INAGURAL-DISSERTATION

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Tag der mündlichen Prüfung:

Locus specific analysis of PcG/TrxG proteins using Bio-tagging technology

Gutachter: Prof. Dr. Renato Paro

Prof. Dr. Christof Niehrs

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Zusammenfassung

In der Entwicklung eines multizellulären Organismus werden verschiedene Zelltypen durch unterschiedliche Transkriptionsprogramme spezifiziert, die durch das Zusammenwirken von Promotoren, genregulatorischen Elementen und Transkriptionsfaktoren entstehen. Die Identität der Zelltypen wird durch ein spezifisches Genexpressionsmuster definiert und dieses wird über die Zellteilung hinweg an die Tochterzellen weitergegeben. Bei diesem Prozeß des "zellulären Gedächtnisses" spielen epigenetische Mechanismen eine wichtige Rolle.
In Eukaryoten wurden verschiedene chromatinbindende Proteinkomplexe identifiziert, insbesondere das System der Polycomb- (PcG) und Trithoraxgruppen (trxG) Proteine. Diese hochkonservierten Proteine sind in der Lage die Expression ihrer Zielgene durch lokale Chromatinmodifikationen zu kontrollieren. Die PcG und trxG Proteine binden als multimere Proteinkomplexe an bestimmte regulatorische Elemente in der Nähe ihrer Zielgene, welche PREs (polycomb regulatory elements) genannt werden. Ein PRE kann demnach als ein epigenetischer Schalter verstanden werden, auf den die PcG und trxG Proteine eine einander entgegengesetzte reprimierende (PcG) bzw. aktivierende (trxG) Wirkung haben.
Bisher konnten nur wenige verschiedene PcG Komplexe biochemisch identifiziert werden, aber es konnte mehrfach *in vivo* gezeigt werden, daß die Zusammensetzung der Proteinkomplexe an PREs sich je nach untersuchtem Zielgen oder -gewebe stark unterscheidet. Außerdem unterschied sich die Zusammensetzung der aufgereinigten Proteinkomplexe je nach verwendeter Aufreinigungsmethode und untersuchtem Zeitpunkt in der Entwicklung. In allen Fällen aber wurden Extrakte aus ganzen Embryonen der Fruchtfliege *Drosophila melanogaster* oder Insekten- und Säugetierzellinien verwendet. Daher repräsentieren die identifizierten Komponenten nur den Durchschnitt an Proteinkomplexen, die an die verschiedenen Zielgene gebunden waren. Bisher ist es noch nicht gelungen eine Methode zu entwickeln, die es ermöglicht, Proteinkomplexe in einer spezifischeren Weise zu isolieren und zu charakterisieren.
Das Ziel dieser Arbeit war die Entwicklung eines Systems zur Untersuchung der Proteinkomposition nativen Chromatins in einer lokus-, zeit- und gewebespezifischen Weise. Hierzu wurde eine Methode aus vier Komponenten entworfen, die die Affinität des Tet Repressors zu seiner Zielsequenz auf der DNA dazu benutzt, die Proteinkomponenten eines spezifischen transgenen PREs aufzureinigen. Die Expression dieses Proteinköders kann nun mit Hilfe des Gal4/UAS Systems gewebe- und zeitabhängig reguliert werden. Ein weitere Regulationsmöglichkeit bestand in der ebenfalls induzierbaren Expression des Enzyms, das den hier verwendeten bio-tag zu Proteinaufreinigung *in vivo* erkennt und biotinyliert.
Alle Komponenten wurden als Transgene in Drosophila melanogaster eingebracht und das so konzipierte System funktionell getestet. Während der Aufreinigung der Proteinkomplexe traten verschiedene methodische Probleme auf, welche die Effizienz des Systems an mehreren Stellen beeinflußten. Jedes Problem wurde separat in einer Serie von Experimenten untersucht, um ein verbessertes Aufreinigungsprotokoll zu entwickeln. Das so optimierte System war trotzdem bisher nicht in der Lage, PRE gebundene Proteinkomplexe in ausreichender Menge spezifisch anzureichern. Dennoch konnten durch die in dieser Arbeit durchgeführten Experimente zur Optimierung der Methoden die wichtigsten Schritte solcher Aufreinigungssysteme identifiziert werden. Zukünftige Alternativen für einzelne Schritte des hier entwickelten Systems werden auf den Erkenntnissen dieser Arbeit beruhen.

