and PREs to address the question of whether PREs also contain a position-specific regulatory function were conducted (Americo *et al.*, 2002), showing that the memory function is not specific to Hox genes.

It was demonstrated that epigenetic memory of transcriptional states could persist from embryonic into larval stages. Additionally, an early developmental stage was defined at which the PRE still showed bivalent potential and could be set to either active or silent (Poux *et al.*, 1996; Sengupta *et al.*, 2004).

The molecular differences between active and silent PREs are not entirely understood. As mentioned above, while PcG proteins are bound to PREs irrespective of the transcriptional state, strikingly H3K27me3 is only found on repressed chromatin (Papp and Muller, 2006).

It is important not to forget the role of the DNA itself. The PRE itself is required to propagate memory. In experiments in which the PRE was removed at different time points during development from a reporter construct using the enzyme Flipase recombinase (FLP), PcG-mediated repression was lost within a few cell generations (Busturia *et al.*, 1997; Sengupta *et al.*, 2004). These experiments indicated that the PRE itself, or something bound to it, is required to propagate the silenced state.

Clearly, an excisable PRE represents a more elegant possibility to look at H3K27me3 in the context of loss of repression, than to remove the underlying enzyme in its entirety. Whether loss of repression, upon removal of the PRE, correlates with loss of the ‘repressive’ H3K27me3 mark is not understood. However, excising the PRE *in vivo* would allow for a better understanding of the molecular mechanism of H3K27me3 propagation.

I. 6. Aim of this work: Understanding the propagation of H3K27me3

Removing the PRE *in vivo* would be a promising way to study its impact on the surrounding chromatin landscape. Although H3K27me3 is commonly referred to as a *repressive* mark, it is currently not understood, whether it is actually cause or consequence of repression. Nonetheless, it was shown to be the crucial substrate for PcG-mediated repression (Pengelly *et al.,* 2013).

Correct maintenance of PcG-mediated repression is critical for many fundamental processes of normal development in many organisms. Proteins involved in the PcG-system in vertebrates are often found misregulated in disease. Deregulation of the PcG system, including incorrect inheritance of the essential H3K27me3 mark, can lead to neoplastic transformation and cancer in multi-cellular eukaryotes.

The pivotal question of PcG research focuses on inheritance of transcriptional states, the very essence of epigenetics. What are the molecular players required to transmit a repressive state through DNA replication and mitosis?

While the mechanisms by which PcG proteins inflict silencing on transcription during cell division have become clearer within the last years, the major challenge in the field of epigenetics remains to elucidate how transcriptional states endure demanding processes such as DNA replication and also how this information can be accurately propagated to daughter cells after mitosis.

We therefore decided to study H3K27me3 levels in the proximity of a transgenic, excisable PRE to specifically dissect the role of the PRE in maintenance of H3K27me3. In particular, I aimed to shed light on one of the most central questions in the field by looking at transmission of H3K27me3 throughout cell divisions.

There are two mechanisms that have to be discriminated, which both fit the current state of knowledge (see *Fig. I.5*). One option would be that H3K27me3 behaves like a self-sustainable mark that is sufficient to tether PcG complexes to their site of action and to reset H3K27me3 on newly incorporated nucleosomes after cell division (*Fig. I.5,* top; Hansen *et al.,* 2008). A second

option, which takes into consideration that the PRE itself is strictly required to maintain repression (Sengupta *et al.*, 2004), would be that in the absence of the PRE PcG complexes can not find their targets anymore or can not persist long enough to fulfill their enzymatic action. This failure to bind or to persist would result in loss of H3K27me3 within a few cell generations (*Fig. I.5,* bottom).

With this study we would like to elucidate the mechanism of H3K27me3 transmission and maintenance from one cell generation to the next in *Drosophila*.

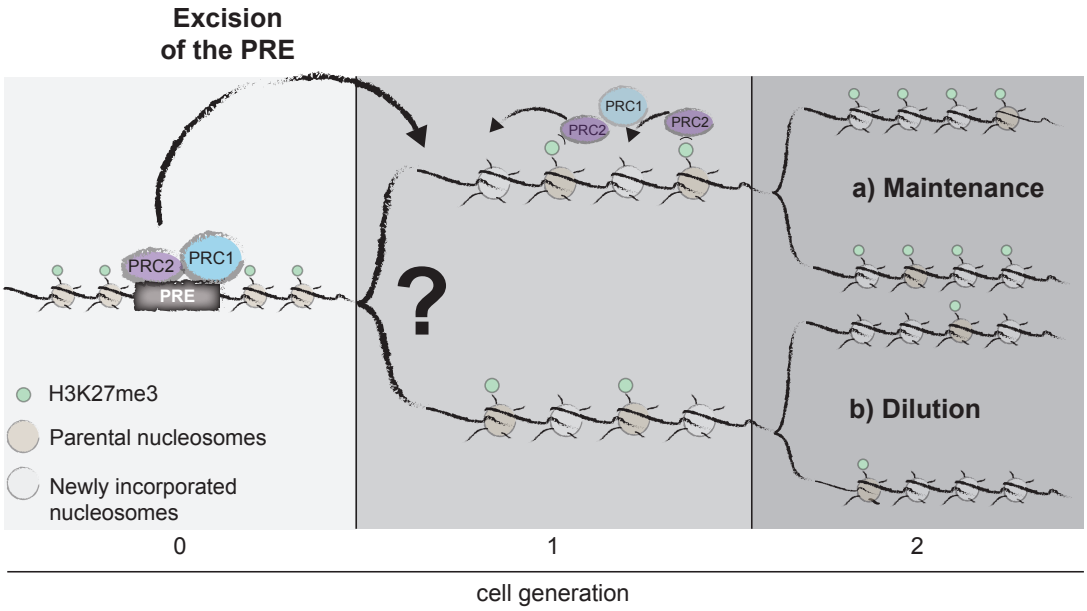

***Fig.* I.5: Potential mechanisms of H3K27me3 dynamics after loss of the PRE**. PcG complexes interact with the PRE. The PRE is excised and DNA is replicated – in the next cell generation chromatin assembles from parental (brown circle) and newly synthesized nucleosomes (beige circle). Parental nucleosomes carry H3K27me3 (green circle). In the absence of the PRE either H3K27me3 levels are maintained, for example through recognition by PRC2 complexes (option a); upper scheme) or diluted down (option b); lower scheme). In the latter case, passage into a new cell generation would be accompanied with lower levels of H3K27me3.

II. Materials and Methods

II. 1. Fly work

II. 1. 1. Fly husbandry

Flies were kept on cornmeal-molasses-yeast medium supplemented with mold inhibitor propionic acid. Stocks were kept at 18°C, crosses were made at 25°C.

II. 1. 2. *Drosophila melanogaster* strains

PREs >PRE>*dpp*^{WE}-*LacZ* (ry^{+}) #7b; cn; ry^{42}
cn; >PRE>*dpp*^{WE}-*LacZ* (ry^{+}) #17b, ry^{42}
cn; >PRE>*dpp*^{WE}-*LacZ* (ry^{+}) #30b, ry^{42}
cn; >PRE>*dpp*^{WE}-*LacZ* (ry^{+}) #36c, ry^{42}
>PRE>*dpp*^{WE}-UZ-*LacZ* (ry^{+}) #17-1, ry^{42}

Enzymes
w^{118}; P(ry[t7.2])70Flp)10 (#6938, Bloomington Stock Center)

II. 2. Genomic DNA preparation from flies

Fifty to hundred young adult flies were narcotized and shock-frozen in liquid nitrogen to promote cell disruption. The frozen flies were then crushed with a pestle in 500 µl solution A (0.1M Tris-HCl pH7.5, 0.1M EDTA, 0.1M NaCl, 0.5% SDS). The crushed fly mixture was then incubated at 70°C for 30 min. The mixture was left to cool down 10 min at room temperature (RT) before addition of 70 µl 8M KAc. After 30 min on ice, debris was spun down for 15 min at 14000 rpm at 4°C. The resulting supernatant was transferred to a fresh tube. This centrifugation step was repeated as often until the supernatant was clear.

250 µl isopropanol was added to the supernatant to precipitate DNA by centrifugation for 5 min at 13300 rpm at RT.

Next, the supernatant was discarded and the pellet was washed using 70% v./v. EtOH. Then, the pellet was cleared by centrifugation for 5 min at 13300 rpm at RT. The supernatant was discarded and the pellet left to dry at 37°C. After 30 min, the pellet was re-suspended in 100 µl double-distilled H_2O (ddH_2O). 1 µl DNA-free RNase (Roche, #11579681001, 10 mg/ml) was added to the solution and left to incubate for 5 min at 37°C. 1/10 volume of 3M NaOAc pH5.2 and 2.5 volumes of cold 100% EtOH were added and vortexed to mix. The mixture was then kept for 30 min at -80°C. The DNA was pelleted by centrifugation for 20 min at 13300 rpm at 4°C. The supernatant EtOH was aspirated off and the pellet was washed in 500 µl 70% EtOH. The mixture was cleared by centrifugation for 10 min at 14000 rpm at RT. Subsequently, EtOH was removed and the DNA was air-dried at 37°C. Finally, the DNA was re-suspended in 30 µl ddH_2O.

II. 3. Inverse PCR of genomic DNA

Genomic DNA (gDNA) was digested for 2.5 h at 37°C using Sau3AI (NEB, #R0169L, 5000 units/ml):

gDNA	10µl
10X NEB buffer 1	2.5µl
BSA	2.5µl
ddH_2O	13.5µl
Sau3AI	2.5µl

The enzyme was inactivated for 20 min at 65°C. Digested gDNA was then ligated using T4 DNA ligase (NEB, #M0202S, 400000 units/ml).

Digested gDNA	10µl
10X ligation buffer	40µl
ddH_2O	344µl
T4 ligase (2.1 Weiss units)	2.17µl
10mM ATP	4µl

The reaction was incubated over night (o/n) at 16°C and subsequently heat-inactivated for 10 min at 65°C. The DNA from the ligation reaction was next precipitated using EtOH:

Ligation product	200µl
3M NaOAc	20µl
100% cold EtOH	660µl

To precipitate, the mixture was incubated for 30 min at 20°C and vortexed. The mixture was next kept for 30 min at -80°C and cleared by centrifugation for 20 min at 4°C. EtOH was aspirated off and the pellet was washed with 70% EtOH. The pellet was cleared by centrifugation for 10 min at 13300 rpm at 4°C and left to dry at 37°C. After 30 min the pellet was re-suspended in 20 µl ddH_2O. Not more than 10^5-10^{10} copies of DNA were used for the PCR reaction (for details on primer sequence, please refer to *Table II.5*):

Template DNA	1-10 µl
Primer 1 (either for 3’ or 5’ end of P-element) 10 µM	2 µl
Primer 2 (either for 3’ or 5’ end of P-element) 10 µM	2 µl
Phusion Master Mix 2X (#M0531, NEB)	25 µl
ddH2O (add up to 50 µl)	

PCR setup:

1	95 °C	5 min
2	95 °C	30 s
3	60/55 °C	1 min
4	68 °C	1 min
5	Go to 2	34 x
6	72 °C	10 min
7	4 °C	∞

The entire PCR reaction for each fly strain was run on a 1.5% agarose gel in TBE. The bands of interest were purified using the QIAquick gel extraction kit (QIAGEN, #28704), following the manufacturer's instructions. DNA was eluted using 30 µl ddH_2O. PCR products were then sent for sequencing to MWG, using the primers that were used for amplification.

II. 4. Chromatin preparation from *Drosophila* larvae

Flies for chromatin preparation were crossed in vials and left at 25°C for a total egg laying period of 12 h. All dissections were performed 120 h after egg-laying (AEL) in the third instar larval stage. In experiments - in which heat shocks were required – heat shocks were carried out at indicated times before dissections. All heat shocks were performed for 1 h at 37°C in a water bath. During dissections larval fat bodies, digestive tracts, and salivary glands were removed. All dissections were carried out in PBS on ice.

After dissection, tissues were cross-linked for 15 min at RT in 1% fresh MeOH-free formaldehyde (Thermo Scientific, #28906) in cross-linking solution (50mM Hepes pH8.0, 1mM EDTA pH8.0, 0.5mM EGTA pH8.0, 100mM NaCl). Supernatant was discarded and cross-linking was stopped by washing with stop solution (125mM glycine in PBS, 0.01% Triton X-100) for 10 min at RT. Tissues were then washed for 10 min in solution A (10mM Hepes pH8.0, 10mM EDTA pH8.0, 0.5mM EGTA pH8.0, 0.25% Triton X-100) and solution B (10mM Hepes

pH8.0, 1mM EDTA pH8.0, 0.5mM EGTA pH8.0, 0.01% Triton X-100, 200mM NaCl), respectively.

Imaginal wing discs were dissected off the remaining cuticles in solution B and collected in fresh tubes. Excess solution B was discarded and imaginal wing discs were transferred into sonication buffer (10mM Hepes pH8.0, 1mM EDTA pH8.0, 0.5mM EGTA pH8.0, 1X complete protease inhibitor (#11873580001, Roche), 1X Pefabloc (#76307, Sigma-Aldrich), 0.1% SDS; per sonication, 120 imaginal wing discs were sonicated in 130 µl sonication buffer). Discs in buffer B were frozen down in liquid nitrogen at this step and kept at -80°C until further use. Before sonication, discs were thawed on ice.

Sonication was performed in AFA microtubes (Covaris, #520045) in a Covaris S220 AFA instrument using the following setup: 105W/ 2%/ 30 min. Afterwards, the samples were adjusted to 0.5% N-Lauroylsarcosine (Sigma-Aldrich, #L7414) and kept rotating at 4°C for 10 min. After processing, samples were centrifuged to pellet insoluble material at maximum speed at 4°C for 10 min.

Following centrifugation, the chromatin samples were dialyzed three times against 1X dialysis buffer (5% glycerole, 10mM Tris-HCl pH8, 1mM EDTA pH8, 0.5mM EGTA pH8), for 2 h (last dialysis step o/n) at 4°C, using dialysis tubes with 1 kDa cut-off (GE Healthcare, #28955966). Chromatin was then transferred to siliconized eppendorf tubes and cleared of insoluble material by centrifugation at maximum speed for 20 min at 4°C. The supernatant was directly used for chromatin immunoprecipitation (ChIP) without further freeze-thawing.

II. 5. Chromatin immunoprecipitation

Protein A^5 sepharose (PAS; #17-0780-01, GE Healthcare) lyophilized powder was swollen in RIPA 140mM NaCl for 1 h, rotating at 4°C. After swelling, PAS beads were washed again for 15 min in the same buffer.

Chromatin (from II. 4) was then adjusted to RIPA buffer conditions in a volume of 1 ml. The pre-swollen PAS beads were used to pre-clear the

chromatin (40 µl 50% slurry per 1 ml) while rotating for 1 h at 4°C. The rest of the pre-swollen beads were kept at 4°C for purification of immuno-complexes later on. After pre-clearing, beads were spun down for 30 s at 4000 rpm at 4°C and supernatant was transferred to a fresh siliconized collection tube. To minimize loss of chromatin material in bead slurry, PAS beads were washed with 300 µl 140mM RIPA and supernatant was transferred to the collection tube. From this supernatant 100 µl were removed and stored at 4°C – this material was used to determine the amount of input chromatin.

Next, chromatin was incubated with primary antibody for 16 h on a rotating wheel at 4°C. On the next day, immuno-complexes were the bound to pre-swollen PAS beads on a rotating wheel for 4 h at 4°C (40 µl 50% slurry per ChIP). Tom remove of unspecifically-bound proteins, beads were then washed once with 140mM RIPA buffer (140mM NaCl, 10mM Tris-HCl pH8, 1mM EDTA pH 8, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1X Pefabloc, 1X Complete protein inhibitor), four times with 500mM RIPA buffer (500mM NaCl, 10mM Tris-HCl pH8, 1mM EDTA pH8, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1X Pefabloc, 1X Complete protein inhibitor), once with LiCl buffer (250mM LiCl, 10mM Tris-HCl pH8, 1mM EDTA pH8, 0.5% NP-40, 0.5% sodium deoxycholate, 1X Pefabloc, 1X Complete protein inhibitor), and twice with TE buffer (10mM Tris-HCl pH8, 1mM EDTA pH8). Each washing step involved rotation of beads in 1 ml of the respective buffer for 10 min at 4°C with several buffer changes, followed by centrifugation of beads for 30 s at 4000 rpm at 4°C. After the last centrifugation step, beads were taken up one more time in 1 ml TE, an the wash buffer was removed down to a volume of 100 µl to ensure that no beads were removed.

The following DNA purification were then performed on both, input, and IP samples: 2 µl DNA-free RNase (#11119915001, Roche) were added, followed by incubation at 37°C for 30 min with soft agitation (300 rpm). After RNAse digestion, 6 µl 10% SDS and 12 µl of 5 mg/ml proteinase K (Promega, #V302B) were added to the mixture. Proteinase K digestion was performed for 10 h at 37°C with agitation (300 rpm), before reversal of formaldehyde cross-links for 6 h by incubation at 65°C at 300 rpm.

For the rest of the DNA purification procedure, Minelute PCR purification kit columns were used (#28004, QIAGEN). First, beads were mixed to detach

eluted material and centrifuged at 4000 rpm for 1 min to pellet beads. Then, supernatant without beads was transferred to a new eppendorf tube. 40 µl of TE was added to the beads to re-suspend them. Material was again collected by mixing and centrifuging before adding the eluate to the first eluate.

To increase binding to the Minelute columns, five volumes of clean PB buffer (QIAGEN), as well as 10 µl 3M sodium acetate pH5.2 were added to the mixture and vortexed well. This solution was next pipetted onto Minelute columns and centrifuged for 1 min at 14000 rpm. Twice, 750 µl clean PE (QIAGEN) were added to wash the membrane and incubated for 5 min at RT, before centrifugation. DNA was eluted by addition of 50 µl 5mM Tris-HCl pH8.5, preheated to 65 °C, incubation on the membrane for 1 min at RT, and centrifugation for 1 min at 14000 rpm. This elution was repeated once more. Pooled DNA eluates were diluted with 400 µl 5mM Tris-HCl pH8.5.

II. 6. Quantitative real-time PCR (qPCR)

ChIP eluate was analyzed by qPCR (StepOne plus, Applied Biosystems) using SYBR Green Power Master Mix (#4367660, Applied Biosystems) and standard settings (#4367660, Applied Biosystems) with primers at a final concentration of 5µM per reaction. Primers used for qPCR analysis are described in II. 11.

qPCR was performed in technical duplicates or triplicates, serial dilutions of gDNA were measured together with the input and immuno-precipitated DNA samples to form the standard curve. This setup allowed calculation of relative amounts of immuno-precipitated chromatin to input chromatin. Each ChIP experiment was performed in biological triplicate with each antibody independently to allow for statistical conclusions.

II. 6. 1. Data analysis of qPCR results

Mean values of biological replicates were calculated from standard means of technical replicates of each individual experiment. Values for

biological triplicates were calculated as mean values of two to three technical read-outs. Error bars indicate the standard deviation (SD) of biological triplicates.

II. 7. Quantification of PRE-excision efficiency in imaginal wing discs

120 h AEL 3rd instar larvae were dissected in 1X PCR-buffer (standard Taq buffer, #M0273L, NEB) to isolate two imaginal wing discs of single larvae. Imaginal wings discs were then taken up in 20 µl 1X PCR-buffer, containing proteinase K (0.25 µl/20 µl reaction) and incubated for 1 h at 50°C. Proteinase K was then inactivated for 10 min at 94°C.

10 µl of crude gDNA extracted from this digestion were used in the qPCR reaction, representing DNA from one imaginal wing disc.

To determine the ratio of cells of wing imaginal discs that retained the PRE versus those that excised it, mean values of qPCR analysis of a primer pair spanning the PRE (PRE*dpp*^{WE} or rosy-PRE) were divided by values from a steady primer pair that remained unchanged upon excision (LacZ). This ratio was normalized to amplification read-out before excision of the PRE. Error bars indicate SD values of n larvae. p-values were determined performing a standard t-test.

II. 7. 1. X-gal staining of wing imaginal discs

3rd instar larvae were dissected in PBS (10mM PO_4, 137mM NaCl, 2.7mM KCl, adjusted to pH7.4 w/ HCl) on ice. Cuticles were transferred to a tube containing PBS, on ice, while other larvae were dissected. Cuticles were fixed for 2 min with 1 ml of 1% glutaraldehyde solution (1% glutaraldehyde in PBS; #G5882, Sigma-Aldrich). The fixing solution was removed after 1.5 min of fixation. Cuticles were washed several times with PBS and twice with C-P buffer (200mM Na_2HPO_4, 100mM citric acid). Next, cuticles were stained in X-gal

staining solution (C-P-buffer, 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$), 1 mg/ml X-Gal (in DMF)) in a humidifying chamber o/n at 37°C, protected from light. Staining was performed in an eppendorf tube, placed in a humidified chamber. The next day, X-gal staining solution was removed and cuticles were washed twice with PBS, once with PBS-30%-glycerol and once in PBS-50%-glycerol, respectively. Imaginal discs were then dissected in PBS-50%-glycerol and mounted in 100% glycerol. Discs were analyzed using a Zeiss AXIO Scope.A1 microscope.

II. 8. Immuno-staining of *Drosophila* wing discs

3rd instar larvae were dissected in PBS on ice. Cuticles were transferred to a tube containing PBS on ice, while other larvae were dissected. Cuticles were fixed for 20 min, in agitation in 4% formaldehyde. At least six washes with BBT (0.2% Triton X-100, 2% BSA in PBS) were performed within 30 min. Cuticles were then incubated with 200 µl of primary antibody diluted in BBT o/n at 4°C. The next day, at least six washes with BBT were performed within 1 h, rotating at RT. Next, cuticles were incubated with 200 µl secondary antibody diluted in BBT, supplemented with Hoechst 33342 (50 ng/ml), o/n at 4°C or at RT for several hours. The next day, two washes with BBT and four with PBT (0.2% tween in PBS) were performed, respectively. Finally, wing discs were dissected in PBT, mounted with Fluoromount-G (#00-4958-02, affymetrix) on microscope slides and analyzed by fluorescence microscopy on a confocal Zeiss LSM780 microscope.

II. 9. Aphidicolin treatment of *Drosophila* larvae

Drosophila larvae were collected 96 h AEL and washed in PBS to get rid of food, sticking to the larvae. After washing, larvae were transferred to 35 x 10 mm Petri dishes (#351008, FALCON), covered with a 500 my polyamid sieve, cut to the same size (Klein & Wieler) or with nitrocellulose membrane filters (#9004-70-0, Merck-Millipore) containing just enough PBS to cover the surface of the dish, ensuring that the larvae were not drowning. After 30 min in

PBS, larvae were transferred to a fresh Petri dish, containing a sieve or membrane and yeast peptone dextrose (YPD), thickened to a final concentration of 1.0M sorbitol, as well as dextran blue to a final concentration of 400 mg/ml (#31393, Sigma-Aldrich). To ensure a minimum dilution of YPD with water or other solvents, dextran blue was dissolved in YPD with 1.0M sorbitol. 5-ethynyl-2'-deoxyuridine (EdU) was used as read-out for on-going cell division; therefore, YPD was supplemented with 1X EdU ClickIt solution (#C10338, Lifetechnologies). The cell cycle inhibitor Aphidicolin was used at a final concentration of 5 µg/ml (#A0781, Sigma-Aldrich; stock solution prepared in 100% EtOH, 1 mg/ml) to pause the cell cycle.

Larvae were incubated in the YPD solution (with or without Aphidicolin, with or without EdU treatment) for 4 h before heat shock (HS). After 4 h of incubation, larvae in the dishes were transferred onto ice, sorted by visual inspection and detection of dextran blue in the gut, and then subjected to a heat shock (or not) for 1 h at 37°C. After heat shock, larvae were again transferred into freshly made YPD solution with sorbitol, dextran blue, and Aphidicolin (same concentrations as before). Larvae were incubated again for 12 h in the solution, transferred in the dishes onto ice, and those that still incorporated the food, as judged by visual inspection of dextran blue in the gut, were dissected and fixed as described for chromatin preparation in II. 4.

II. 10. Antibodies

Name	Description	Amount [µl]/ Dilution	Use
Hoechst 33342	DNA staining	1:500	IS
α-Cy3 anti-rabbit (Jackson)	#111-025-003 goat anti-mouse IgG	1:500	IS
α-E(z)	3TAF affinity purified, rabbit, full length E(z), 5th bleed	2	ChIP
α-H3 (Abcam)	#1791 rabbit, polyclonal, C-terminus	0.5	ChIP
α-H3 (Active Motif)	#39763 mouse, monoclonal, N-terminus	5	ChIP
α-H3K27me2 (Millipore)	#07-452 rabbit, polyclonal	1	ChIP
α-H3K27me3 (Millipore)	#07-449 rabbit, polyclonal	0.25	ChIP
α-Pc	PCCD2 08.2005 affinity purified, rabbbit	20	ChIP
α-Ph	Ph2 affinity purified, aa766984, clone from R. Paro	10	ChIP
α-β-galactosidase	mouse	1:200	IS

Table II.1: List of antibodies with respective amounts that were used for ChIP or dilution that was used for immunostainings (IS).

II. 11. Primers used for qPCR analysis of ChIP experiments

II. 11. 1. Primers in Ultrabithorax, heterochromatin, and euchromatin

Primer pair C1-C3 are located in Ultrabithorax (*Ubx*), their localization to the bithoraxoid (*bxd*) PRE is indicated in Table II.2. Primer pair C4-C5 are located in euchromatic (eu) and heterochromatic (het) regions. The position of C4 and C5 is relative to the transcription start site (TSS).

Name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Position [kb]
C1	GCCGTGGAGCAGTTCAAAGTA	TCGTTGGTCGTGTCCTCTTAATT	+26.8
C2	CCATAAGAAATGCCACTTTGC	CTCTCACTCTCTCACTGTGAT	+31.5
C3	GTCCTGGCCAAGGCAAATATT	CGAAAGGAGAACGGAGAATGG	+34.4
C4	TCAAGCCGAACCCTCTAAAAT	AACGCCAACAAACAGAAAATG	-12.5
C5	CCGAACATGAGAGATGGAAAA	AAAGTGCCGACAATGCAGTTA	-3.1

Table II.2: Primer pairs used for qPCR analysis of endogenous regions. Sequences are given from 5' to 3'. The position is given according to next transcription start site (C4-C5) or to the next PRE (C1-C3).

II. 11. 2. Primers in transgenic region

Primer pairs that are located in the transgenic region of the >PRE>-*dpp*^{WE}-*LacZ* flies (T2-T10), of the >PRE>-*dpp*^{WE}-*LacZ*-UZ (T12-T13) and primers directly up- or downstream of the transgenes (T0, T0', T11, T11', T12, T13) are given with their positions relative to the border of the transgenic *bxd* PRE. T0 is specific for 36c, T0' and T11' for 7b, T1 and T11 for 17b, and T5' and T11' for 17-1.

Name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Position [kb]
T0'	ACTGCCCCTAATGTCAGCAT	CACCAGCAACTAGGCGATTC	-5.2
T0	AAACCCAGAGGGCTTTGGTC	TCGTCGACATCATCATACAGTCC	-5.2
T1	TGTGCTCAAATC%CGTTGCT	GCAGGAGCGTTTTTCTGAGG	-5.4
T2	TCATAGCTTGGAGTTTGCTTGC	AGCTCAACAGGTTTTAGGAACCA	-4.1
T3	ATCGCGATGAGGACATGCTT	ACTCTTACCGAAAATGACAGATCCA	-1.4
T4	GCGGAAAACGAAAGAGAGCGCC	TGGATGTGCGACTGACTGGCTG	0
T5	GAATGGGACGCGAATTCGATA	ACAAGTGCTTGGAGTTTCCTA	+0.9
T5'	TCGGCGTTTCATCTGTGGTG	TCAGGTCAAATTCAGACGGCA	+1.4/5.0
T5''	TGTGAAATCGGTCAAGCCCC	CGTTGTAAAACGACGGGATCG	7.3
T6	TAATCACGACGCGCTGTATC	CCAGGTAGCGAAAGCCATTT	+2.4/6.0
T7	GATGCGGTGCTGATTACGAC	GTGTATCGCTCGCCACTTCA	+3.4/7.0
T8	CGGTCGCTACCATTACCAGTT	TCGATCGAAACATTCTTATCAGTCT	+3.7
T9	ACACTGATATGGTCGCTCGC	ATTTCGCAGAACAAGCTGGC	+4.3
T10	ACACTGATATGGTCGCTCGC	ATTTCGCAGAACAAGCTGGC	+4.3
T11	TCTCGCGCTGAGATTGTGAAA	CTGCCCGCAGTTGTTAAACC	+5.3
T11'	TCGCCCAGTTGAATTTGTCCT	GACACGCTTTGCTGCTTCTT	+5.2

Table II.3: Primer pairs used for qPCR analysis of transgenic regions. Sequences are given from 5' to 3'. The position is given according to the border of the *bxd* PRE inside of the transgene. The following primers were specific for distinct transgenes: T0 for 36c, T0', T11' for 7b, T1, T11 for 17b, T5,' T11' for 17-1. Primer T4 corresponds to the *bxd* PRE in the transgene.

II. 12. Primers for PRE-excision efficiency determination

Name	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
LacZ	AGCGGAAAACGAAAGAGAGC	CGCTCTAGCCAGCTTCAAAAG
PRE*dppWE*	GCGGAAAACGAAGAGAGCGCC	TGGATGTGCGACTGACTGGCTG
RyPRE	AGTTGAGCAAGTTTTCGATGAA	ACCTGCAGCCAAGCTTCTTT

Table II.4: **Primer pairs used for PRE-excision efficiency determination.** These primers were used to assess PRE-excision by qPCR on imaginal wing discs. Sequences are given from 5' to 3'. PRE*dppWE* and RyPRE span the *bxd* PRE in the transgene. From these primers DNA can only be amplified in the presence of the PRE.

II. 12. 1. Primers used for inverse PCR

Name	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
Plac4/1	ACTGTGCGTTAGGTCCTGTTCATTGTT	CACCCAAGGCTCTGCTCCCACAAT
Pry4/1	CAATCATATCGCTGTCTCACTCA	CCTTAGCATGTCCGTGGGGTTTGAAT
Pry2/Plw3-1	CTTGCCGACGGGACCACCTTATGTTATT	TGTCGGCGTCATCAACTCC

Table II.5: Primer pairs used for inverse PCR. Sequences are given from 5' to 3'. All primers are P-element specific primers.

III. Results

III. 1. Inheritance of H3K27me3 upon excision of a PRE

III. 1. 1. Transgenic fly lines

III. 1. 1. 1. >PRE>*dpp^{WE}-LacZ*

Transgenic flies, containing an excisable PRE of the BX-C have been described before (Sengupta *et al.*, 2004). Aditya K. Sengupta had generated and used these flies in the laboratory many years ago to show that a PRE from *Ubx* in *Drosophila* represents a general silencer element that works on heterologous enhancers and promoters in a PcG-dependent manner.

The transgene that was used to generate these fly lines is depicted in Figure III.1. The PRE in the transgene corresponds to the 1.6 kb *bxd* PRE (Fritsch *et al.*, 1999) of the *Drosophila* BX-C. In the transgene it is encompassed by two Flipase recombinase target (FRT) sites. The FRT sequences were cloned from the plasmid J33R (Struhl *et al.*, 1993). A 3 kb fragment of the *LacZ* reporter gene is located downstream of the *bxd* PRE and is expressed under control of the TATA box minimal promoter from *hsp70*, coupled to the decapentaplegic wing disc enhancer (*dpp^{WE}*) (Müller and Bienz, 1991). The rosy sequence, which is included 3' of the PRE, corresponds to the wild-type rosy gene. The entire cassette is flanked by P-element repeats permitting P-transposase-mediated integration into the genome. The transgenes are kept in a rosy mutant background (*ry^{42}*), in which 16 bp of the cell non-autonomous eye marker are replaced by 5'-CCAAGAG-3'.

ry loss of function mutants fails to produce drosopterin in the eye, resulting in a brownish eye color. *ry^{+}* was used as a transgene marker to identify successful integration events.

III. 1. 1. 2. PREs in >PRE>*dpp^{WE}-LacZ* repress *LacZ* expression *in vivo*

Four independent >PRE>*dpp^{WE}-LacZ* fly strains, in which the transgene had been introduced by random P-element insertion into different genomic

localizations, were analyzed in this study. The fly strains >PRE>*dpp*^{WE}*-LacZ* 7b, >PRE>*dpp*^{WE}*-LacZ* 17b, >PRE>*dpp*^{WE}*-LacZ* 30b, and >PRE>*dpp*^{WE}*-LacZ* 36c will be referred to as 7b, 17b, 30b, and 36c, in the following.

The *dpp*^{WE} enhancer in the construct drives expression of *LacZ* in a stripe of cells in the anterior compartment of the *Drosophila* wing imaginal disc (as to be seen in *Fig. III.2*). Importantly, expression of *LacZ* can only be observed in fly strains in which the PRE had been excised from the transgene. In animals carrying the full transgene (i.e. including the PRE), *LacZ* expression is repressed by the *bxd* PRE. This repression is PcG-dependent: in larvae that are trans-heterozygous for hypomorphic mutations in Suppressor of zeste (Su(z)12) and thus have compromised PRC2 activity, *LacZ* becomes de-repressed (Sengupta *et al.*, 2004).

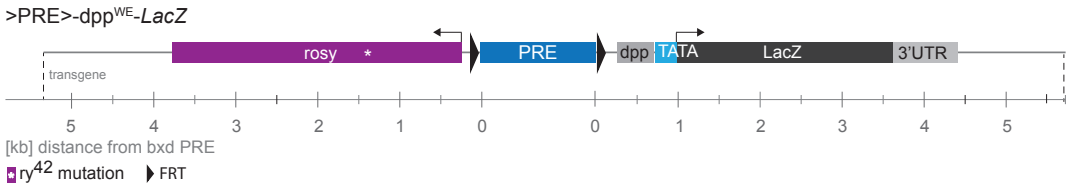

Fig.* III.1: Schematic drawing of >PRE>*dpp*^{WE}*-LacZ Scale bar indicates distances in kb from the PRE. Black arrow heads next to the PRE represent FRT sites. Arrows mark TSS. The asterisk in rosy marks the site where the native ry^{42} allele contains the lesion described in the text. Borders of the transgene are symbolized by a dotted line. 3'UTR is 3' untranslated region.

As proof of principle for the inducible system, fly strains 7b, 17b, 30b, and 36c were tested for *LacZ* expression in the presence and in the absence of the PRE, respectively. To this aim, transgenic flies were crossed to flies carrying a transgene that allows expression of FLP recombinase under the control of a *heat shock*-inducible *hsp70* promoter (*hsp70*-FLP). In the progeny, excision of the PRE induced by heat shock, resulted in de-repression of *dpp*^{WE}*-LacZ* that could be visualized by X-gal staining of wing imaginal discs.

Figure III.2 shows *Drosophila* wing imaginal discs from such animals in which *LacZ* expression was analyzed 24 h after heat shock. The stripe pattern which could be observed in heat-shocked larvae corresponds to *dpp*^{WE}-mediated *LacZ* expression.

Expression of *LacZ* upon heat shock indicates that in all four fly strains that were analyzed, the PREs were efficiently excised upon induction of *hsp70*-FLP as previously reported (Sengupta *et al.*, 2004). I therefore conclude that in all four fly strains the PRE can be removed by heat shock.

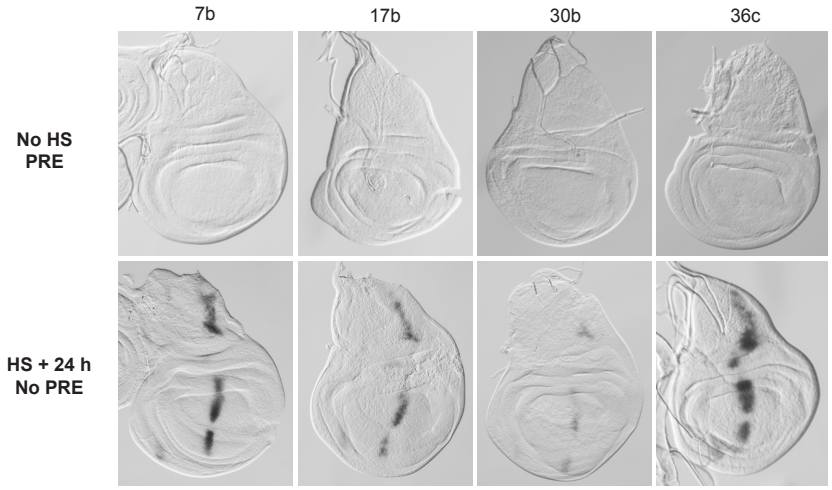

***Fig.* III.2**: **X-gal staining of wing discs before and after HS.** Wing discs were from progenies of yw122; FN9 F40 flies crossed to >PRE>*dppWE-LacZ* flies 7b, 17b, 30b, and 36c (ID of fly strain is indicated above the pictures). Upper row depicts wing imaginal discs in which the PRE is present (No HS), lower row shows discs from which the PRE had been excised 24 h before dissection by HS (HS + 24 h). Accordingly, *LacZ* becomes de-repressed in the discs from which the PRE had been removed.

III. 1. 1. 3. Only a small fraction of cells in wing discs express β-galactosidase

It should be noted that the dpp^{WE} activates expression of β-galactosidase, the *LacZ* product only in a small fraction of wing disc cells. Analyses by X-gal staining to monitor the enzymatic cleavage of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside by ß-galactosidase is an assay to detect presence of ß-galactosidase protein, which might even detect protein that accumulated over time. To complement these analyses, I also stained imaginal discs with an antibody against ß-galactosidase to directly detect the protein (*Fig. III.3*). Together, these analyses suggest that the fraction of cells that

express ß-galactosidase corresponds to less than 5% of all cells in a wing imaginal disc (*Fig. III.3*).

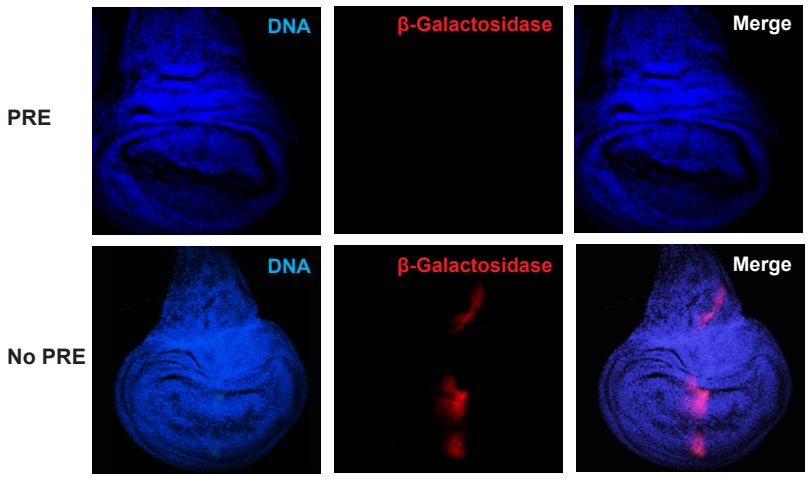

***Fig.* III.3**: **β-galactosidase IS in the presence and the absence of the PRE in wing discs.** Antibody staining a of 3rd instar wing imaginal discs of >PRE>*dppWE-LacZ* 36c (upper panels) or >*dppWE-LacZ* ΔPRE 36c #2 (lower panels) gainst DNA in blue (Hoechst) or β-galactosidase in red.

To calculate this fraction, 3rd instar larval wing imaginal discs were dissected, fixed, and stained for β-galactosidase as well as DNA (Hoechst 33342). The fraction of cells expressing *LacZ* was calculated from the number of pixels that were positive for β-galactosidase staining in the respective microscopic channel in the absence (>*dpp*^{WE}*-LacZ* ΔPRE 36c #2) or in the presence of the PRE (>PRE>*dpp*^{WE}*-LacZ* 36c). Numbers represent positively stained pixel as assessed by Leica TCS SP8 imaging software.

In discs that lack the PRE (lower panel), approximately 3.7% of cells showed nuclear *LacZ* expression (mean number of *LacZ*-expressing cells per disc: 20073 ± 2308 SD, mean number of Hoechst-stained cells per disc: 540438 ± 9862 SD).

I could thus confirm that only a small number of imaginal disc cells actually express β-galactosidase under control of the *dpp* enhancer. This finding is important, since presence of β-galactosidase is an indication for transcribed and translated *LacZ.* Active transcription can affect histone modifications (Schmitt *et al.,* 2005; Erokhin *et al.,* 2015), therefore it was important to clarify that the fraction of *LacZ* transcribing cells in the entire wing disc is negligible

compared to the fraction of cells that do not show *LacZ* transcription as assessed by presence of β-galactosidase.

III. 1. 1. 4. >PRE>-*dpp*^{WE}-*LacZ* insertion sites were mapped to chromosome 3R and the X chromosome

As mentioned before, the four fly strains 7b, 17b, 30b, and 36c had been generated using random P-element insertion. To determine the genomic context and chromatin environment of the transgenes in the four strains, insertion sites were mapped using inverse PCR.

For inverse PCR, gDNA was purified from transgenic flies and digested using the restriction endonuclease Sau3AI, which recognizes the sequence ^GATC_. This digestion step resulted in countless pieces of DNA, which were subsequently re-ligated randomly into plasmids, using T4 DNA ligase. Some of these newly generated circular pieces of DNA contained the flanking P-elements, from which inside-out PCR could be performed using P-element-specific primers. Analysis of PCR products by agarose gel electrophoresis revealed bands that could be analyzed by sequencing (see Materials and Methods for details).

From the sequencing data, genomic sites of insertion could be identified for all transgenic fly strains. In three of the fly strains (17b, 30b, and 36c), insertions occurred in chromosome 3R. In strain 7b the transgene was inserted into the X chromosome. Exact genomic localizations, including genes that are affected by the insertions, are given in Table III.1.

For verification of insertion sites, combinations of P-element specific primers with localization-specific primers were used in PCR on gDNA. Only in primer combinations were localization-specific and insertion-site specific primers matched (7b reverse (R) with gDNA from 7b, 30 forward (F) with gDNA from 30b, 36 R with gDNA from 36c) specific amplification was observed, as shown in Figure III.4. This test confirmed that the previously identified insertion sites were correct.

	Insertion site		
>PRE>*dppWE-LacZ*	**Chromosome**	**Localization**	**Gene affected**
7b	X	511711	CG13366, intronic region
17b	3R	27545450	*tramtrack*, intronic region
30b	3R	10902373	*dpr9*, intronic region
36c	3R	27569655	CG1890, exon 2

Table III.1: Insertion sites of >PRE>*dpp^{WE}-LacZ* constructs (in 7b, 17b, 30b, and 36c fly lines). Chromosomes, localizations, and genes, into which the insertions occurred, are listed.

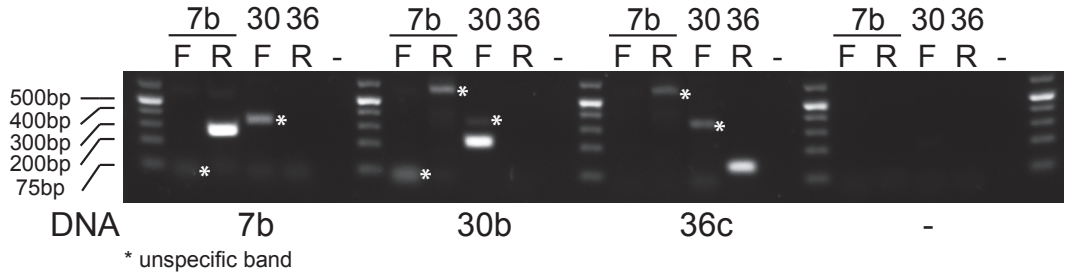

***Fig.* III.4: PCR verification of insertion sites in 7b, 30b, and 36c.** gDNA that was used in the different PCR reactions is indicated below the figure P-element specific primer Pry2 was combined with localization-specific primers. Localization-specific primers are noted above the PCR lanes. Unspecific background bands are marked with asterisks. F forward, R reverse. 17b was analyzed in earlier experiments in is therefore not included in this gel.

III. 1. 1. 5. Endogenous >PRE>-*dpp^{WE}-LacZ* insertion sites are not occupied by H3K27me3 domains

In order to be able to assess a possible role of flanking chromosomoal regions when analyzing transgene regulation by the *bxd* PRE, it is crucial to know the presence of regulatory sequences and chromatin modifications at the transgene insertion site. Specifically, levels of H3K27me3 in endogenous regions – in the absence of the transgenic PRE – have to be known. Defining the genomic chromatin environment ascertains that H3K27me3 levels at these localizations can be entirely accounted for by the presence of the PRE.

To define the chromatin environment, in which the transgenes were inserted, I compared H3K27me2 and H3K27me3 levels in *Drosophila* nuclear chromatin from 3rd instar larval or adults and chromatin from embryos (age 14 - 16 h) *in silico*. The genomic context of all four transgenic fly lines was determined using *modEncode*, comparing H3K27me2 and H3K27me3 levels as determined in Oregon-R strain 14-16 h embryos as well as chromatin prepared from adult Oregon-R heads. *ModEncode* is a research network that provides, among other information, details on genome-wide histone modifications in the fly (www.modencode.org).

ChIP profiles of different chromatin types are depicted in Figure III.5-8. Arrows in the figure mark insertion sites in each of the fly lines. Profiles comprise regions of 50 kb around the insertion sites of the transgenes (lower panels) and a close-up of the insertion sites, showing 1 kb of the genomic region (upper panels), respectively. Areas in which H3K27me2 or H3K27me3 levels are higher than background represent specific histone modification domain and are highlighted in yellow.

None of the profiles shows specific H3K27me3 signals in areas surrounding the insertion sites. The four fly lines differ with respect to distances between transgenic insertion sites to the closest H3K27 trimethylation peak (above background), with respect to H3K27me3 levels at these peak regions, as well as with respect to the distances over which H3K27me3 domains spread.

All genomic regions are devoid of endogenous PREs and – considering the low background H3K27me3 levels in all fly strains – are well suited for ChIP experiments that aim to characterize transgene-induced H3K27me3 patterns.