Summary

During development many cell types are generated by specific transcription programs that involve activation of gene expression at the level of promoters, enhancers and transcription factors. The identities of these different cell types are characterized by distinct sets of active and inactive genes, and need to be maintained through cell division. To achieve this, the cell type specific expression pattern has to be stably transmitted to the daughter cells. Epigenetic "cellular memory" mechanisms are often involved. In eukaryotes several chromatin-associated protein complexes have been identified, one of these systems comprises the Polycomb group (PcG) and the trithorax group (trxG) complexes. These widely conserved proteins act by locally modifying chromatin to maintain the transcriptional status of their target genes. The PcG and trxG bind as multi-protein complexes to regulatory elements called PREs (Polycomb response elements) in or near their target genes. A PRE can thus be considered an epigenetic switchable element upon which the PcG and trxG act antagonistically to maintain either silencing (PcG) or activation (trxG). Until now only a few distinct PcG complexes have been biochemically identified, but there is ample *in vivo* evidence that the composition of the PcG is different at different target genes and in different tissues. In addition, the purified complexes composition varies in some extent at different developmental times and depending on the protocol used for the purification. In all cases, biochemical isolations of complexes were performed using whole *Drosophila* embryos, *Drosophila* cell lines or mammalian cell lines; thus the purified complexes represent an average of all complexes at all target genes. Altogether, the memory system seems to be much more complicated than previously thought. Additional factors that come in to action at certain points in development might exist, giving a degree of specificity to Pc/TrxG action. So far, little has been done to establish procedures for the differential isolation and characterization of tissue specific, developmental time specific, or locus specific PcG or TrxG complexes.

The aim of this thesis work was to design a system to look at the components of chromatin when still bound to the DNA in a locus, time and tissue specific manner.

During this work a four-component system was developed based on the utilization of a protein bait and its binding site in the chromatin to co-purify proteins bound to transgenic PRE, giving locus-specificity to the system. To control time or tissue specificity, the protein bait expression was induced by Gal4. One more level of control was introduced by tagging the bait with a Bio-tag recognized and *in vivo* biotinylated by an enzyme that is also inducible expressed. All system components were introduced in *D. melanogaster* flies and the generated system was tested proving to be biologically feasible. When tested for protein purification many methodological problems were encountered that affected the efficiency of the system at various levels. Each problem was confronted separately and many experiments were conducted to find a better solution for each experimental step that would lead to a better output. However, protein pull-downs after system optimization resulted in no quantitative enrichment of PRE bound proteins. Nevertheless, thanks to the characterization and system optimization performed in this work, critical steps for functioning of this type of systems were identified. Alternative solutions to some aspects of the system designed will be based in the future on the findings of this work.

Introduction

1. Introduction

One of the most remarkable characteristics of a multicellular organism is that each and every single cell contains exactly the same genetic information and yet there are many different cell types. From the moment of fertilization, the development of an adult organism requires the specification of distinct cell types all originated from a single cell. This generation of cellular diversity, known as differentiation, is achieved through the establishment of characteristic patterns of gene expression initiated by specific transcription factors.

The changes in cellular biochemistry and function that occur during differentiation are preceded by the commitment of the cell to a certain fate. At this point, even though the cell does not differ phenotypically from its uncommitted state, its developmental fate has become restricted. Once a cell has reached this determined condition, the characteristic gene expression profiles must be maintained and passed on to the daughter cells for many cell divisions. The maintenance relies on epigenetic "cellular memory" mechanisms (Paro and Harte 1996), which involve changes at the chromatin level, through histone modifications, DNA methylation, and binding of protein complexes to the DNA. Although great progress has been made in the past years, many questions remain unanswered regarding the molecular mechanisms underlying this epigenetic memory.