Fly line 7b and 17b were chosen for further analyses. Because the chromatin-immunoprecipitation analyses were performed in heterozygots, the insertion site of 7b on the X chromosome (which is present in female flies twice but only once in male flies) was particularly useful. In male larvae, this allowed to determine the H3K27me3 signal in the chromatin flanking the transgene insert, without the complication of also detecting ChIP signal from (non-modified) histones on the homologous chromosome. The 17b line was chosen, because the transgene insert is located more than 20 kb away from the nearest H3K27me3 domain, minimizing any potential contribution from PREs in the vicinity of the transgene insertion site.

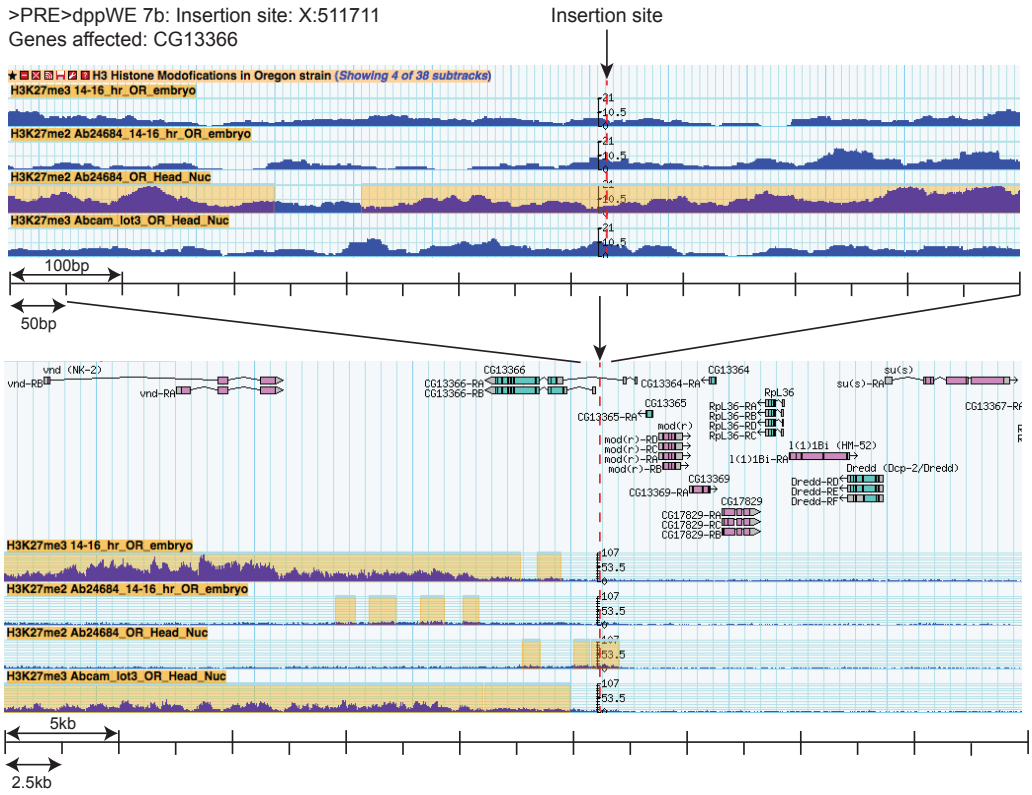

***Fig.* III.5: Chromatin environment of genomic insertion site in >PRE>*dpp^{WE}-LacZ* 7b.**

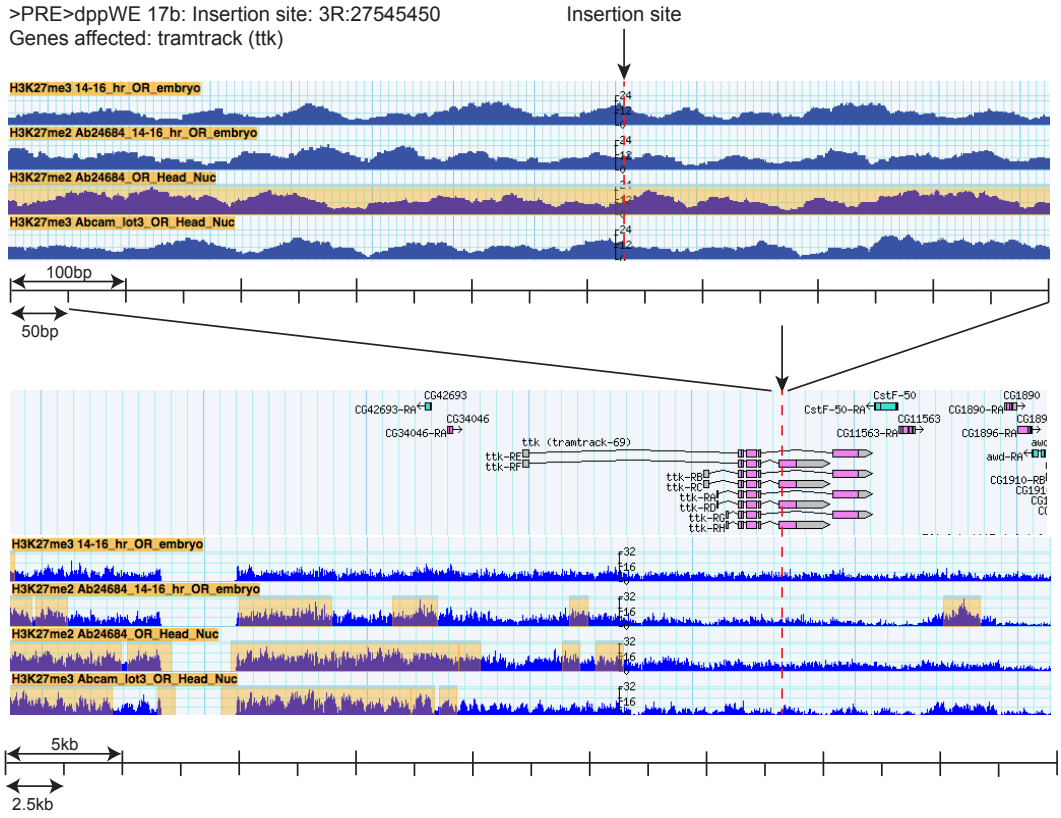

***Fig.* III.6: Chromatin environment of genomic insertion site in >PRE>*dpp^{WE}-LacZ* 17b.**

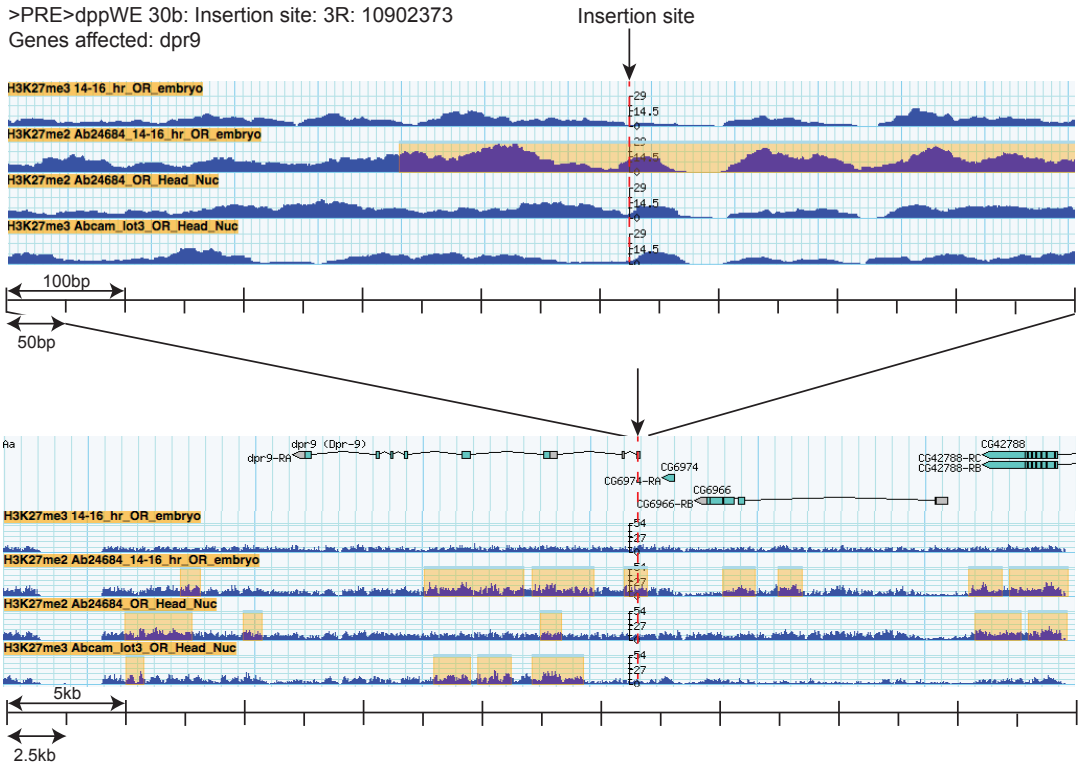

***Fig.* III.7: Chromatin environment of genomic insertion site in >PRE>*dpp*^{WE}-*LacZ* 30b.**

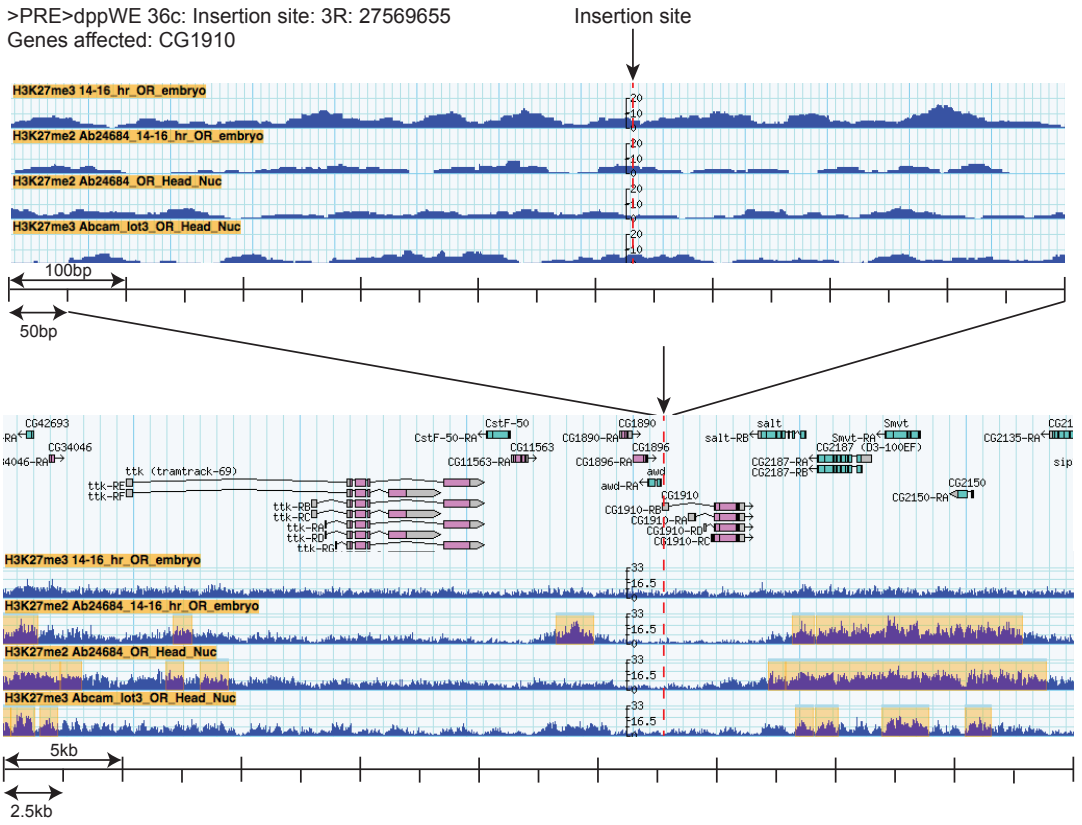

***Fig.* III.8: Chromatin environment of genomic insertion site in >PRE>*dpp*^{WE}-*LacZ* 36c.**

III. 1. 1. 6. Generation of stable flip-out strains >*dpp*^{WE}-*LacZ* ΔPRE

As shown above, *LacZ* expression under control of the *dpp* enhancer is repressed in imaginal wing discs in the presence of the *bxd* PRE, in all four generated fly lines.

In order to generate derivative transgenic lines, containing the transgene lacking the PRE, the PRE was excised in the germline of transgenic animals and derivative *dpp*^{WE}-*LacZ* ΔPRE transgenic lines were established. Flip-out was verified by X-gal staining of larval progenies of these flies. In *dpp*^{WE}-*LacZ* ΔPRE transgenic larvae high level of *LacZ* expression in the wing discs was detected in all animals, as expected (see *Fig. III.9*).

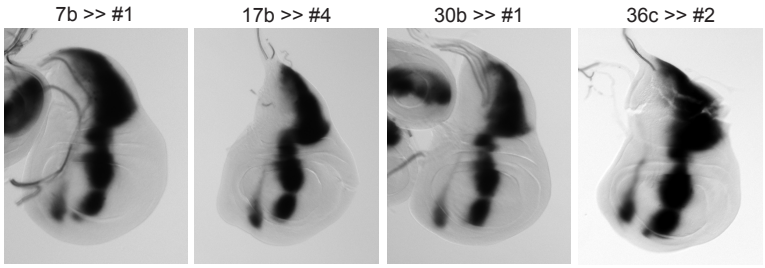

***Fig.* III.9: X-gal staining of imaginal wing discs of stable flip-out fly strains.** All imaginal wing discs show strong *LacZ* expression and therefore indicate stable excision of PREs from these strains. Strain numbers are indicated above the figure.

III. 1. 2. Excision dynamics of PRE removal by heat shock

III. 1. 2. 1. Determination of the presence or the absence of PRE

In a next series of experiments I investigated the efficiency of PRE-excision within the population of cells in wing imaginal discs. To this end males containing the >PRE>*dpp*^{WE}-*LacZ* transgene (7b, 17b, 30b, and 36c, respectively) were crossed with females containing the *hsp70*-FLP transgene.

Upon *heat shock* induced excision of the PRE, I determined the fraction of cells lacking the PRE using the qPCR strategy depicted in Figure III.10. The ratio of the amount of amplified DNA labeled in blue (PRE amplicon) to the amount of amplified DNA labeled in black (*LacZ* amplicon) in wing imaginal

discs from non-heat shocked and heat shocked animals permitted to calculate the fraction of cells from which the PRE had been excised after heat shock. Figure III.10 depicts the results from determining the ratio of PRE amplicon to *LacZ* amplicon when comparing >PRE>*dpp*^{WE}-*LacZ* and >*dpp*^{WE}-*LacZ* Δ*PRE* transgenics.

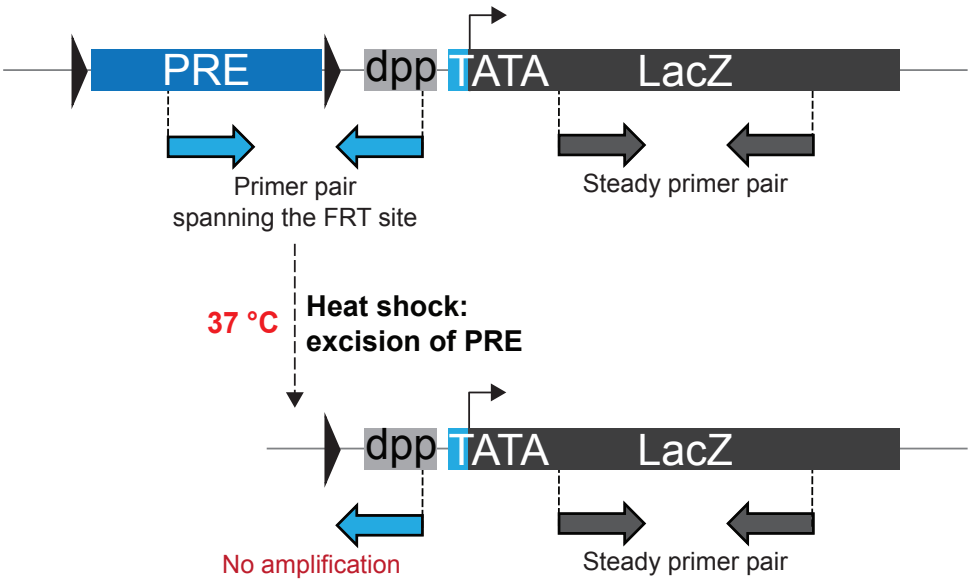

***Fig.* III.10: Schematic illustration of DNA elements in >PRE>*dpp*^{WE}-*LacZ* transgene with respective primer localization.** Primers are represented as arrows below the DNA elements. Primer pair PRE*dpp*^{WE} spanning the FRT is incomplete after excision of the PRE by heat shock. The steady primer pair *LacZ* can amplify unchanged after heat shock.

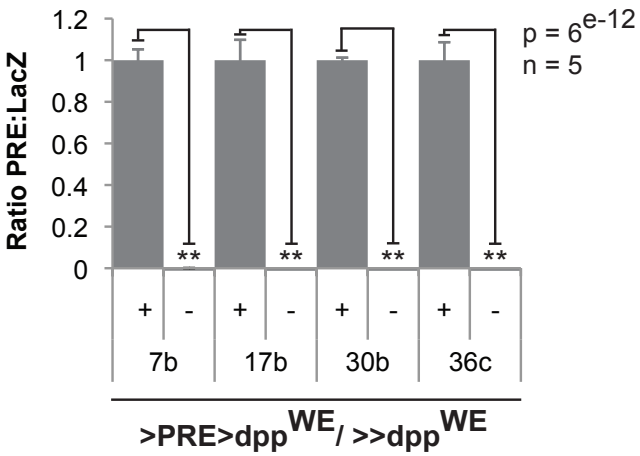

***Fig.* III.11: Ratio of PRE to *LacZ* in the presence and the absence of the PRE.** Ratios of PRE*dpp*^{WE}-amplifying cells to cells that only amplify *LacZ* in stable fly strains with PRE (+) and without (-),

respectively. Strain numbers are indicated below the graph. Ratios were calculated from mean values of 5 independent gDNA preparations all run in technical duplicate. Error bars indicate SDs: p-value = 6e-12 (n = 5).

III. 1. 2. 2. A single heat shock is sufficient to excise the PRE from >95% of cells in imaginal wing discs

For our experimental approach it was crucial to achieve complete excision in all cells of the imaginal wing discs. At the same time, I had to be certain that the FLP enzyme is not leaky, which means that excision should exclusively occur upon heat shock and not independently of it. Several *hsp70*-FLP transgenic lines were tested for their potential to completely excise the PRE after a single heat shock. Best efficiencies were achieved with line Flp10.

Using the above described qPCR strategy; I induced PRE-excision in all four >PRE>*dpp*^{WE}-*LacZ* strains by heat shock and analyzed in each case wing discs from multiple individual larvae 24 or 48 h later. As shown in Figure III.12, if the fly strains were not subjected to a heat shock (No HS), the ratio of PRE*dpp*^{WE} to *LacZ* amplicons were approximately equal to, as expected. If a single heat shock was applied to induce FLP expression and wing discs were analyzed 24 h later the ratio of PRE*dpp*^{WE} to *LacZ* template DNA dropped to less than 0.05, indicating that less than 5% of wing disc cells retained the PRE. Ratios of PRE to *LacZ* did not decrease further 48 h after heat shock, suggesting that the process of PRE-excision efficiency was completed within 24 h. I therefore concluded that *hsp70*-FLP effectively excised the PRE in >95% of cells within 24 h after heat shock in all fly lines.

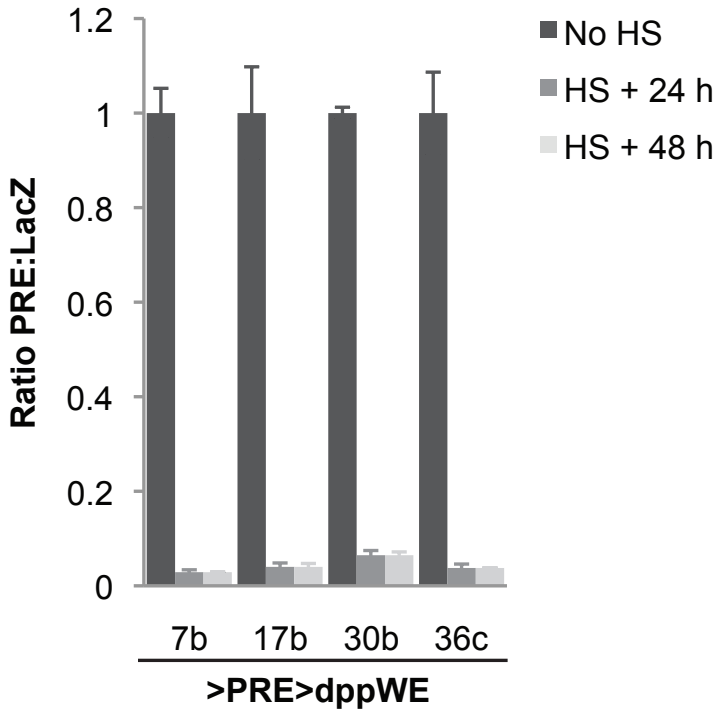

***Fig.* III.12: PRE-excision efficiencies** - assessed as ratio of amplification of PRE*dpp*^{WE} primers to amplification of primers in *LacZ* region (ratio one indicates equal values). Ratios are given for all fly strains before heat shock and 24 h/ 48 h after one heat shock, respectively. Error bars indicate SDs of n = 8 independent experiments.

III. 1. 2. 3. The PRE if efficiently excised within eight hours after heat shock

In order to draw conclusions on how H3K27me3 levels change after excision of the PRE in and around the transgene, it was important to define a time point zero for these experiments. Therefore, I analyzed by what time point after heat shock the PRE would be excised from >95% of wing disc cells. As shown in Figure III.13, two hours after heat shock the ratio of PRE*dpp*^{WE} to *LacZ* amplification had already dropped to less than 40%. Eight hours after heat shock, excision had occurred in more than 95% of the wing disc cells. These observations suggest that FLP-mediated excision is a relatively rapid process.

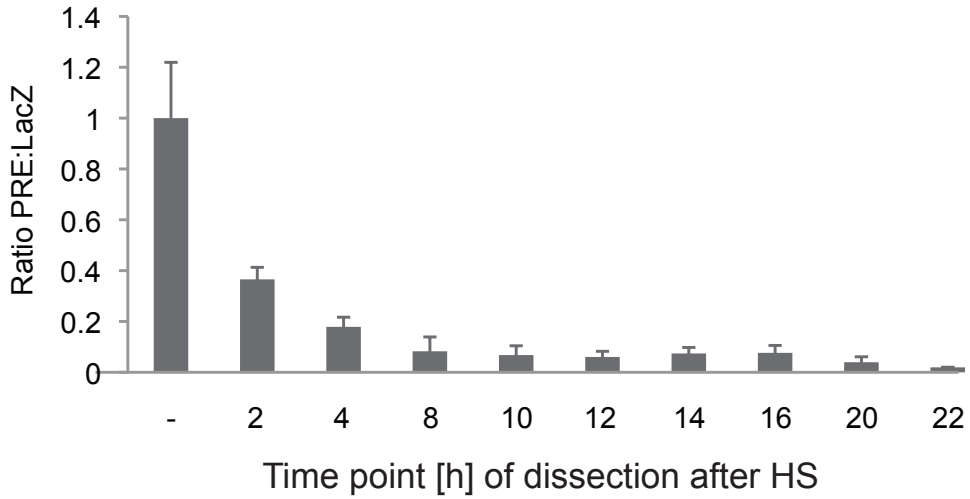

***Fig.* III.13: Time course of PRE-excision after heat shock**. PRE-excision was assessed as ratio of amplification of PRE*dpp*^{WE} to amplification of *LacZ* primers by qPCR. Dissection time points are given as hours after heat shock. Error bars represent SDs of n = 6 larvae.