The fruit fly *Drosophila melanogaster* is one of the most valuable of organisms in biological research, particularly in genetics and developmental biology. It has been used as a model organism for research for a century and many of the genes involved in epigenetic regulation have been originally described in the fly. Furthermore, a broad variety of genetic and molecular tools to influence gene expression exist in *Drosophila*, making this fly an excellent model organism to study the molecular aspects of the cellular memory mechanisms.

1.1 Early Drosophila development: the specification of embryonic cells

Following fertilization, mitosis begins; however in the early *Drosophila* embryos mitosis is not accompanied by cytokinesis, but instead results in the formation of a syncytial blastoderm. Cellular membranes do not form until after the thirteenth nuclear division, and all the nuclei share a common cytoplasm where material can diffuse throughout the

embryo. This common cytoplasm is not homogeneous; on the contrary, different morphogenetic protein gradients are established across the embryo that are responsible for the specification of cell types along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes.

The specification of individual cell fates begins already before fertilization, in the oocyte (Ingham 1988; St Johnston and Nusslein-Volhard 1992). The nurse cells of the ovary deposit mRNA in the developing oocyte, and these mRNAs are allocated to different regions of the cell (Frigerio et al. 1986; Berleth et al. 1988). Critical for the determination of the A-P axis are *bicoid* (*bcd*) and *hunchback* (*hb*) mRNAs, for head and thorax formation; and *nanos* (*nos*) and *caudal* (*cad*) mRNAs, for abdominal segments formation. The products of these maternal effect genes reside at the top of four genetic hierarchies that control the regional specification and result in the subdivision of the embryo into fourteen distinct parasegments (PS) (Figure 1.1).

In the fertilized embryo the maternal mRNAs are translated into proteins. These proteins, mostly transcription factors, are responsible for the activation of the gap genes, the first class of segmentation genes. Bicoid protein, forms a gradient in the anterior half of the embryo, where it represses the translation of *caudal* mRNA needed for posterior specification (Rivera-Pomar et al. 1996; Chan and Struhl 1997) and activates the transcription of the embryonic *hunchback* (Driever and Nusslein-Volhard 1988; Driever and Nusslein-Volhard 1989; Struhl et al. 1989), also a transcription factor. Together Bicoid and Hunchback activate the gap genes necessary for anterior formation (Figure 1.1).

Similarly, the posterior is defined by the activity of *nanos* gene (Lehmann and Nusslein-Volhard 1991; Wang and Lehmann 1991; Wharton and Struhl 1991). Nanos protein forms a gradient that is highest at the posterior end and it functions by inactivating *hunchback* mRNA translation. In addition, Caudal functions as transcription factor at the posterior to activate posterior-specific gap genes.

During early development the Drosophila embryo is metamerized into parasegments. The gap genes divide the embryo into broad regions, each containing several parasegment primordial (Driever and Nusslein-Volhard 1989; Struhl et al. 1989; Gaul and Jackle 1990; Rivera-Pomar et al. 1995). The proteins products of these genes interact with neighbouring gap gene protein products activating the transcription of pair-rule genes. In turn, the pair-rule gene products subdivide the broad gap gene regions to

originate fourteen parasegments (St Johnston and Nusslein-Volhard 1992; Rivera-Pomar and Jackle 1996; Huang et al. 2002). Finally, the segment polarity genes, which encode besides transcription factors many proteins constituents of signal transduction pathways, reinforce the parasegmental periodicity and establish the boundary between anterior and posterior compartments (Martinez-Arias and Lawrence 1985) (Figure 1.1).