Since the mechanism of FLP recombination is not entirely understood yet, I also addressed the question of whether the 5% of cells which retained the PRE after heat shock, might have been exposed to the enzyme in a specific phase of the cell cycle that was incompatible with FLP-mediated recombination. I performed a second heat shock four hours after the first one to test whether excision efficiency could be further increased.

In fact, as shown in Figure III.14, I observed that ratios of PRE to *LacZ* were further decreased by applying a second heat shock four hours after the first heat shock (compare the PRE:*LacZ* ratio signal in the one heat shock + 8 h panel with the two consecutive heat shocks + 4 h panel; also compare the one heat shock + 6 h panel with the two heat shocks + 2 h panel). In this experiment, an additional primer pair spanning the FRT site upstream of the PRE (RyPRE) was used to calculate the ratio between cells that retained the PRE versus those in which the PRE was excised. Both primer pairs imply a highly similar excision profile.

This experiment shows that it is unlikely that cells that retained the PRE failed to excise because they were exposed to FLP at a specific cell cycle stage that would interfere with recombination and, thus, excision of the PRE.

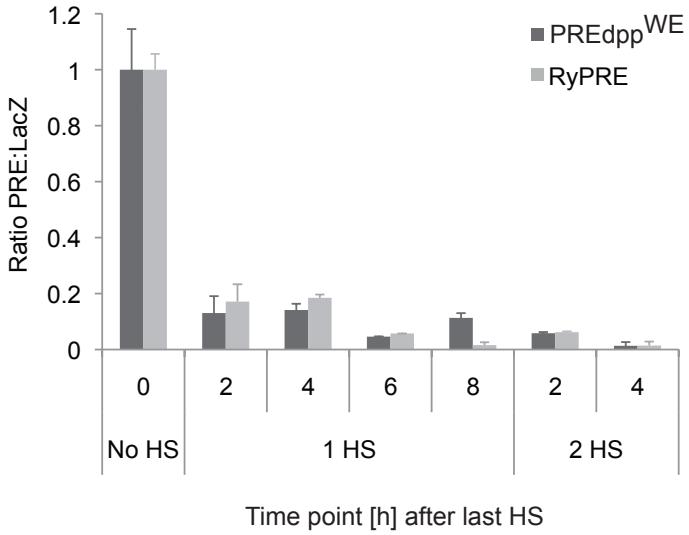

***Fig.* III.14: PRE-excision efficiency comparing one or two heat shocks.** At indicated time points before dissection, qPCR amplification of PRE*dppWE* and *LacZ* was performed to assess excision efficiency. The second heat shock was performed 4 h after the first one. Ratios are normalized to *No HS* ratio. Error bars indicate SDs of n = 10 larvae.

Taken together, these excision quantification experiments suggests that one heat shock is sufficient to excise the PRE from more than 95% of imaginal wing disc cells within eight hours. A second heat shock does not further ameliorate excision efficiency. Therefore, a single heat shock of one hour was applied and time point 'zero' was set at eight hours after heat shock induction.

III. 1. 3. Chromatin landscape surrounding the trans-genes

III. 1. 3. 1. The presence of a transgenic PRE establishes repressive H3K27me3 domains

In order to analyze the levels of H3K27me3 at the transgene, chromatin was prepared from wing imaginal discs from >PRE>*dpp^{WE}-LacZ* transgenic larvae (line 7b), and from larvae of the derivative transgenic line >*dpp^{WE}-LacZ* ΔPRE (line 7b) (*Fig. III.3*). Next, ChIP was performed using an H3K27me3 antibody to determine H3K27me3 occupancy at the transgene. As a control, ChIP against H3 was performed.

Chromatin immunopreciptiates were analyzed by qPCR using primer pairs that specifically amplified sequences from the transgene but not from the endogenous *hsp70*, *dpp*, and *ry* genes at their native location. Additional amplicons were used to monitor the presence of H3K27me3 and H3 at regions in the flanking chromatin at the transgene insertion site. In fly line 7b with the insertion of the transgene on chromosome X (see *Fig. III.5*) chromatin was prepared from male larvae, containing a single copy of transgene-flanking chromatin. In case of the other transgenic lines chromatin was prepared from transgene heterozygotes. In all cases, ChIP experiments were performed from at least three independently prepared batches of chromatin and the ChIP signals were represented as percentage of input chromatin with error bars indicating the standard deviation (SD) of the signal at each region.

Figure III.15 shows H3K27me3 and H3 profiles at the >PRE>*dpp*^{WE}*-LacZ* 7b (upper panel) and at the corresponding >*dpp*^{WE}*-LacZ* Δ*PRE* 7b (lower panel) transgene insertion.

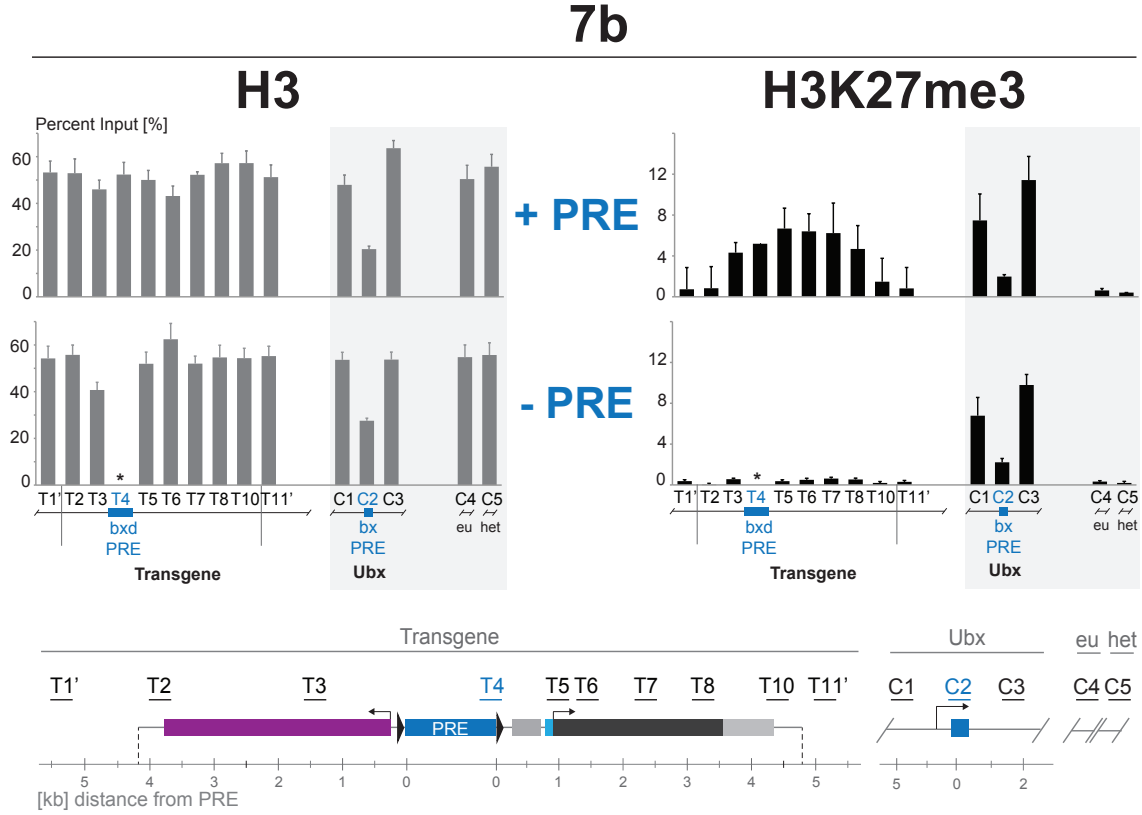

***Fig.* III.15**: **H3 and H3K27me3 levels in 7b in the presence and in the absence of the PRE.** Percent input levels as determined by ChIP-qPCR in the presence and the absence of the PRE inside and surrounding the transgene in >PRE>*dpp*^{WE}*-LacZ* 7b (+ PRE) and >*dpp*^{WE}*-LacZ* ΔPRE 7b #1 (- PRE).

Basic H3K27me3 levels in + PRE graph are due to the presence of the PRE; in their derivative flip-out line (- PRE) H3K27me3 levels are as low as in negative control regions (C4, C5). A scheme of primer localizations in the transgene is shown below the graphs. PREs are highlighted in blue. Control regions are displayed over grey background. Please note that in the absence of the transgenic PRE (- PRE), primer pair T4 does not yield any signal (due to the absence of its forward primer). Primer pair T1' and T11' are in the genomic region and specific for the insertion site on the X chromosome. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

At the >PRE>*dpp*^{WE}*-LacZ* transgene insert, H3K27me3 is detected spanning a domain of at least 6.5 kb (*Fig. III.15:* upper panel, right), whereas no H3K27me3 is detected at the same transgene lacking the PRE (*Fig. III.15*: >*dpp*^{WE}*-LacZ* ∆*PRE,* right panel). As a positive control for H3K27me3 enrichment in the same chromatin, primer pairs amplifying regions directly up- and downstream of the *bx* PRE at the native *Ubx* gene (C1-C3) were used; these showed comparable enrichment of H3K27me3 in >PRE>*dpp*^{WE}*-LacZ* and in >*dpp*^{WE}*-LacZ* ∆*PRE* transgenic animals. In addition, I also measured the H3K27me3 signal at an euchromatic (eu) and a heterochromatic (het) region known to lack H3K27me3 enrichment (C4 and C5; cf. Papp and Müller, 2006).

The H3 profile was comparable in the >PRE>*dpp*^{WE}*-LacZ* and in >*dpp*^{WE}*-LacZ* ∆*PRE* animals. Note the dip of H3 and H3K27me3 at the *bx* PRE (*Fig. III.15*) that reflects reduced nucleosome occupancy at the center of PREs, as previously observed (Papp and Müller, 2006; Kahn et al, 2006). Reduced nuclesome occupancy is also observed at the endogenous *bxd* PRE (Papp and Müller, 2006) but likely not detected here because the T4 amplicon is offset and located more then 0.5 kb from the center of the *bxd* PRE in the transgene.

Taken together, these data show that the *bxd* PRE inserted into a naïve location in the genome is sufficient to establish an H3K27me3 domain that spans several kilobases (kb).

III. 1. 3. 2. PRE-imposed H3K27me3 domains are not specific for insertion sites

I next investigated whether such H3K27me3 domains are also generated at other transgene insertion sites. To this end I generated the H3K27me3 and

H3 ChIP profiles at the 17b and 36c transgene inserts. At both sites, the profiles were generated in >PRE>*dpp*^{WE}*-LacZ* and in >*dpp*^{WE}*-LacZ* Δ*PRE* animals (*Fig. III.16* and *III.17*). At both transgene inserts, a domain of H3K27me3 was observed that required the presence of the *bxd* PRE in the transgene. In both transgene inserts, the H3K27me3 domain spanned about 10 kb (*Fig. III.16* and *III.17*). In both lines, the H3K27me3 enrichment signals tended to be higher than in the 7b line, not only at the transgene but also at the endogenous *bx* PRE. This is likely due to an improvement of the ChIP procedures during the course of the project.

These data show that the *bxd* PRE is able to generate H3K27me3 domains in very different chromosomal environments.

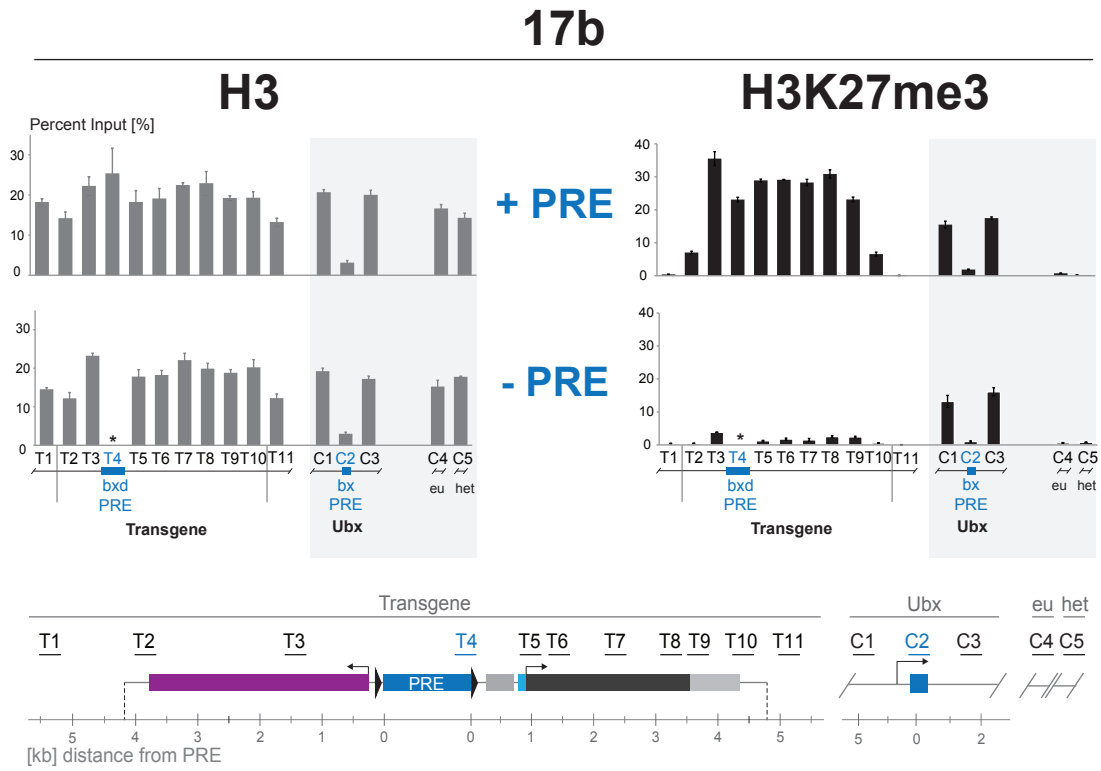

***Fig.* III.16: H3 and H3K27me3 levels in 17b in the presence and in the absence of the PRE.** Percent input levels as determined by ChIP-qPCR in the presence and the absence of the PRE inside and surrounding the transgene in >PRE>*dpp*^{WE}*-LacZ* 17b (+ PRE) and >*dpp*^{WE}*-LacZ* ΔPRE 17b #4 (- PRE). Basic H3K27me3 levels in + PRE graph are due to the presence of the PRE; in their derivative flip-out line (- PRE) H3K27me3 levels are as low as in negative control regions (C4, C5). A scheme of primer localizations in the transgene is shown below the graphs. PREs are highlighted in blue. Control regions are displayed over grey background. Please note that in the absence of the transgenic PRE (- PRE), primer pair T4 does not yield any signal (due to the absence of its forward primer). Primer pair T1 and T11 are in

the genomic region and specific for the insertion site on chromosome 3R. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

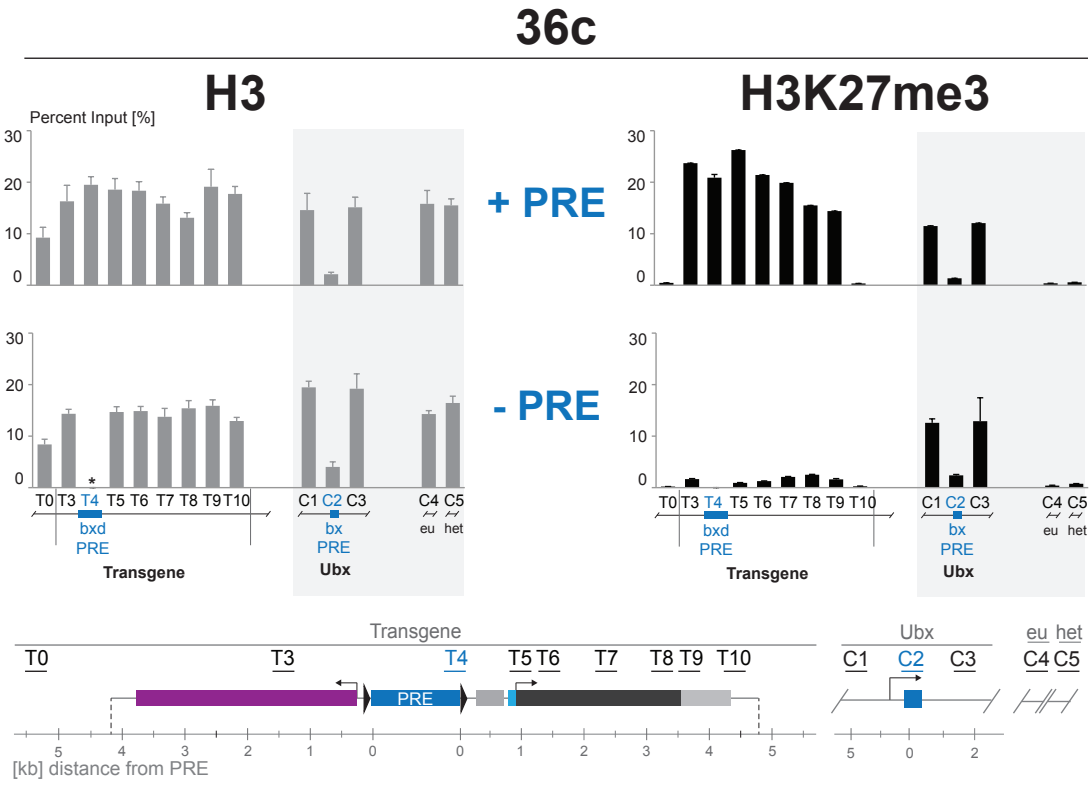

***Fig.* III.17: H3 and H3K27me3 levels in 36c in the presence and in the absence of the PRE.** Percent input levels as determined by ChIP-qPCR in the presence and the absence of the PRE inside and surrounding the transgene in >PRE>*dpp*^{WE}-*LacZ* 36c (+ PRE) and >*dpp*^{WE}-*LacZ* ΔPRE 36c #1 (- PRE). Basic H3K27me3 levels in + PRE graph are due to the presence of the PRE; in their derivative flip-out line (- PRE) H3K27me3 levels are as low as in negative control regions (C4, C5). A scheme of primer localizations in the transgene is shown below the graphs. PREs are highlighted in blue. Control regions are displayed over grey background. Please note that in the absence of the transgenic PRE (- PRE), primer pair T4 does not yield any signal (due to the absence of its forward primer). Primer pair T0 is in the genomic region and specific for the insertion site on 3R. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

III. 1. 4. PRE-imposed, transgenic H3K27me3 domains encompass up to 12 kb

Intriguingly, the H3K27me3 domain, emanating symmetrically from the *bxd* PRE does not appear to invade the flanking chromatin at the insertion site.

Figure III.18 shows H3K72me3 ChIP-qPCR data in the genomic context of the 17b transgene insertion and indicates distances of the H3K27me3 peaks to the transgenic *bxd* PRE. The H3K72me3 domain spreads over a distance of 12 kb (approximately 6 kb up- and downstream of the PRE, respectively). This is highly comparable to what has been found before around transgenic PREs (Comet *et al.,* 2011). In its native chromosomal location in the *Ubx* locus, the H3K27me3 domain spans approximately 100 kb (Schwartz *et al,* 2006). The width of these endogenous domains may require the presence of multiple strong PREs (e.g., such as the *bxd* PRE together with the *bx* PRE or also additional, weaker PREs) or other genomic features that are less well understood.

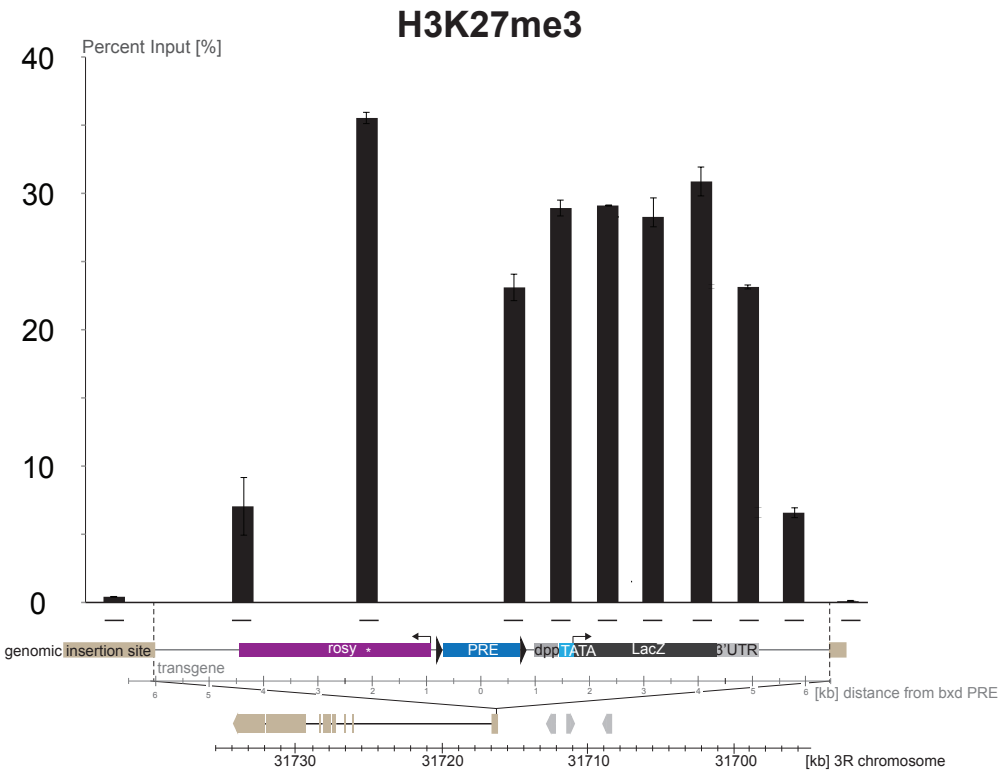

***Fig.* III.18: H3K27me3 domain size in 17b** (as determined by ChIP-qPCR). The PRE-imposed domain spreads over approximately 12 kb and abruptly discontinues. Below the graph, DNA elements of the transgene are depicted with genomic insertion site in *tramtrack.* The *tramtrack* gene with intronic and exonic regions is illustrated in beige below, grey filled elements are undefined genes downstream of *tramtrack*. Composition of the transgene of endogenous regions only allows for qPCR analysis of borders between DNA elements (which are specific for the transgene) or differences of the genetic background (rosy).

Insertion in 17b occurred in an exonic region of a gene known as *tramtrack* (enrolled in neural cell fate (Guo *et al.*, 1995)), whose exons are illustrated below the graph as beige, filled arrows. Only few undefined, small genes are located downstream of the insertion site, illustrated as grey, filled arrows.

Predominant H3K27me3 peaks abruptly discontinue in about 6 kb distance from the PRE on each side. Highly similar domain sizes are observed in the other transgenic fly trains (compare *Fig. III.16* and *III.17*). The very large H3K27me3 domain of 50 kb identified in another study in the native context could be caused by the large number of clustered PREs present in the BX-C locus analyzed there (Schwartz *et al.,* 2006).

III. 1. 5. Excision of transgenic *bxd* PRE *in vivo* results in loss of H3K27me3 domains within a few cell generations

Previous experiments in mammalian cells that tethered PRC2 to DNA using a Gal4-EED fusion protein, suggested that after establishment of an H3K27me3 domain, PRC2 could propagate this domain in the absence of the Gal4-EED DNA-tether through time and cell division. Specifically, in these experiments, tetracycline-induced binding of Gal4-EED upstream of a luciferase reporter gene did not only result in H3K27 trimethylation and luciferase repression, but also in the maintenance of H3K27me3 after washout of tetracycline. This property has been ascribed to the ability of the native EED subunit of PRC2 to bind to H3K27me3 (Hansen *et al,* 2008, Margueron *et al,* 2009).

Considering the fact that in *Drosophila,* PcG-repression cannot be maintained in the absence of PREs (Chan *et al.,* 1994; Chiang *et al.*, 1995; Busturia *et al.*, 1997; Sengupta *et al.*, 2004), I next asked whether H3K27me3 at the transgene would be maintained and propagated after excision of the *PRE* in >PRE>*dpp*^{WE}*-LacZ* transgenic animals. To this end, I generated animals carrying both >PRE>*dpp*^{WE}*-LacZ* and *hsp70*-FLP, excised the PRE by heat shock induction of FLP expression, and analyzed the H3K27me3 profile in wing

imaginal disc cells at different time points after the heat shock (see above under III. 1. 2). Specifically, the chromatin was prepared 12 h, 32 h, and 56 h after heat shock. Taking into consideration that the average cell cycle takes between eight and twelve hours in third instar larvae (Martin *et al*, 2009), the cells had thus undergone at least one cell division at the 12 h time point, at least two cell divisions at the 32 h time point, and approximately four cell divisions at the 56 h time point. The developmental stage at which larvae were dissected was kept constant at 120 h AEL, and hence the time points of excision were progressively set earlier to separate PRE-excision and ChIP analyses. For time point zero, chromatin was prepared from larvae that were not exposed to a heat shock.

Figure III.19 shows percent input data of a time-course experiment in >PRE>*dpp*^{WE}*-LacZ* (line 17b) for H3 and H3K27me3, respectively. Graphs on the left hand side show data for H3 ChIP, H3K27me3 is shown on the right. Time points (12 h, 32 h, 56 h after heat shock) are indicated in the center between the graphs. As in previous Figures, a scheme of primer localization is depicted below the graphs and, as mentioned above, amplicon T4 can no longer be analyzed after heat shock because of lack of the PRE.

As described above, at time point zero (0, upper panels) H3K27me3 forms a broad domain at the >PRE>*dpp*^{WE}*-LacZ* transgene insert 17b that extends from the 3' end of the *ry* gene to the *hsp70* UTR of the 3' end of *LacZ* and the domain is absent in the >*dpp*^{WE}*-LacZ* ∆*PRE* version of this line (No PRE, lower panels).

12 h after induction of FLP expression (i.e., initiation of excision of the PRE), the H3K27me3 signal across the transgene were relatively uniformly reduced by two to three fold (*Fig. III.19*). 32 h after FLP induction, H3K27me3 levels were further reduced by a factor of two to three, and they reached background levels 56 h after FLP induction. Thus, at each time point, H3K27me3 levels across the transgene were reduced by approximately 50%. Importantly, H3 levels remained unchanged throughout the course of the experiment. Moreover, H3K27me3 levels at the control regions at the *bx* PRE of *Ubx* (C1-C3), and at the euchromatic and heterochromatic region (C4, C5) did not show any change in H3K27me3 levels upon excision of the PRE. The reduction of H3K27me3 was thus specifically linked to the removal of the PRE

in the transgene. Concomitant with the loss of H3K27me3, *LacZ* became de-repressed, and ß-galactosidase activity became detectable 12 h after initiation of PRE-excision (*Fig. III.20*), as previously reported (Sengupta *et al.*, 2004). Possible explanations for the lag between removal of the PRE from cells by 8 h and the detection of strong ß-galactosidase activity by 32 h will be discussed in the *Discussion* section.

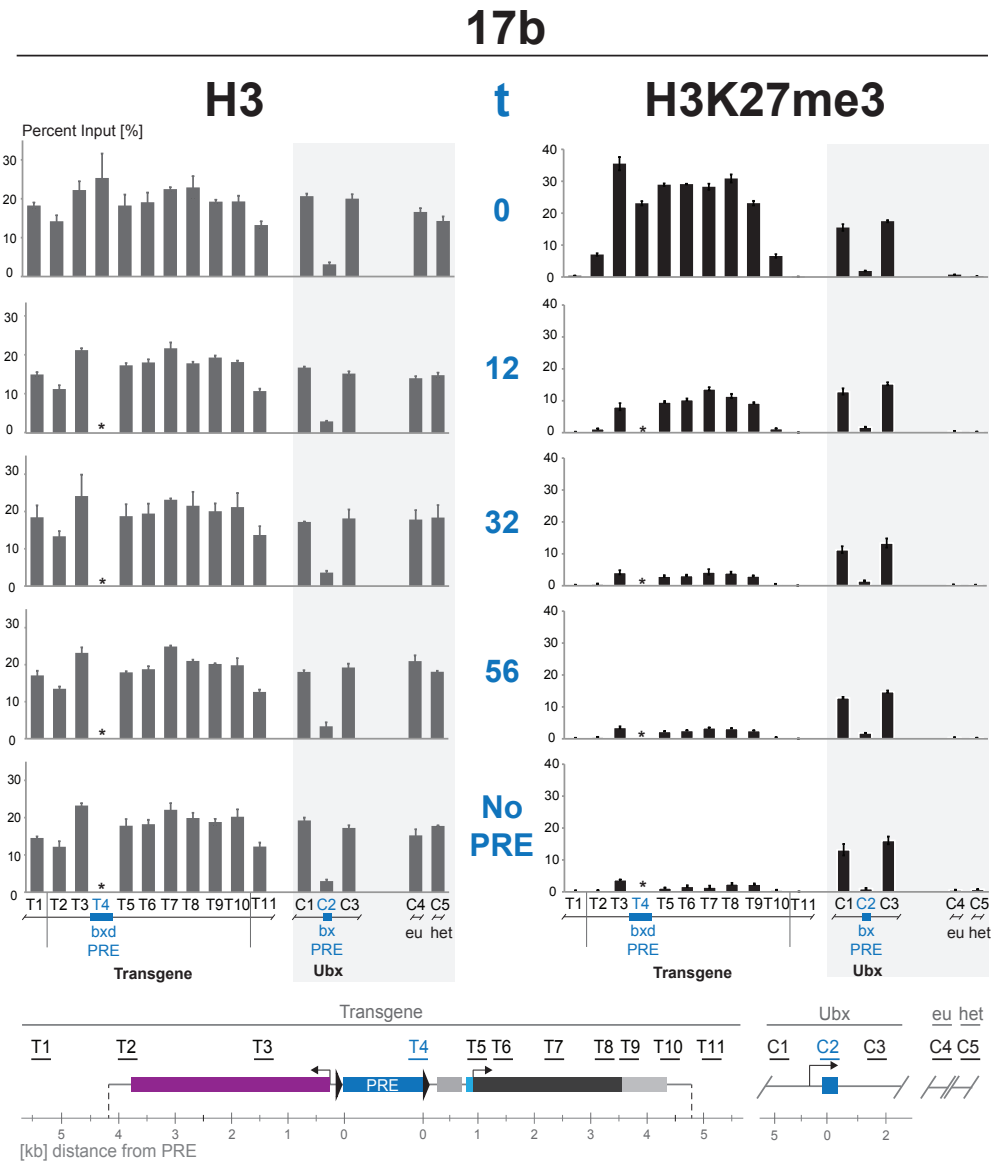

***Fig.* III.19**: **Time course PRE-excision in 17b.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}*-LacZ* 17b or >*dpp*^{WE}*-LacZ* 17b ΔPRE #4 together with *hsp70-FLP* as determined by ChIP-qPCR. Heat shock was induced at indicated time points (t) before dissection, 120 h AEL. Left panels show ChIP against H3, right panels against H3K27me3. The uppermost panels show ChIP from larvae that were not subjected to a heat shock (0), the panels at the bottom show ChIP from larvae from a stable flip-out strain >*dppWE-*

LacZ 17b ΔPRE #4 (No PRE). The other graphs show H3 and H3K27me3 levels at indicated time points after heat shock. Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T1-T11 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

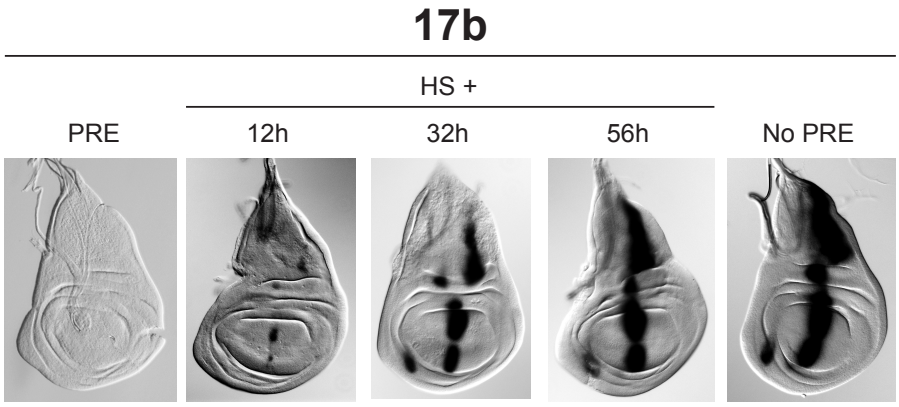

***Fig.* III.20: Time course X-gal staining of wing imaginal discs in 17b** (crossed to *hsp70*-FLP). Discs either contained the PRE (PRE), were subjected to a heat shock at indicated times (12 h, 32 h, 56 h) or did not contain the PRE (No PRE).

It is important to note that reduction of H3K27me3 levels is not due to transcription through *LacZ* in imaginal wing discs. As shown in Figure III.3, only about 5% of cells actually transcribe *LacZ*. Therefore, the effect of transcription on H3K27me3 decay is negligible.

Together, these data show that the PRE is not only strictly required to maintain repression, but is also essential for maintaining H3K27me3 across the entire chromatin domain encompassing the transgene. This finding suggests that in the absence of the PRE, PRC2 is unable to generate and propagate H3K27 trimethylation by the proposed positive feedback loop involving Esc/EED binding to H3K27me3.

III. 1. 6. Decay dynamics of H3K27me3 upon excision of the *bxd* PRE are independent of its genomic localization

The decay of H3K27me3 seen in >PRE>*dpp*^{WE}-*LacZ* line 17b demonstrated that PRE-imposed H3K27me3 domains are lost within a few cell generations after excision of the PRE. This rapid decay could potentially be due to specific effects at the genomic insertion site harboring 17b, such as effect of transcription or chromatin organization in the flanking genomic regions (Schmitt *et al.,* 2005). To address this issue, I also analyzed H3K27me3 dynamics after PRE-excision in the fly strains 7b and 36c. As illustrated in Figures III.21 and III.22, after excision of the PRE in these two transgene inserts, H3K27me3 was lost with similar kinetics like in the 17b line. Together, these experiments suggest that the chromosomal environment does not affect the loss of H3K27me3 and that the rate of H3K27me3 decay is likely a direct consequence of removal of the PRE.

Altogether, time course PRE-excision experiments were performed in three independent fly lines. Decay rates in all experiments were highly comparable and indicated that excision of the PRE results in loss of H3K27me3 within the subsequent four cell generations.

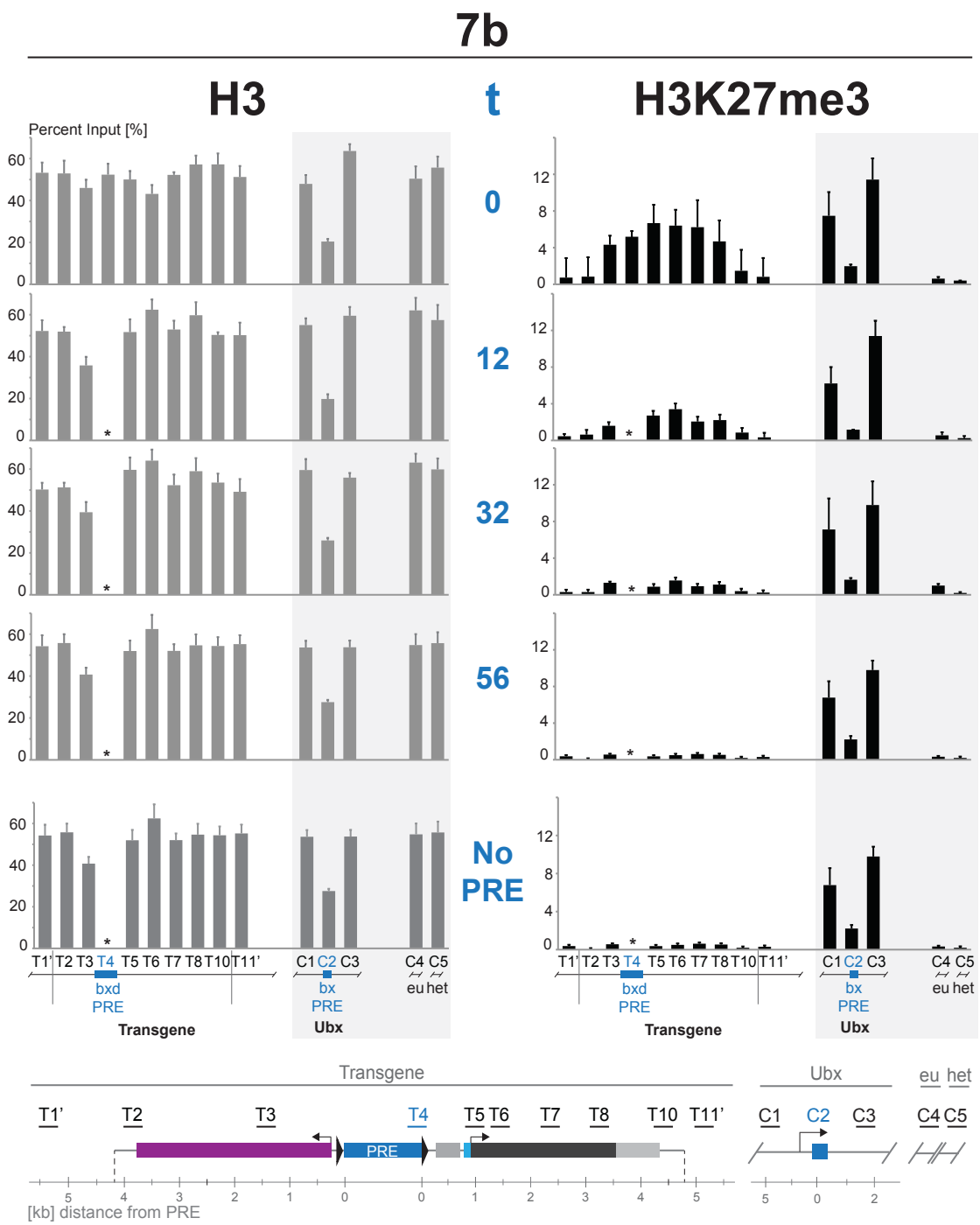

***Fig.* III.21: Time course PRE-excision in 7b.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}*-LacZ* 7b or >*dpp*^{WE}*-LacZ* 7b ΔPRE #1 together with *hsp70-FLP* as determined by ChIP-qPCR. Heat shock was induced at indicated time points (t) before dissection, 120 h AEL. Left panels show ChIP against H3, right panels against H3K27me3. The uppermost panels show ChIP from larvae that were not subjected to a heat shock (0), the panels at the bottom show ChIP from larvae from a stable flip-out strain >*dpp*^{WE}*-LacZ* 7b ΔPRE #1 (No PRE). The other graphs show H3 and H3K27me3 levels at indicated time points after heat shock. Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T1'-T11' refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in

the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

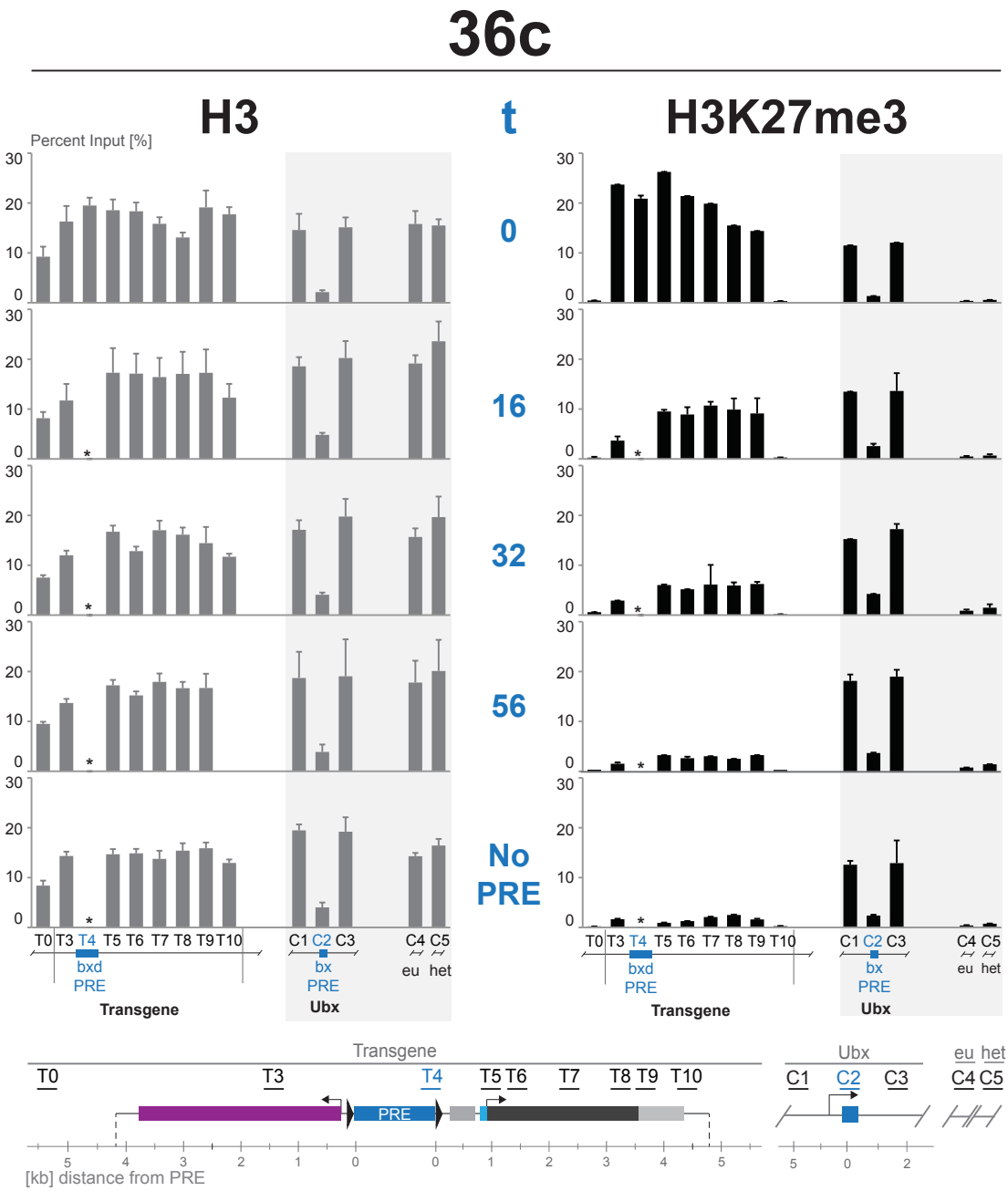

***Fig.* III.22: Time course PRE-excision in 36c.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}-*LacZ* 36c or >*dpp*^{WE}-*LacZ* 36c ΔPRE #1 together with *hsp70-FLP* as determined by ChIP-qPCR. Heat shock was induced at indicated time points (t) before dissection, 120 h AEL. Left panels show ChIP against H3, right panels against H3K27me3. The uppermost panels show ChIP from larvae that were not subjected to a heat shock (0), the panels at the bottom show ChIP from larvae from a stable flip-out strain >*dpp*^{WE}-*LacZ* 36c ΔPRE #1 (No PRE). The other graphs show H3 and H3K27me3 levels at indicated time points after heat shock. Control regions are above grey background. Schematic illustrations of the transgene with

localization of PREs (blue, rectangular boxes) is given below the graphs. T0-T10 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

III. 1. 7. Nucleosomes in the transgene are saturated with trimethylation

In a next set of experiments, I investigated whether the nucleosomes across the transgene array are mainly trimethylated at H3K27 or whether the region also contains a substantial fraction of nucleosomes that are in the dimethylated state (H3K27me2). The rationale for testing this was that saturating levels of H3K27me3 nucleosomes might be a pre-requisite for the propagation of H3K27me3 by the postulated positive feedback loop involving PRC2-binding to H3K27me3. A simple explanation for the rapid loss of H3K27me3 might be that a substantial fraction of nucleosomes in the 12 kb chromatin interval might be in the H3K27me2 or H3K27me1 state. It should be noted, that in bulk histones from Drosophila approximately 50-60 % of histone H3 is mono- or dimethylated at K27 (Ebert *et al.*, 2004). Indeed, H3K27me3 shows a broad distribution throughout the genome in both Drosophila (*modEncode*) and in mammalian cells (Ferrari *et al.,* 2014).

I performed ChIP analyses to monitor the levels of H3K27me2 in >PRE>*dpp*^{WE}*-LacZ* 17b and in the corresponding >*dpp*^{WE}*-LacZ* ∆*PRE* 17b line (*Fig. III.23*). In the presence of the PRE, H3K27me2 levels in the transgenic regions were substantially lower than at the eu- and heterochromatic control regions (C4, C5) that are highly decorated with H3K27me2 as previously reported (Nekrasov *et al*., 2007). H3K27me2 levels at the >PRE>*dpp*^{WE}*-LacZ* indeed were at the low level that is detected at the *Ubx* gene, which in wing imaginal disc cells is known to be extensively trimethylated at H3K27 (*Fig. III.2.3*, see above). In contrast, at the >*dpp*^{WE}*-LacZ* ∆*PRE* transgene, H3K27me2 levels at the transgene (T2-T11) approached the levels see in

control regions C4 and C5, likely reflecting dimethylation of H3K27 by untargeted PRC2.

In summary, comparison of these patterns to the ones seen for H3K27me3 in the same fly strains (compare to III. 1. 3. 1, *Fig. III.16*) suggests that H3K27 residues were saturated for H3K27me3 in the presence of the PRE. This assumption is based on previous studies that showed that approximately 50% of bulk histone H3 in *Drosophila* is dimethylated at K27 and that this modification is present genome-wide but reduced at repressed PcG target genes where H3K27 is predominantly trimethylated (Nekrasov *et al*., 2007). This suggests that the transgenic *bxd* PRE in the transgene mediates formation of a saturated H3K27me3 domain across the surrounding chromatin. The rapid loss of H3K27me3 after excision of the PRE in all fly strains can thus not be explained by an inability of PRC2 to perpetuate H3K27me3 because of sub-saturating levels of H3K27me3 nucleosomes in the area.