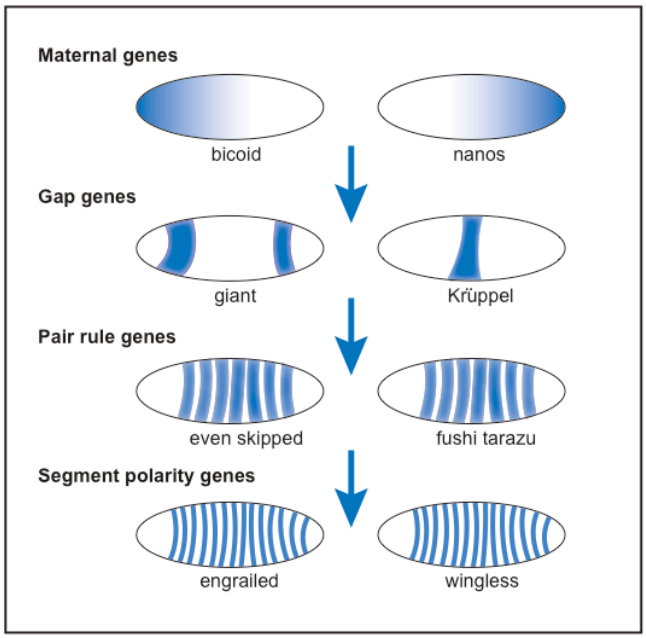

Figure 1.1. Four genetic hierarchies during embryonic specification. After fertilization the products of maternal effect genes, first hierarchical step, are translated into proteins that form gradients in the embryo. Bicoid and Nanos (among others) regulate the expression of the embryonic Gap genes in specific regions of the embryo. The Gap genes, mainly transcription factors regulate the expression of the Pair rule genes (*even skipped, fushi tarazu* and others), which divide the embryo in fourteen parasegments and regulate the Segment polarity genes, such as *engrailed* and *wingless*. These constitute the last step of the hierarchy and reinforce segmental periodicity. Gap and Pair rule genes activate expression of homeotic genes that determine segment identity. (Adapted from flymove; *http://flymove.uni-muenster.de/)*

In the *Drosophila* adult, these fourteen parasegments will form the three head, the three thoracic and the eight abdominal segments. Although they are similar in number, segments and parasegments are shifted relative to another; each segment is composed of the posterior part of a parasegment and the anterior portion of the next one. The identity of these segments is determined by the homeotic selector genes, which are regulated by pair-rule and gap genes products (Lewis 1978; Qian et al. 1991; Zhang et al. 1991; Muller and Bienz 1992; Shimell et al. 1994; Maeda and Karch 2006). Once the expression patterns of the homeotic genes have become stabilized, the transcription

factors regulating them disappear (Frasch et al. 1987; Gaul et al. 1987; Tautz 1988) but the homeotic genes transcriptional states become "locked" by epigenetic cellular memory mechanism (Ingham and Whittle 1980; Mc Keon and Brock 1991; Simon et al. 1992; Maeda and Karch 2006) (Figure 1.2).

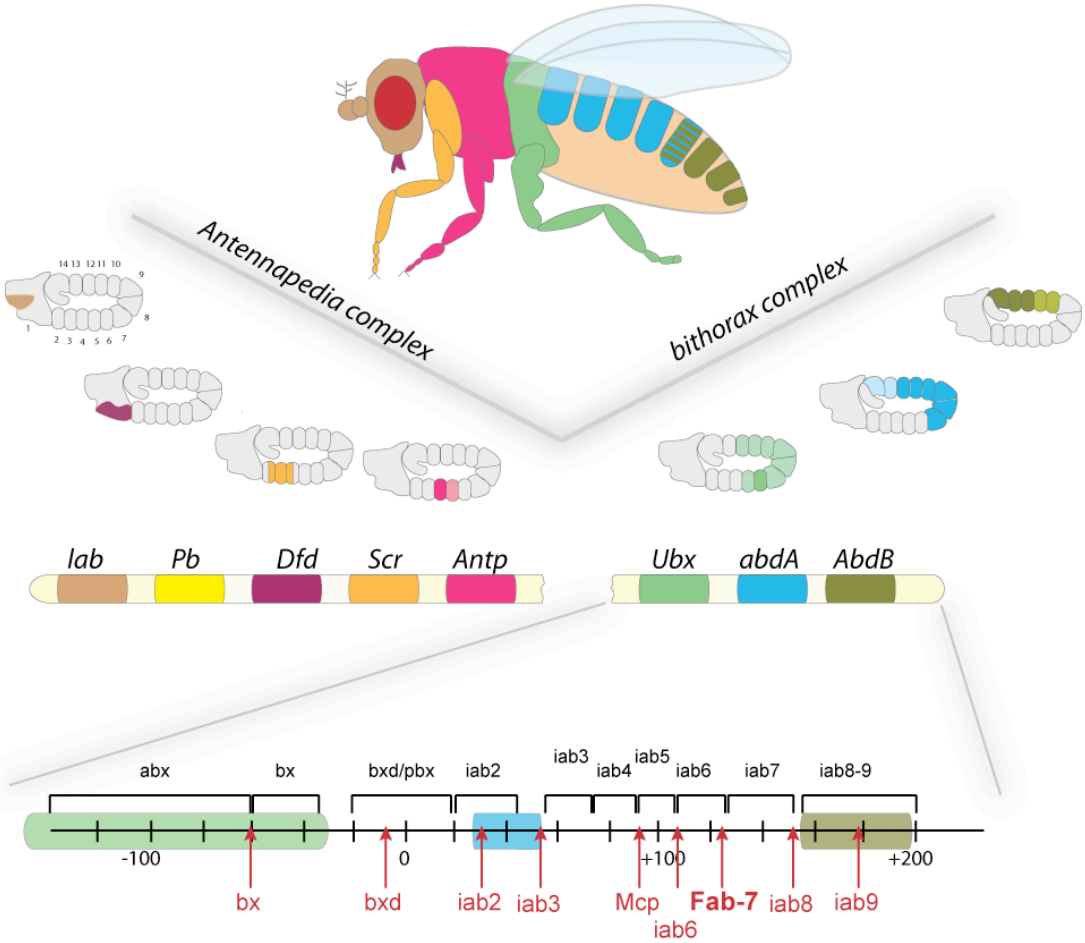

Figure 1.2. Bithorax complex regulatory regions. Homeotic genes specify segment identity via differential expression along A-P axis of Drosophila embryo. The pattern of expression of the homeotic *Antennapedia* and *bithorax* complexes is shown. The regulatory regions of the BX-C are shown in detailed. Interspersed in the complex are PS-specific enhancers (*abx/bx, bxd/pbx* and *iab 2-9*), which are responsible for establishing homeotic gene transcription in response to segmentation gene products during early embryogenesis. The maintenance of the expression patterns after transcription factors have disappeared is regulated by PcG and TrxG of proteins through binding to PREs (*bx*, *bxd, iab2, iab3, Mcp, iab6, Fab-7and iab8; iab9* is a putative PRE*)* indicated with red arrows in the *bithorax complex*. (Adapted from Gilbert 2003).

1.2 Maintaining cell identity: the cellular memory components

Cellular memory is the system responsible for the maintenance of the transcriptional state of the homeotic genes and many other genes involved in cell proliferation, signalling and cell cycle progression. The main components of the cellular memory are the Polycomb group (PcG) and the Trithorax group (TrxG) proteins, which were first genetically characterized in *Drosophila*. Mutations in PcG genes lead to the ectopic expression of homeotic genes, resulting in posterior transformations of segments and body structures. This suggested that the PcG genes are part of a specific repressor system that maintains the target genes in the silent state (Lewis 1978; Struhl and White 1985; Glicksman and Brower 1990; Jones and Gelbart 1990; Simon et al. 1992). The TrxG acts antagonistically to PcG and is responsible for the maintenance of the active transcriptional state (Kennison and Tamkun 1988). Mutants of this group of genes show mostly anterior transformations, caused by reduced expression of homeotic genes. Both group of proteins exert their function through specific cis-regulatory DNA elements; the Polycomb/Trithorax response elements (PRE/TRE), which have dual potential for epigenetic maintenance of both active and silence states (Busturia et al. 1989; Simon et al. 1990; Simon et al. 1993; Chan et al. 1994; Chiang et al. 1995). For simplification these elements will be referred to as PREs.