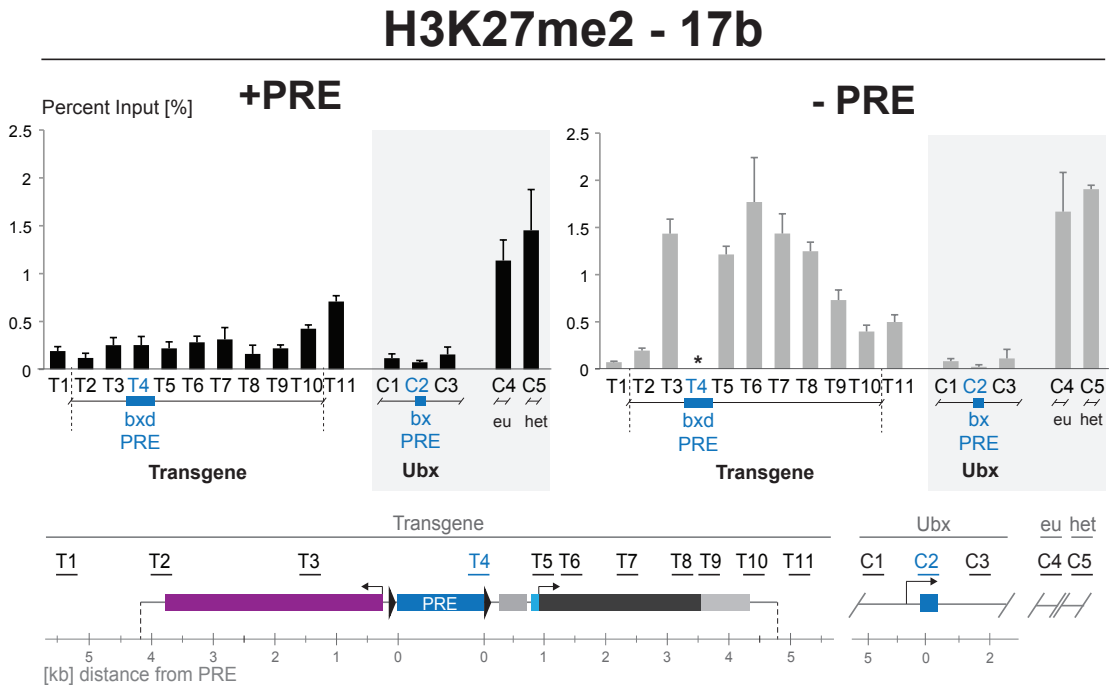

***Fig.* III.23: H3K27me2 levels in the presence or the absence of the PRE in 17b.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}-*LacZ* 17b (+ PRE) or >*dpp*^{WE}-*LacZ* 17b ΔPRE #4 (- PRE) as determined by ChIP-qPCR. Panels show ChIP against H3K27me2. Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T1-T11 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

III. 1. 8. Decay of H3K27me3 upon excision of the *bxd* PRE is independent of the promoter

In order to exclude promoter-specific effects on decay rates of H3K27me3 after excision of the PRE, PRE-excision experiments were repeated in a strain carrying the transgene with a different promoter. The >PRE>*dpp*^{WE}-*Ubx-LacZ* line 17-1 contains a 4.1 kb fragment from the *Ubx*-promoter instead of the TATA box minimal promoter from *hsp70*. The construct was described before (Sengupta *et al.*, 2004). The >PRE>*dpp*^{WE}*-Ubx-LacZ* construct is depicted in Figure III.24. Like in the case of the >PRE>*dpp*^{WE}*-LacZ* transgene, I found a domain of H3K27me3 spanning the entire >PRE>*dpp*^{WE}*-Ubx-LacZ* transgene and only background signal of H3K27me3 at the *dpp*^{WE}*-Ubx-LacZ* ΔPRE version of this transgene insert (*Fig. III.25*, compare top and bottom panel).

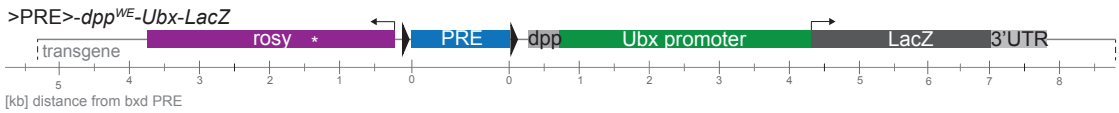

***Fig.* III.24: Schematic drawing of >PRE>*dppWE*-Ubx-*LacZ*.** (drawn to scale). Scale bar indicates distances in kb from the PRE. Black arrow heads next to the PRE represent FRT sites. Arrows mark TSS. The asterisk in rosy marks the site of mutation in ry[42]. Borders of the transgene are symbolized by a dotted line. 3'UTR is 3' untranslated region.

I next generated animals carrying *hsp70*-FLP together with the >PRE>*dpp*^{WE}*-Ubx-LacZ* transgene and excised the PRE by heat shock induction of FLP expression at different time points during larval development. Analysis of chromatin 12 h, 32 h, and 56 h after PRE-excision showed that loss of H3K27me3 at this transgene with the *Ubx* promoter occurred at a similar rate like in the case of the transgene with the *hsp70* TATA box promoter. I conclude that the promoter of *Ubx*, a *bona fide* PcG target gene, does not contribute to sustain H3K27me3 following removal of the PRE.

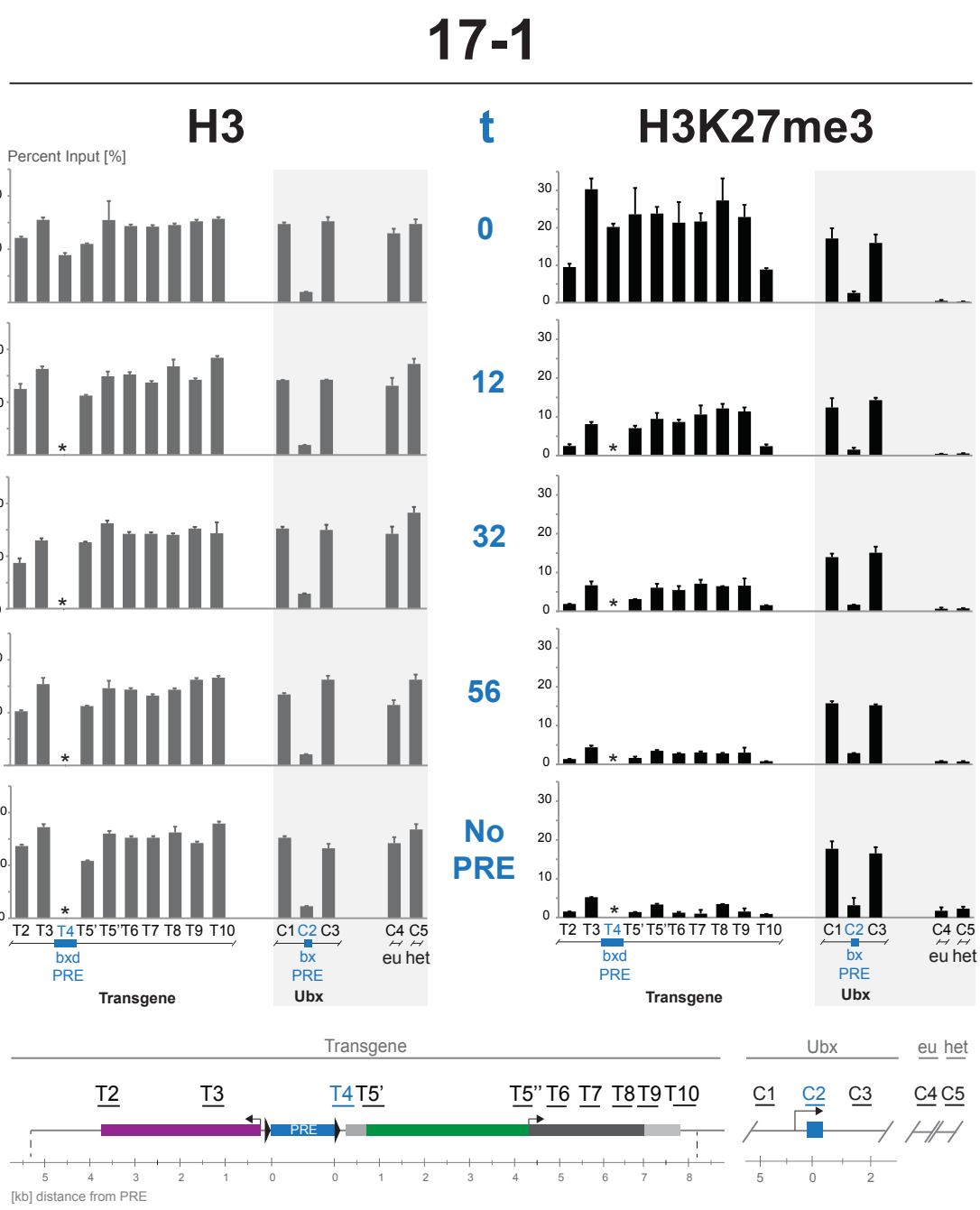

***Fig.* III.25: Time course PRE-excision in 17-1.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}-UZ-*LacZ* 17-1 or >*dpp*^{WE}-*LacZ* 17-1 ΔPRE #3 together with *hsp70-FLP* as determined by ChIP-qPCR. Heat shock was induced at indicated time points (t) before dissection, 120 h AEL. Left panels show ChIP against H3, right panels against H3K27me3. The uppermost panels show ChIP from larvae that were not subjected to a heat shock (0), the panels at the bottom show ChIP from larvae from a stable flip-out strain >*dpp*^{WE}-*LacZ* 17-1 ΔPRE #3 (No PRE). The other graphs show H3 and H3K27me3 levels at indicated time points after heat shock. Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T2-T10 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene,

drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

III. 1. 9. Loss of H3K27me3 requires passage through S-phase

III. 1. 9. 1. Aphidicolin can reversibly inhibit cell division *in vivo*

The rapid reduction of H3K27me3 upon removal of the PRE in all experimental situations described above suggested that the loss reflects dilution of existing H3K27me3 nucleosomes with every cell division. The cell cycle length in the wing imaginal disc changes during larval development (Martin *et al.,* 2009) but, as discussed above, the time points of PRE-excision were chosen to roughly correspond to at least one (12 h), at least two (32 h), or more than three (56 h) cell divisions. I therefore next investigated whether excision of the PRE in cells where DNA replication was blocked would change the kinetics of H3K27me3 loss.

To inhibit the cell cycle in developing larvae I fed larvae the tetracyclic diterpene antibiotic Aphidicolin, which inhibits DNA polymerase A and D and therewith imposes a cell cycle block in early S-phase (Spadari *et al.*, 1985). To this end, I developed a protocol to rear larvae in liquid medium to which I could add Aphidicolin (see Materials and Methods). Inhibition of DNA replication was monitored by feeding larvae EdU for 16 h and analyzing EdU incorporation by immunodetection of EdU in fixed imaginal disc tissues. The feeding scheme is presented in Figure III.26. In the experimental set up, Aphidicolin was added for 4 h to allow for all cells to arrest in S-phase before transferring the larvae to medium containing EdU and Aphidicolin for 12 h (*Fig. III.26* B). A control setup included treating the larvae in the same way, followed by a period of 4 h where the larvae were reared in food that only contained EdU and no Aphidicolin (*Fig. III.26* C). This latter experiment allowed to assess whether the cells would then enter S-phase and continue the cell cycle, thereby showing that they were still viable following the preceeding Aphidicolin treatment. To ensure that the larvae took up the Aphidicolin- (and EdU) containing medium, dextran-blue was added

to the food and all larvae were analyzed for the presence of dextran blue signal in the gut, prior to immunostaining or ChIP analysis.

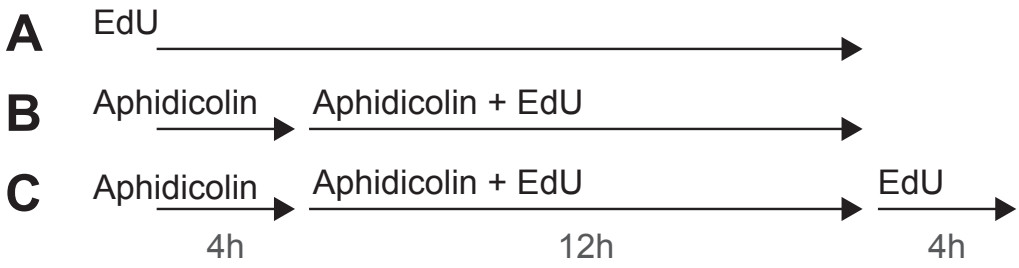

***Fig.* III.26: Treatment scheme of EdU incorporation assay with and without Aphidicolin treatment**. Larvae were fed with Aphidicolin only (A), first fed with Aphidicolin only, then with Aphidicolin plus EdU (B) or as in treatment B plus release of larvae in EdU-containing food without EdU.

Figure III.27 shows the EdU incorporation assay. Larvae efficiently took up EdU with the food (panel on the left, pink staining). The presence of Aphidicolin in the food completely abolished incorporation of EdU into the cells, which indicated that S-phase was stopped. Importantly, Aphidicolin concentration was optimized to permit inhibition of the cell cycle (panel in the center) without causing lethality of the larvae (panel on the right). Cell viability was confirmed by incorporation of EdU following a 12 h-Aphidicolin treatment (*Fig. III.27*). The EdU incorporation after Aphidicolin treatment shows that the Aphidicolin block is fully reversible.

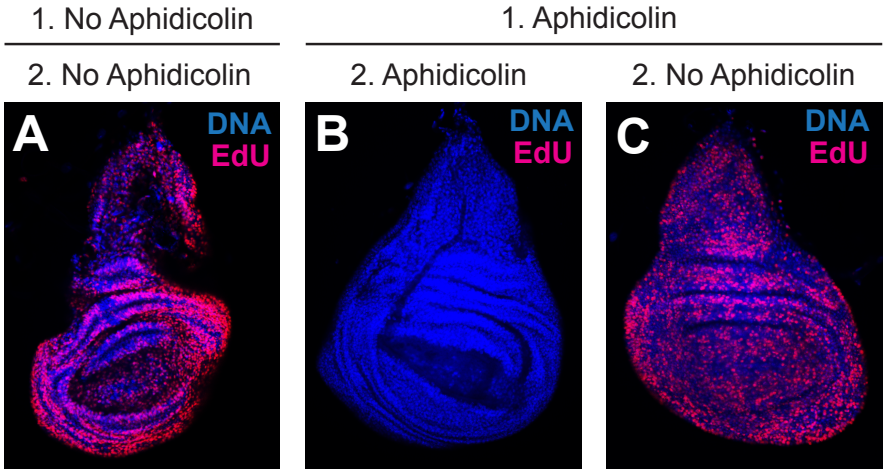

***Fig.* III.27: EdU incorporation assay** with indicated treatments (refer to *Fig. III.26* for a treatment scheme). Wing imaginal discs of larvae that were fed with EdU for 16h. B and C: Larvae were additionally

fed with Aphidicolin for 12 h. In C, Aphidicolin block was released by an additional feeding period of 4 h in EdU only. Discs were stained against DNA (Hoechst, in blue) and EdU (cell division, in pink).

III. 1. 9. 2. Aphidicolin treatment does not interfere with PRE-excision

I next tested whether PRE-excision efficiency was in any way affected by Aphidicolin treatment. I therefore fed larvae that contained *hsp70*-FLP and the >PRE>*dpp*^{WE}-*LacZ* 17b transgene with Aphidicolin-containing medium for 4 h and then applied a 1-h heat shock. After the heat shock, larvae were again transferred into Aphidicolin-containing medium for 12 h and then dissected and analyzed for excision of the PRE by qPCR of imaginal wing discs as describe above. As control, I used (a) larvae that were not fed with Aphidicolin and (b) larvae that were not subjected to a heat shock.

As illustrated in Figure III.28, in Aphidicolin-treated larvae approximately 80% of the cells had excised the PRE. Even though excision was not as complete as in non-Aphidicolin-treated larvae the PRE was thus nevertheless removed from the majority of cells. Please note that high error bars are due to the crude preparation of DNA from single wing imaginal discs by proteinase K digestion. Amplification of heat shock and no-heat shock samples represent independent experiments that vary largely in absolute values, due to variance in cell number in the setup (tissue size and number vary in independent experiments). In order to allow for comparison between samples, values were normalized to mean amplification of all discs in the *LacZ* control region, causing great differences in the ratios. This results in a substantial variation in the ratios between independent measurements. PRE-excision was thus not drastically impaired in Aphidicolin-arrested cells.

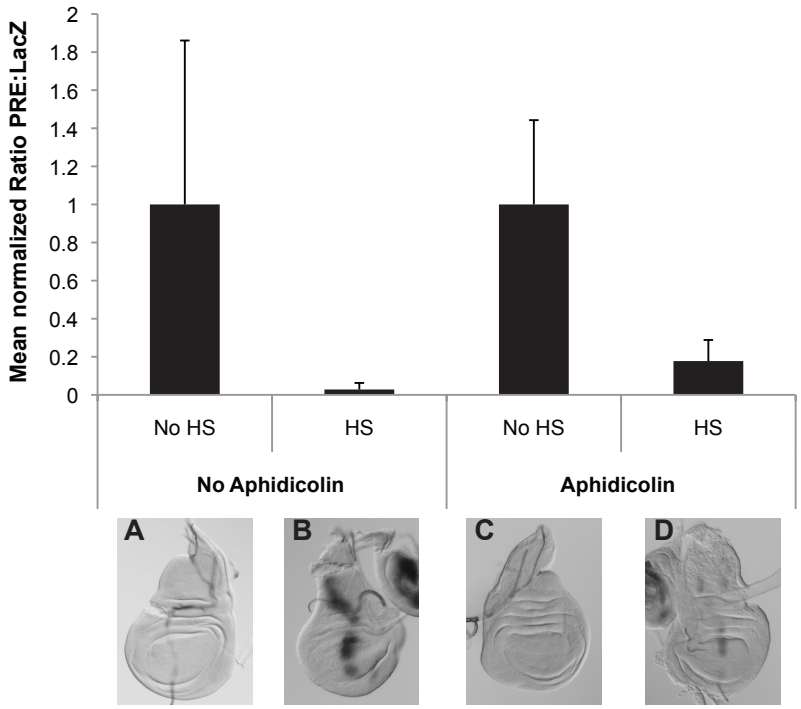

***Fig.* III.28: PRE-excision verification upon Aphidicolin treatment.** Upper panel: qPCR analysis of DNA from imaginal wing discs measured amplification from PRE*dppWE* and *LacZ*. Ratio of these primer pairs allows for conclusions about fraction of cells that completely excised the PRE. Larvae were either fed with Aphidicolin-containing food (Aphidicolin) or non-Aphidicolin-containing food (No Aphidicolin) and subjected to a heat shock (HS) or not (No HS). Excision of the PRE was slightly reduced after heat shock in larvae that were treated with Aphidicolin. Bottom panel: X-gal staining of wing imaginal discs that were subjected to the same treatments, but released to non-Aphidicolin-containing food for an additional 12 h.

To complement this analysis, I also assessed de-repression of *LacZ* in the wing discs by X-gal staining. For this experiment, larvae were treated as above and then transferred into non-Aphidicolin-containing medium for an additional 12 h; they were thus dissected 24 h after the heat shock. As shown in Figure III.28, ß-galactosidase expression was also clearly detected in Aphidicolin-treated larvae, albeit at a lower level compared to non-Aphidicolin-treated larvae (*Fig. III.28,* compare D to B). This shows that not only cell division but also transcriptional activation (i.e., the de-repression of the dpp^{WE}-*LacZ* Δ*PRE* transgene) remains functional after Aphidicolin treatment.

III. 1. 9. 3. In the absence of DNA replication, H3K27me3 persists after PRE-excision

The experiments described above suggested that Aphidicolin-treatment was a suitable approach to block the cell cycle in S-phase without interfering with FLP-mediated excision of the PRE. I therefore next examined how H3K27me3 levels change after PRE-excision in Aphidicolin-treated larvae using the same strategy as described above. Again, animals carrying one copy of >PRE>*dpp*^{WE}*-LacZ* 17b were used for this experiment, and chromatin was prepared 12 h after the heat shock-induced expression of *hsp70*-FLP. As control, I also prepared chromatin from larvae that were reared the same way in liquid medium lacking Aphidicolin and were subjected to the same heat shock to induce PRE-excision.

The results of this experiment are shown in Figure III.29. The panel on the left demonstrates that the experimental setup (i.e., culturing and growing the larvae in liquid medium), resulted in the same loss of H3K27me3 as observed in Figure III.19 – 12 h after heat shock, H3K27me3 levels were reduced to 50% compared to animals containing the intact >PRE>*dpp*^{WE}*-LacZ* transgene (compare black bars (no HS)) to green bars (HS + 12 h). Moreover, H3K27me3 levels in control regions remained undiminished (*Fig. III.29*). In contrast, in Aphidicolin-treated larvae (*Fig. III.29,* panel on the right) H3K27me3 levels remained high after PRE-excision (*Fig. III.29,* panel on the left). As expected, H3K27me3 levels also remained unchanged in control regions with both treatments.

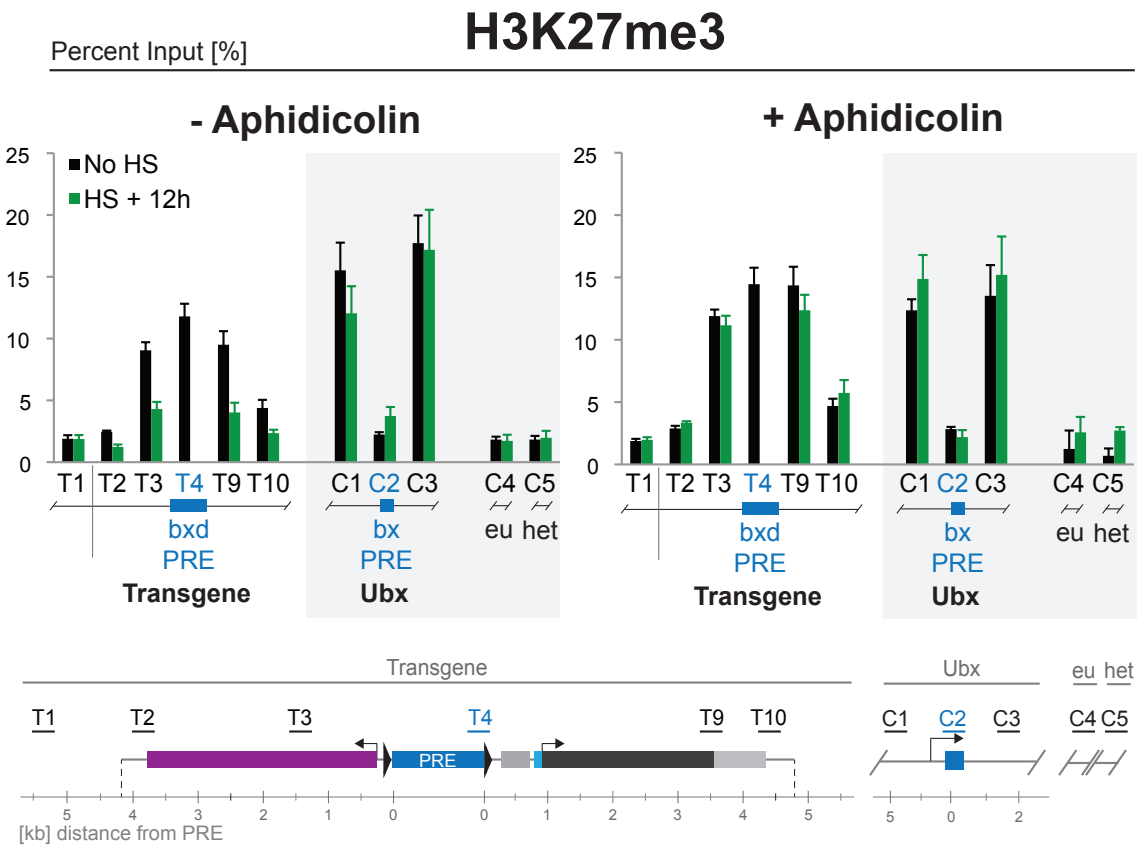

***Fig.* III.29: PRE-excision with or without Aphidicolin treatment.** Percent input levels of H3K27me3 of larvae that carry >PRE>*dpp*^{WE}*-LacZ* 17b together with *hsp70*-FLP as determined by ChIP-qPCR. Black bars represent no heat shock samples (No HS), green bars represent larvae that were subjected to a heat shock and dissected 12 h afterwards (HS + 12 h). Left panel shows non-Aphidicolin treated larvae (- Aphidicolin), compared to the panel on the right, where larvae were fed with Aphidicolin 4 h prior to and 12 h after HS (+ Aphidicolin). Control regions are above grey background. A schematic illustration of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T1-T10 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localization at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

These results show that in the absence of DNA replication, H3K27me3 is not lost. This leads to the important conclusion that under normal growth conditions, the reduction of H3K27me3 levels after excision of the PRE is caused by dilution of H3K27me3 nucleosomes during DNA replication. In animals in which DNA replication was inhibited by Aphidicolin, the H3K27me3-containing nucleosomes that were modified by PRE-bound PRC2 remain at the gene after excision of the PRE.

III. 2. PcG protein binding is lost together with excision of the PRE

H3K27me3 is lost upon removal of the PRE due to dilution by DNA replication. This implies that binding of PRC2 and other PRE-bound PcG proteins is likely lost following PRE-excision. I therefore also examined the loss of PcG protein binding at different time points after PRE-excision

By ChIP, most PcG complex components are highly localized at PREs (Papp and Muller, 2006; Schwartz *et al.,* 2006) and no binding can be detected up- and downstream of the PREs. This is indeed the case for E(z) or the PRC1 subunit Ph; both proteins are readily detected at the >PRE>*dpp*^{WE}*-LacZ* transgene but only at the PRE (T4 region) and not at the neighboring T3 and T5 regions (*Fig. III.30*). As expected, no binding is detected at the *dpp*^{WE}*-LacZ* ∆*PRE* transgene (*Fig. III.30*). This also means that after PRE-excision, it is not possible to monitor maintenance of binding of these proteins at the transgene.

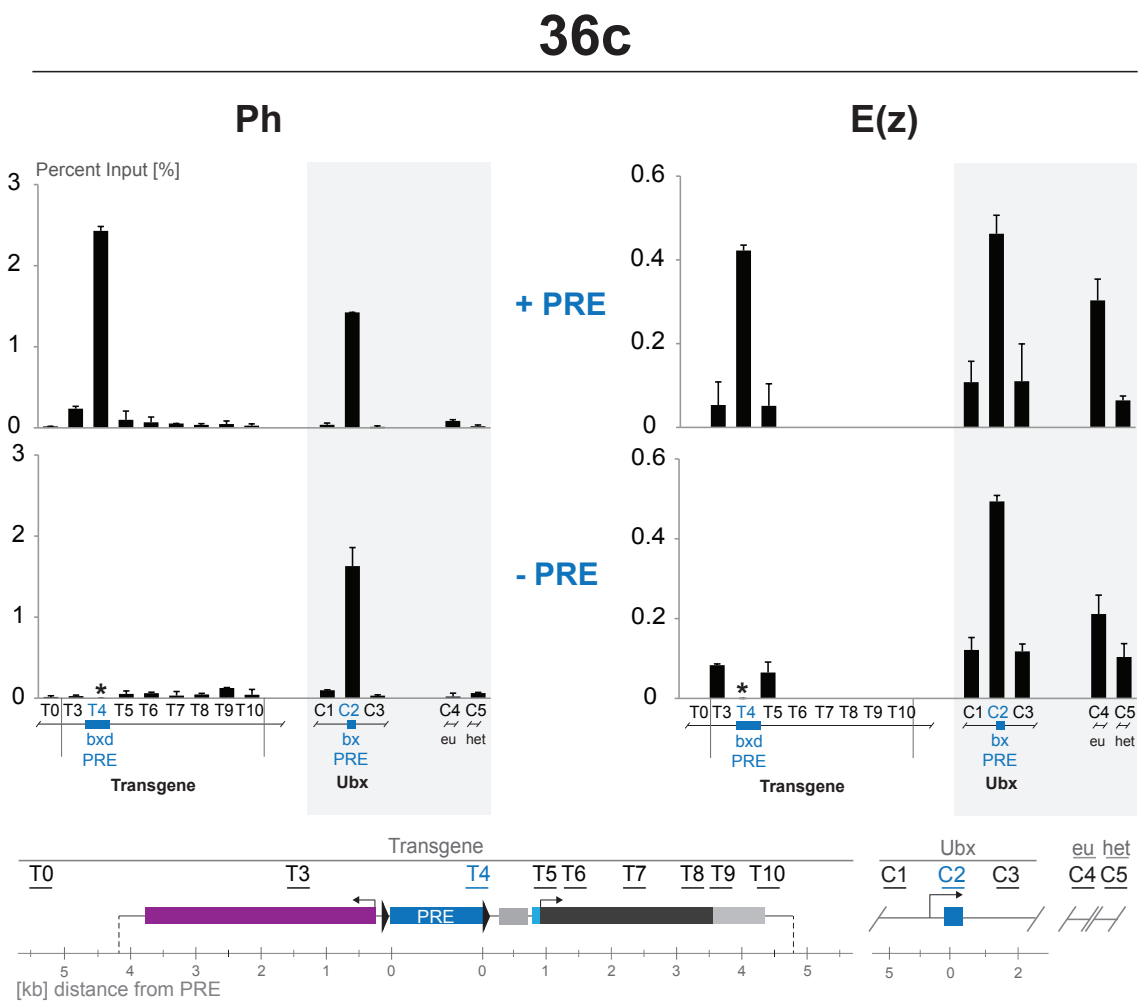

***Fig.* III.30: PcG proteins bind to transgenic PRE.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}-*LacZ* 36c (upper panels; + PRE) or >*dpp*^{WE}-*LacZ* 36c ΔPRE #1 (lower panels; - PRE) together with *hsp70*-FLP as determined by ChIP-qPCR. Left panels show ChIP against Ph, right panels against E(z). Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T0-T10 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

In contrast to E(z) and Ph, binding of the PRC1 subunit Pc is not only detected at the PRE, but also in the flanking T3 and T5 regions (*Fig. III.31*, compare top panel (0) and bottom panel (no PRE)). The chromodomain of Pc can interact with H3K27me3 marks and ChIP assays are thus thought to capture such interactions with H3K27me3 modified nucleosomes. Figure III.31 shows the Pc and, for comparison, the H3K27me3 profile at different time points after PRE-excision. Interestingly, Pc was lost immediately after excision

of the PRE: 12 h after heat shock, Pc signals at regions T3 and T5 were at the level of background signal. This stands in clear contrast with H3K27me3, where the modified nucleosomes were still present at this time point (i.e., 12 h) after induction of PRE-excision. Together, this suggests that Pc is primarily targeted to PREs and that the Pc ChIP signals detected at T3 and T5 in the >PRE>*dpp*^{WE}*-LacZ* transgene likely reflect interaction of PRE-anchored Pc with H3K27me3 modified nucleosomes; an interaction that no longer occurs after excision of the PRE.

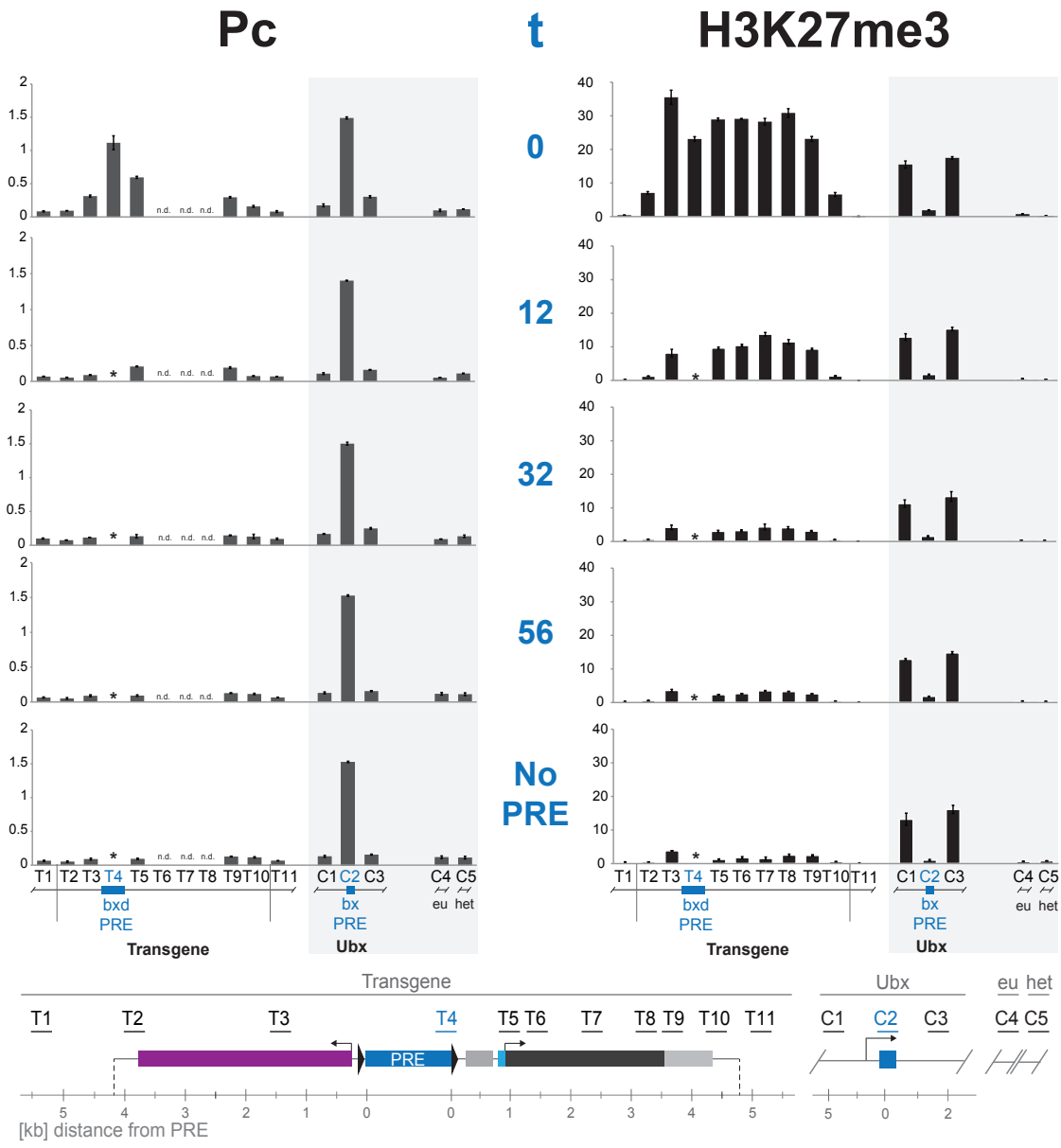

***Fig.* III.31: Pc time course after PRE-excision in 17b.** Percent input levels of larvae that carry >PRE>*dpp*^{WE}*-LacZ* 17b or >*dpp*^{WE}*-LacZ* 17b ΔPRE #4 together with *hsp70*-FLP as determined by ChIP-

qPCR. Heat shock was induced at indicated time points (t) before dissection, 120 h AEL. Left panels show ChIP against H3, right panels against H3K27me3. The uppermost panels show ChIP from larvae that were not subjected to a heat shock (0), the panels at the bottom show ChIP from larvae from a stable flip-out strain >*dpp*^{WE}-*LacZ* 17b ΔPRE #4 (No PRE). The other graphs show H3 and H3K27me3 levels at indicated time points after heat shock. Control regions are above grey background. Schematic illustrations of the transgene with localization of PREs (blue, rectangular boxes) is given below the graphs. T1-T11 refer to primer pairs that were used in or around the transgene. C1-C5 represent control primers, which are located elsewhere in the genome and should not be influenced by excision of the PRE. The transgene, drawn to scale with primer localizations at distinct DNA elements is depicted at the bottom. Error bars indicate SDs of biological triplicates, read out in technical duplicates.

IV. Discussion

A number of studies have focused on dynamics of histone modifications in the past years. Maintenance of transcriptional states is one of the biggest questions in the field of epigenetics. Trimethylation of H3K27 is of particular interest, since it had been directly linked to transcriptional repression (Pengelly *et al.,* 2013). Interestingly, PcG-mediated repression of reporter genes is lost in *Drosophila* within a few cell generations after removal of a transgenic PRE and therewith the binding platform of the PcG complexes (Sengupta *et al.*, 2004). Since the underlying molecular mechanisms are still elusive, we were eager to elucidate the question of how the epigenetic information provided by H3K27me3 is inherited.

The work presented here demonstrates that loss of a transgenic *bxd* PRE in *Drosophila* wing discs – accompanied with loss of repression – results in loss of H3K27 trimethylation, as assessed by ChIP analyses. I was able to show that decay of H3K27me3 levels upon PRE-excision is a general feature, which occurs independently of the genomic localization and irrespective of the promoter, under which the reporter gene is expressed. Furthermore, in this study, I revealed that decay of H3K27me3 in our system is halted in the presence of Aphidicolin by *in vivo* inhibition of the cell cycle. Finally, I could demonstrate that the Pc protein is unable to remain associated with repressed chromatin domains in the absence of the causative PRE.

The data presented in this study allows for conclusions about the mechanism of propagation of H3K27me3. Combined data of H3K27me3, in the presence and in the absence of Aphidicolin treatment, lead us to infer that decay of H3K27me3 owes its dynamics to a simple dilution mechanism during cell division. Our findings further point out that H3K27me3 is not maintained by a plain self-propagation mechanism in which histone modifications are readily duplicated to neighboring new histones (Probst *et al.,* 2009; Margueron and Reinberg, 2010; Alabert and Groth, 2012). Failure of PcG proteins to remain associated with the transgenic PRE emphasizes the important role of a binding platform to maintain PcG-mediated repression. This finding adduces strong

evidence that, in the absence of the PRE, repression and its hallmark: H3K27me3 are irrecoverably lost.

IV. 1. Implications of this study

A few aspects of this work should be clarified in the context of the current state of the art in the field of epigenetics. In the following, I will therefore discuss (1) general implications of our findings in accordance with recent suggestions about H3K27me3 inheritance, (2) saturation of PRE-imposed H3K27 domains in trimethylation, (3) histone turnover and its impact on replication-uncoupled incorporation of nucleosomes, (4) cell cycle dependency of H3K27me3 dilution, (5) the contribution of active demethylation on decay rates of H3K27me3, and finally (6) the effect of transcription through H3K27me3 domains on histone replacement. This allowed me to come up with a novel implication of H3K27me3 maintenance.

IV. 1. 1. Loss of repression correlates with loss of H3K27me3 – implications of new insights

In 2008, a study performed by Hansen *et al.* suggested that merely the presence of the H3K27me3 mark was sufficient to recruit the PRC2 complex to its site of action (Hansen *et al.,* 2008). The goal of that study was to define a model for transmission of H3K27me3 throughout subsequent cell divisions. In their study, Hansen *et al.* analyzed the maintenance of H3K27me3 using a heterologous reporter assay in mammalian cells. The system they used allowed for tethering of a Gal4-fusion human ortologue of Esc: Gal4-EED to the transcription start site of a luciferase reporter gene. Gal4-EED was under control of a tetracycline-regulated promoter, which allowed for induction of transcriptional repression of luciferase by treatment with tetracycline. Hansen *et al.* found that H3K27me3 was maintained at the TSS and up to 1600 bp downstream of the TSS for up to four days after washout of tetracycline.

Importantly, while not investigated, during the entire experiment of Hansen *et al.*, endogenous proteins were still present. They concluded that once the H3K27me3 is established, it recruits the PRC2 complex to maintain the histone mark at sites of DNA replication. Similar experiments were performed looking at H3K9me3, a mark for heterochromatin formation in murine ES cells (Hathaway *et al.,* 2012).

However, *in Drosophila*, the PRE as binding platform is strictly required to maintain PcG-mediated repression (Busturia *et al.*, 1997; Sengupta *et al.*, 2004). Moreover, the artificial tethering of a protein to a regulatory region, as it was done by Hansen *et al.* (Hansen *et al.,* 2008), might mimic the presence of an endogenous binding platform. The fact that repression is lost upon removal of a PRE (Busturia *et al.*, 1997; Sengupta *et al.*, 2004) challenges the model of H3K27me3 self-sustainability (Hansen *et al.,* 2008). We therefore decided to revise their findings in a less artificial system and thereby shed light on H3K27me3 inheritance.

The system I used for the study allows endogenous recruitment of PcG proteins to their site of action – a genomic copy of the *bxd* PRE, integrated into a new localization that is endogenously not subjected to PcG-mediated repression (compare *Fig. III.1*). Removal of the PRE allowed to specifically re-enact the question of whether in the absence of the endogenous binding platform, merely the presence of the H3K27me3 mark is sufficient to recruit the PRC2 complex to its site of action, as suggested by Hansen *et al.* (Hansen *et al.,* 2008).

With our system I found that removal of the PRE leads to complete loss of H3K27me3 within 56 h after heat shock, and therewith 56 h after removal of the PRE. Importantly, I could show beforehand that the PRE was removed within 8 h after heat shock. This allowed for us to infer a time point zero to actually look at H3K27me3 dynamics during cell divisions. To define a time point zero, I tried to estimate cell division timings in the wing disc. It is important to note that, during larval development, time frames of cell divisions change and cells presumably divide in an asynchronous manner in a complex tissue such as the wing disc (Martin *et al.,* 2009).

In time course excision experiments that were presented in this study, I could demonstrate that the PRE, which is strictly required to maintain

repression, is moreover required to maintain the hallmark of repression – H3K27me3 – at its place.

Two recent studies, which were both performed in *Schizosaccharomyces pombe,* determined H3K9me3 dynamics, more specifically H3K9me3 maintenance. H3K9me3 is a conserved mark of heterochromatin, which is catalyzed by the Suv39h homolog Clr4 (Audergon *et al.*, 2015; Ragunathan *et al.*, 2015). In their assays, the Clr4 methyltransferase was tethered to a bacterial tetracycline repressor, which allowed for establishment of heterochromatin domains, marked by the presence of H3K9me3. They found that in the absence of the demethylase Epe1, H3K9me3 persisted over many cell generations. Both publications concluded that H3K9me3 is an epigenetically inherited mark.

So what is the difference between inheritance of H3K27me3 and H3K9me3? Clearly, there could be differences in epigenetic features of pro- and eukaryotes, but, more obviously, H3K9me3 marks completely different kinds of chromatin domains compared to the ones marked by H3K27me3 on facultative heterochromatin. In facultative heterochromatin, exogenous effects or different requirements during development allow for changes in the transcriptional on and off states. In contrast, H3K9me3 is a mark of constitutive heterochromatin domains in pericentromeric chromatin, which does not require dynamic features or adaptation to developmental stages or to environmental effects. Although the Clr4 chromodomain can recognize H3K9me3, there is no strict requirement to read and propagate a signal from constitutive heterochromatin – it should remain silent at all times. Moreover, mutations in Clr4 solely de-repress a single mating type locus in yeast (mat3) (Ekwall and Ruusala*,* 1994). The major role in epigenetic inheritance of H3K9me3 is therefore questionable.

The fast decay that was observed in my experiments seems to be the opposite of what has been found by Hansen *et al*., who demonstrated that H3K27me3 was sufficient to stably persist in the absence of its initial trigger, and also of what has been found for the constitutive heterochromatin mark H3K9me3. These discrepancies could simply disguise disparities in different species, different experimental procedures, or purely general divergence between constitutive and facultative heterochromatin.

Nevertheless, these papers indicate possible implications of an active removal mechanism of histone marks. Therefore, active demethylation will be discussed in more detail later on, in the context of cell cycle dependency of H3K27me3 decay and potential mechanisms of H3K27me3 propagation.

IV. 1. 2. H3K27me3 domain structure, as defined by the absence of dimethylation

Before mechanistic differences of H3K9me3 inheritance in yeast and of H3K27me3 inheritance in *Drosophila* are discussed, we first need to understand technical details of the experiments that I performed.

The transgene that I used for my assays contains a single, endogenous PRE of the *Ubx* locus, which was shown to be sufficient to initiate and to maintain transcriptional repression outside of its endogenous context (Sengupta *et al.,* 2004). This is of special importance, since under native conditions several PREs are clustered in the *Ubx* locus – forming part of an even bigger region: the BX-C.

In the native context, PREs are frequently associated with large H3K27me3 domains, spanning regions of more than 50 kb. By definition, an H3K27me3 domain is of repressive nature and bears high levels in trimethylation of H3K27. As mentioned before, saturating levels of H3K27me3 nucleosomes might be a pre-requisite for the maintenance of H3K27me3.

In order to test saturation of H3K27me3 of nucleosomes in proximity of the transgenic PRE I looked at levels of H3K27 dimethylation in these regions. The latter modification shows a much broader and more unspecific distribution over the genome (Ferrari *et al.,* 2014). Mostly in strongly trimethylated domains, such as in the endogenous *Ubx* region, dimethylation levels of H3K27 are lower than elsewhere. Therefore, H3K27me2 could be an indicator of saturation of nucleosomes in H3K27me3.

Spreading from the center of the transgene to its borders, I found low levels of H3K27me2 in the presence of the *bxd* PRE. These levels are comparable to what I detected around the endogenous *bx* PRE at the *Ubx*

locus. Furthermore, levels of H3K27me2 levels were high in the absence of the PRE and directly up- and downstream of the transgene in the genomic chromatin. The higher levels outside of the transgene were found for both, the presence and the absence, of the PRE. The substantial differences of H3K27me2 levels in the absence versus in the presence of the transgenic PRE strongly suggest that the H3K27me3 domain around the transgenic *bxd* PRE is saturated. Hence, it presents a potentially heritable H3K27me3 domain, which could serve as short-term memory of Polycomb-repressed domains in the cell.

Nevertheless, one has to bear in mind that the comparably small size of the H3K27me3 domain around the transgenic PRE might influence the stability of the domain or even the capability to convey epigenetic information from one cell generation to the next. This means that the dynamics of H3K27me3 I observed in my experiments might differ from dynamics of a bigger H3K27me3 domain.

IV. 1. 3. Histone turnover and its contribution to propagation of a repressed state

The replication-independent histone turnover is another cellular feature, which might affect the heritability of the H3K27me3 mark. What I interpret as dilution could simply represent replication-uncoupled replacement of histones (Dion *et al.*, 2007). If histones – and associated histone modifications – turned over faster than the cell cycle, we would potentially observe the same decay rates of H3K27me3 that we see, independent of cell division.

Recently, several studies have addressed the question of how fast histone turn over during DNA replication (Alabert *et al.,* 2014; Alabert *et al.*, 2015), of how long chromatin assembly takes (Loyola *et al.,* 2006; Scharf *et al.,* 2009) and of general histone dynamics during chromatin maturation (Scharf *et al.,* 2009). These studies suggest that histone modifications are added and removed during chromatin assembly in a highly regulated manner. Interestingly, it was found that particular modifications have considerably different kinetics until they have established a modification pattern that cannot be distinguished

from the parental nucleosomes anymore (Scharf *et al.,* 2009.II). More precisely, using nascent chromatin capture and triple-SILAC (stable isotope labeling with amino acids in cell culture) Alabert *et al.* observed a twofold dilution of histone modification upon DNA replication, highly comparable to what I found in this study. This dilution can be attributed to incorporation of newly synthesized histones. By being able to distinguish and to follow new and old histones throughout several rounds of DNA replication, they identified two distinct mechanisms of histone propagation across the cell cycle (Alabert *et al.*, 2015). On the one hand, H3K9me3 and H3K27me3 are modified by continuous adaptation to resemble parental histone modifications over several cell generations. On the other hand, other histone modifications are generally restored within one cell generation.

These results specifically favor replication-coupled histone incorporation as the major mechanism of dilution of histone modifications and underline the importance of understanding not only the mechanism of propagation of H3K27me3, but also its establishment.

My finding, that H3K27me3 levels stay at a high level in the presence of the cell cycle inhibitor Aphidicolin, places further emphasis on the hypothesis that we observe cell cycle-coupled dilution and not replication-uncoupled histone turnover. In line with the Aphidicolin data, the data from Alabert *et al.* question the simple self-propagation mechanism in which histone modifications are readily duplicated to neighboring new histones (Probst *et al.,* 2009; Margueron and Reinberg, 2010; Alabert and Groth, 2012).

Distinct to the model discussed above, there is also discussion about a second prospective mechanism of inheritance of repressive chromatin states in which instead of methylated histones PRC2 is passed on to daughter cells (Petruk *et al.,* 2012). Both models were compared recently in a study in *Caenorhabditis elegans* germ cells, which presented evidence that H3K27me3 transmits the repressed state transgenerationally and on a short-term scale in embryos (Gaydos *et al.,* 2014). The finding that histone modifications are transmitted with high efficiency at replication forks explains the observation that H3K27me3 can be transmitted through several cell generations in the absence of the active enzyme (Gaydos *et al.*, 2014; Alabert *et al.*, 2015). Importantly, these experiments were performed on another scale, assessing chromatin-wide

levels of H3K27me3, which do not provide the same insight into H3K27me3 inheritance as my experiments.

IV. 1. 4. H3K27me3 is lost in a cell cycle-dependent manner

In order to illuminate the mechanism of H3K27me3 decay, I paused the cell cycle for 12 h using Aphidicolin and subsequently analyzed H3K27me3 levels in the absence and the presence of the transgenic PRE. This cell cycle inhibition experiment demonstrated that the fast decay rates of H3K27me3 after PRE-excision are in fact cell cycle-dependent: in non-replicating cells H3K27me3 levels stayed at a high level even in the absence of the PRE. I verified on different levels that the essence of PRE-excision is not influenced: I verified that the *hsp70*-FLP is fully functional, works efficiently in the presence of Aphidicolin and showed that wing disc cells are viable and able to reenter the cell cycle after Aphidicolin treatment. However, the presence of such an unspecific chemical compound can interfere with more cellular processes than tested here. Therefore, results from this experiment should be interpreted with caution. One should always keep in mind that Aphidicolin could have unforeseen side effects in cells, which could influence the experimental outcome. Nonetheless, inhibition of the cell cycle represents a main feature of Aphidicolin treatment and it is widely accepted as cell cycle inhibitor in tissue culture. The PRE-excision experiment during a paused cell cycle strongly suggests that the mechanism by which H3K27me3 is gradually decreased is dilution.

IV. 1. 5. Does demethylation contribute to H3K27me3 decay?

These findings argue against active demethylation of H3K27me3, but do not exclude contribution of a demethylase.

In the case of H3K9me2/3, it was shown that the mark persists for over 20 cell generations in the absence of its demethylase Epe1 in yeast (Audergon *et*

al., 2015; Ragunathan *et al.,* 2015). A similar mechanism could also be true for H3K27me3. The only specific histone demethylase of H3K27me3 in *Drosophila* is *Utx*. *Utx* and the related JmjD3 protein were identified as histone demethylases of H3K27me3 *in vitro* and *in vivo* (Agger *et al.,* 2007; de Santa *et al.,* 2007; Hong *et al.*, 2007; Swigut and Wysocka, 2007).

Another good way to reassure the hypothesis that H3K27me3 is removed in the absence of the PRE due to dilution, and not due to active removal, would be to analyze H3K27me3 domains upon PRE-excision in the absence of a functional *Utx* protein. A ∂Utx fly strain, in which the *Utx* coding region was replaced by the *miniwhite* marker gene, was generated by Ömer Copur in our lab (Copur and Muller, 2013). *∂Utx* homozygotes develop into adult flies, if hand-sorted from the pool of *∂Utx* heterozygotes, isolated and looked after thoroughly (Copur and Muller, 2013). Within a few hours of hatching, these flies die, thus exhibiting the sensitive nature of their genetic background. First attempts to perform the PRE-excision experiment in *∂Utx* homozygous larvae have unfortunately failed. Therefore, so far I was unable to revise the finding that loss of H3K27me3 is cell cycle dependent, by showing that it is not actively removed by its demethylase *Utx*.

Overall, our PRE-excision experiment in the presence of Aphidicolin strongly suggests that we observe passive dilution of H3K27me3 and not active removal by the demethylase *Utx*. Yet, I cannot draw conclusions about the potential contribution of *Utx* to this process. Therefore, again, results have to be interpreted with caution, bearing in mind that *in vivo* – in the absence of Aphidicolin – *Utx* might contribute to removal of H3K27me3. Moreover, the effect of Aphidicolin has not been specifically tested towards potential inhibitory function of *Utx*. Therefore, one has to take into consideration that Aphidicolin might impact *Utx*'s proper function. To exclude potential effects of *Utx* on H3K27me3 decay performing the PRE-excision experiment in a *∂Utx* background is assigned with absolute priority.

IV. 1. 6. Effect of transcription on H3K27me3 domains

The >PRE>*dpp*^{WE}-*LacZ* transgene comprises *LacZ* under control of the *dpp* enhancer. Numerous articles have suggested that transcription through a PRE can induce switching from a silencing to an activating element (Cavalli *et al.*, 1998; Cavalli, 1999; Rank *et al.,* 2002; Schmitt *et al.*, 2005). Even though it is currently under debate to what extent constitutive transcription through such an element is required to swap fates (Erokhin *et al.*, 2015; Kassis and Muller, 2015), I have to take into account that transcription could convert repression to activation. Therefore, I would like to point out again that the effect of transcription through the transgenic PRE in my experiments is negligible, as assessed by antibody staining against β-galactosidase. Only a very small number of cells transcribe through the PRE under control of the *dpp*^{WE} enhancer (compare *Fig. III.3)*. Unfortunately, I was not able to address the role of transcription in my experiments, but I infer that if there is an effect of transcription through the H3K27me3 domain, the effect is constricted to less than 5% of wing disc cells and can therefore not explain the massive reduction of H3K27me3.

IV. 2. Pc does not bind H3K27me3 domains in the absence of the PRE – novel repercussions on H3K27me3 inheritance

The finding that H3K27me3 decays upon removal of the PRE implies that the H3K27me3 mark is not a self-sustainable mark as it was suggested (Hansen *et al.*, 2008). I assume that H3K27me3 is not sufficient to recruit PcG complexes to their site of action, as demonstrated for the PcG component Pc. Pc fails to bind H3K27me3 in the absence of the PRE.

Although I assume that this holds true in general for PcG complexes, I can only emphasize that Pc is unable to be retained at – and in proximity of – the *bxd* PRE. As mentioned above, Pc is the only PcG protein whose binding is not restricted to the PRE itself, but trails in vicinity of the PRE. Therefore we can follow its binding capacity even after removal of the PRE sequence.

Nevertheless, the incapability of Pc to bind to H3K27me3 12 h after excision of the PRE avers an additional argumentation. Although shown to recognize and bind H3K27me3 with its chromodomain (Doerks *et al.,* 2002; Fischle *et al.*, 2003), Pc fails to remain attached to H3K27me3 domains in the absence of the PRE (compare *Fig. III.31*). Therefore, the presence of H3K27me3 cannot be sufficient to recruit Pc to its site of action, where it is thought to promote interaction of PRC1 with chromatin along the length of repressed genes and thereby to reinforce H2A mono-ubiquitination (Margueron and Reinberg, 2011; Simon and Kingston, 2013).

One hypothesis that could explain these observations is that the PRE itself is required as general binding platform. The PRE would allow for PcG complexes to remain attached with the region long enough to perform their enzymatic activites on the surrounding chromatin. In other words, in the absence of the PRE, Pc – and likely other PcG members – cannot remain in its position on the repressed region around the PRE long enough to recognize H3K27me3 domains (*Fig. IV.1*) (or even find it at all). Developing this hypothesis further, without remaining attached to the repressed chromatin domains, Pc cannot mono-ubiquitinate H2A. Hypothetically speaking, the lack of H2Aub nucleosomes would result in a disturbed positive feedback loop that would in turn promote PRC2's activity and H3K27 trimethylation under native conditions (Kalb *et al.,* 2014).

Clearly, at this stage, I cannot distinguish between effects that are due to the absence of the PRE or due to the subsequent reduction in H3K27me3 levels. Yet, overall, the findings that Pc is unable to persist in the absence of the PRE strongly suggests that PcG components interplay with one another is truly compromised without the PRE being present.

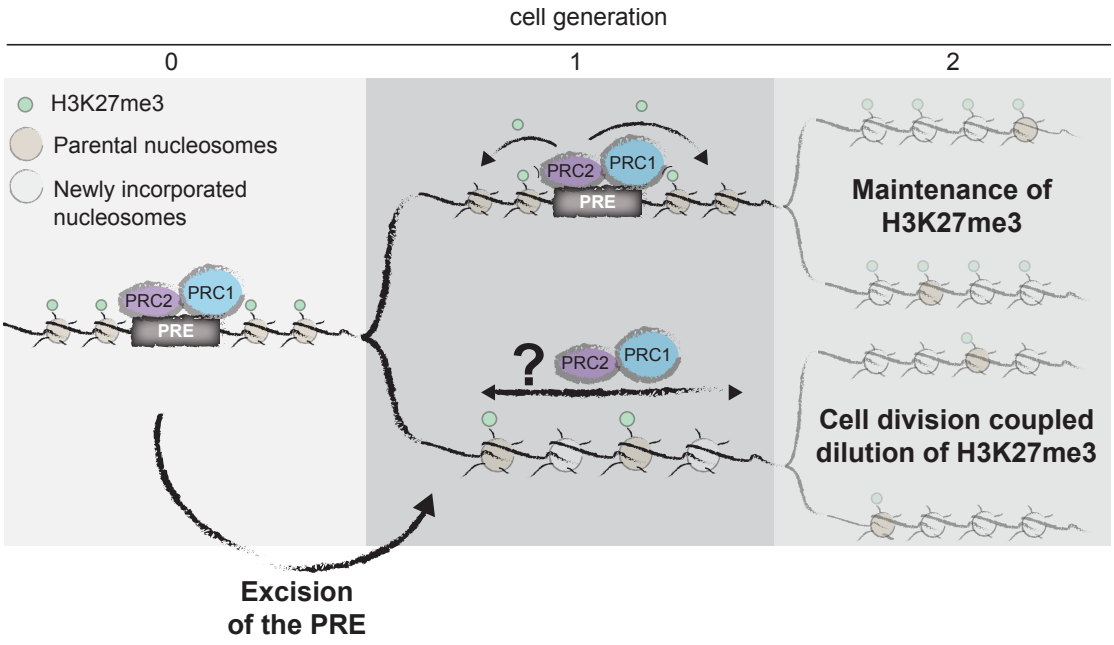

***Fig.* IV.1: Potential mechanism of H3K27me3 decay**. In the absence of the PRE (bottom) PcG complexes fail to recognize or pause at a specific region to reset H3K27me3. This results in cell division coupled dilution of H3K27me3 in the absence of the PRE. In the presence of the PRE (top) PcG complexes recognize parental H3K27me3 marks and maintain the repressed state.

IV. 3. Silencing elements in organisms other than *Drosophila*

This study sheds light on unknown parameters of cellular mechanisms of propagation of the repressive H3K27me3 mark from one cell generation to the next in *Drosophila.* I find that the PRE is strictly required as binding platform to convey epigenetic information of a repressed state.

One of the most compelling arguments for H3K27me3 being a self-propagating mark comes from the Gal4-EED-tethering experiments in mammals, presented earlier (Hansen *et al.,* 2008). Reviewing these data in the context of my findings that H3K27me3 is lost in the absence of the PRE in a cell cycle-dependent manner in *Drosophila*, I find similar arguments that could explain the data set from Hansen *et al.* with a different perspective. Importantly, in mammals, CGIs are associated with gene promoters and there is mounting evidence that specific CGIs may overtake the role of PREs in these organisms

(Mendenhall *et al.*, 2010; Lynch *et al.,* 2012). Comparably in yeast, silencer elements are continuously required for maintenance of the repressed state (Holmes and Broach, 1996).

Independent of the model system, there is likely consensus in requirements for a sequence- or structure-specific pattern that allows silencer complexes to persist in a given region. Tethering of a protein to a specific site, such as in the Hansen paper (Hansen *et al.,* 2008), might provide an artificial binding platform for PcG proteins and therefore permit their prolonged residence at the luciferase promoter and substitute for a proper binding platform as the PRE in *Drosophila* or a CGI in mammals.

IV. 4. Concluding remarks

To conclude my findings, I was able to show that H3K27me3 is not a self-sustainable mark as it was suggested. I showed that the PRE is strictly required for maintenance of the repressed chromatin mark H3K27me3 – which could also hold true for a different binding platform in mammals. Furthermore, I demonstrated that loss of repression upon loss of the PRE is a gradual and moreover passive process of dilution, which is cell cycle-dependent.

I therefore propose a distinct model, which is illustrated in Figure IV.1. PcG complexes require a binding platform, such as a PRE, to persist in a specific region. Pausing at these loci allows for them to interact with the surrounding chromatin marks, such as PRC1, that can interact through the chromodomain of Pc with H3K27me3 to provide a positive feedback loop for H2A ubiquitination (Kalb *et al.,* 2014) or such as PRC2, which can read and write H3K27me3.

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VI. Appendix

VI. 1. List of abbreviations

α	antibody
°C	degree Celsius
+	wild-type
∝	infinity
3'UTR	3'-untranslated region
7b	>PRE>*dppWE-LacZ* 7b
17b	>PRE>*dppWE-LacZ* 17b
17-1	>PRE>*dppWE*-UZ-*LacZ*
30b	>PRE>*dppWE-LacZ* 30b
36c	>PRE>*dppWE-LacZ* 36c
3R	right arm of chromosome III
A	adenine
A-P	anteroposterior
AEL	after egg laying
ATP	adenosine tri phosphate
bp	base pairs
BSA	bovine serum albumine
BBT	0.2% Triton X-100, 1% BSA in PBS (buffer)
bx	bithorax
BX-C	bithorax complex
bxd	bithoraxoid
C	cytosine
CG	undefined *Drosophila melanogaster* protein-coding gene
CGI	CpG island
ChIP	chromatin immunoprecipitation
Clr4	histone H3 methyltransferase in *S. pombe*
cn	cinnabar, eyecolor marker in *Drosophila*
CpG	cytosine followed by guanine base
ddH_2O	double-distilled H_2O
DMF	di-methyl-formamine
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
dppWE	decapentapletic wing disc enhancer
dpr9	defective proboscis extension response 9
dRAF	dRing-associated factor complex
Drosophila	*Drosophila melanogaster*
E(z)	enhancer of zeste
E3	E3 ubiquitin ligase
EDTA	ethylene-diamine-tetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EED	embryonic ectoderm development
EGTA	ethylene-glycol-tetraacetic acid
Epe1	histone H3 lysine 9 demethylase in *S. pombe*
Esc	extra sex combs
EtOH	ethanol

eu	euchromatic
F	forward primer
FLP	Flipase recombinase
Flp10	P(ry[t7.2])70Flp)10
FRT	Flipase recombinase target site
G	guanine
GAF	GAGA factor
gDNA	genomic DNA
h	hours
H1	histone 1
H2A	histone 2 A
H2Aub	histone 2 A mono-ubiquitylation
H2B	histone 2 B
H3	histone 3
H3.3	histone variant 3.3
H3K27	histone 3 lysine 27
H3K27me2	histone 3 lysine 27 dimethylation
H3K27me3	histone 3 lysine 27 trimethylation
H3K36	histone 3 lysine 36
H3K36me2	histone 3 lysine 36 dimethylation
H3K36me3	histone 3 lysine 36 trimethylation
H3K4me3	histone 3 lysone 4 trimethylation
H3K9	histone 3 lysine 9
H3K9me1	histone 3 lysine 9 monomethylation
H3K9me2	histone 3 lysine 9 dimethylation
H3K9me3	histone 3 lysine 9 trimethylation
H4	histone 4
H4K20me3	histone 4 lysine 20 trimethylation
HCl	hydrogen chloride
het	heterochromatic
HMTase	histone methyltransferase
HS	heat shock
hsp70-FLP	*heat shock*-inducible Flipase recombinase
hsp70	heat shock protein 70
IS	immuno staining
J33R	plasmid reference
K	lysine
KAc	kalium acetate
$K_4Fe(CN)_6$	potassium ferrocyanide
$K_3Fe(CN)_6$	potassium ferricyanide
kb	kilo base pair
KCl	potassium chloride
kDa	kilo dalton
LacZ	structural gene of the lac operon
LiCl	lithium chloride
M	molar
MeOH	methanol
n	number
NaCl	potassium chloride
NaH_2PO_4	monosodium phosphate

NaOAc	sodium acetate
NP-40	nonidet P-40
o/n	over night
Ogt	O-glycosyltransferase
PAS	protein A^5 sepharose
PB	phosphate buffer
PBS	phosphate buffered saline
Pc	Polycomb
PcG	Polycomb group
Pcl	Polycomb-like
PCR	polymerase chain reaction
PE	QIAGEN wash buffer
Ph	polyhomeotic
Pho	pleiohomeotic
PhoRC	Pho-repressive complex
PO_4	phosphate
PR-DUB	Polycomb repressive deubiquitinase complex
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PRE	Polycomb Response Element
qPCR	quantitative real-time PCR
R	reverse primer
Ring1a/b	PRC1 subunit
RIPA	radioimmunoprecipitation assay (buffer)
rpm	rounds per minute
RT	room temperature
ry	rosy, eye color marker in *Drosophila*
ry^{42}	mutated rosy gene
s	second
S-phase	synthesis phase (of the cell cycle)
Sce	sex combs extra
SD	standard deviation
SDS	sodium dodecyl-sulfate
SET	Su(var)3-9 and E(z) protein domain
Su(var)3-9	suppressor of variegation 3-9
Su(z)12	Suppressor of zeste
Suv39h	human suppressor of variegation 3-9
SV40	simvian vaculoating virus 40
T	thymine
T4	primer pair spanning the transgenic PRE
TBE	Tris/ Borate/ EDTA buffer
TE	Tris EDTA (buffer)
TRE	trithorax response element
trxG	trithorax group
TSS	transcription start site
Ubx	Ultrabithorax
v./v.	volume percent
w/	with
w^{118}	wild-type *Drosophila* strain
X	X chromosome

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast peptone dextrose

VI. 2. List of Figures

VI. 3. List of tables

VI. 4. Curriculum vitae

Education and Training

04/2012 – 12/2015 **PhD thesis** at the Max-Planck-Institute of Biochemistry, Martinsried, Germany
Supervisor: Dr. Jürg Müller
Title of PhD thesis: Inheritance of the H3K27me3 Modification in the Absence of a PRE

11/2011 – 04/2012 **PhD student** at the German Cancer Research Centre (DKFZ), Heidelberg, Germany
Supervisor: Dr. Thomas Hofmann
Research project aiming to understand the role of HIPK2 upon DNA damage in stem cells.

10/2010 – 10/2011 **M.Sc. thesis** at Harvard Medical School, Boston, USA
Supervisor: Peter Howley, MD
Title of thesis: Study of interaction of viral and host cells factors in transcriptional regulation of human papillomavirus

10/2009 – 11/2011 **M.Sc.** in Molecular Medicine at the Friedrich-Schiller-University, Jena, Germany
Grade: 1.2

06/2009 – 09/2009 **B.Sc. thesis** at Singapore Immunology Network, Singapore, Singapore
Supervisor: Subhra Kumar Biswas, PhD
Title of thesis: The effects of Norrin in gram-negative sepsis

09/2008 – 09/2009 **B.Sc.** equivalent: Trinational study course in Biotechnology
Ecole Superieure de Biotechnologie de Strasbourg, France
Biozentrum Basel, Switzerland
Albert-Ludwigs-University, Freiburg, Germany
Karlsruhe Institute of Technology, Karlsruhe, Germany

10/2006 – 09/2008 **Pre-diploma** in Biotechnology at Berlin Institute of Technology, Berlin, Germany
Grade: 2.1

07/2005 – 07/2006 **Voluntary year of social service** at 15th civic hospital, Moscow, Russia

08/1996 – 06/1005 **University entrance diploma** at Georg-Büchner-Schule, Darmstadt, Germany
Grade: 1.4

Fellowships

11/2011 – 04/2012 **Helmholtz-fellowship**
granted by the DKFZ International PhD program.

09/2008 – 08/2009 **Erasmus-fellowship**
granted for studies abroad

09/2008 – 09/2011 **Eucor-fellowship**
granted by the European Confederation of Upper Rhine Universities

09/2009 – 12/2009 **DAAD-stipend**
granted for research project abroad

06/2009 – 09/2009 **Bourse d'Alsace**
granted for B.Sc. research project in Singapore

Publication

Smith, J.A., **Haberstroh, F.S** ., White, E.A., Livingston, D.M., DeCaprio, J.A., and Howley, P.M. (2014). SMCX and components of the TIP60 complex contribute to E2 regulation of the HPV E6/E7 promoter. Virology, 468-470:311-21.

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I also wish to thank all members of the Müller group for fruitful scientific discussions and advice, especially Dr. Reinhard Kalb, who always helped out, took his time to discuss with me, as well as Dr. Maria Gambetta who provided very useful protocols and technical advice.

I would like to thank IMPRS, my PhD program, for great soft skill courses and opportunities to grow as part of the program.

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Last, I would like to thank my family and friends for their care and support.

VI. 6. Eidesstattliche Erklärung

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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*) Nichtzutreffendes streichen