1.2.1 PcG, TrxG and their Protein Complexes

The Polycomb group and the Trithorax group of proteins bind their target genes as multi-protein complexes. The biochemical purification and characterization of these complexes and the studies of the physical and regulatory interactions between them and their target genes has contributed greatly to the understanding of how the cellular memory system works. For instance, it has been found that many of the proteins in these complexes have enzymatic activities responsible for modifying the chromatin at the target sites. These findings shed light on the molecular mechanisms involved in PcG and TrxG regulation.

A. Polycomb group repressive complexes

Polycomb group silencing involves at least three kinds of multi-protein complexes that work together, the Polycomb Repressive Complexes type 1 and type 2 (PRC1 and PRC2) and, most recently purified, the Pho Repressive Complex (PhoRC) (for review see Ringrose and Paro 2004; Schwartz and Pirrotta 2007). All three complexes contain multiple protein subunits and are crucial for Hox gene silencing. The PRC2 complex is

well conserved in evolution; components of this complex are found in plants and animals, but not in fungi *S. cerevisiae* and *S. pombe* (Schuettengruber et al. 2007). However, PRC2 components are also present in *N. crassa*, showing that these proteins might have an ancient function in transcriptional repression. In contrast, PRC1 core component genes are not found in fungi and plants (Springer et al. 2002), and although they have originated very early en animal evolution, many genes have alternately been lost from some species of the animal kingdom (Schuettengruber et al. 2007). Pc itself is missing in many species. It is probable that in these species other, jet unidentified factors, perform PRC1 function. In plants, for instance, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is necessary for the maintenance of the epigenetically repressed state of some euchromatic genes (Mylne et al. 2006; Sung et al. 2006), and could be the factor replacing PRC1.

The PRC1 complex

The PRC1 complex from *Drosophila* is composed of four core PcG proteins found in stoichiometric amounts: Polycomb (Pc), Posterior sex comb (PSC), Polyhomeotic (Ph) and *Drosophila* Ring1 (dRing1) (Shao et al. 1999; Saurin et al. 2001; Levine et al. 2002) (Figure 1.2). In addition to the core proteins, the 1-2MDa complex has been found to be associated to more than 30 other polypeptides, among them are Sex combs on midleg (SCM), Zeste TBP (TATA-box binding protein)-associated factors, and several transcription factors like TAFII 250, TAFII110, TAFII85 and TAFII62 (Francis et al. 2001; Saurin et al. 2001). The fact that TBP and transcription factors can be purified with PRC1, and that Pc has been found to co-localize with TBP at the promoters of the PcG regulated genes in *Drosophila* SL2 cells, suggest that this complex might silence its targets by direct inhibition of the transcriptional machinery. Furthermore, *in vitro* experiments have shown that the PRC1 complex can block transcription by RNA POLII and the sliding of nucleosomes on chromatin templates mediated by human SWI/SNF ATPase complex (Shao et al. 1999; Francis et al. 2001; King et al. 2002; Levine et al. 2002). Curiously, the SWI/SNF complex is homologue of the *Drosophila* BRM complex, known to be necessary for TrxG dependent activation (Shao et al. 1999; Francis et al. 2001). The remodelling inhibiting function can be reconstituted in a recombinant complex of PSC, Ph, Pc and dRing1, showing that these 4 proteins form a functional PRC1 core complex that can mediate gene repression. The addition of Zeste to the core complex enhances the inhibition of transcription and the binding to Zeste target

sites present on PREs (Mulholland et al. 2003) suggesting a possible role for associated factors that might modulate PRC1 activity. Although PRC1 core complex can block nucleosomes remodelling, it has no preference for a particular DNA sequences. *In vivo*, the specific binding to PREs is probably assisted by other proteins such as Zeste, GAF, PHO, DSP1, GRH and Pipsqueak (PSQ), which have been shown interact with PRC1 (Sato and Denell 1985; Poux et al. 2001; Mahmoudi et al. 2003; Huang and Chang 2004; Dejardin et al. 2005; Blastyak et al. 2006). Finally, PRC1 core complex can also induce *in vitro* condensation of polynucleosomal chromatin templates, as assayed by electron microscopy (Francis et al. 2004).

Another functional feature of PRC1 is given by the chromodomain of Pc protein, which binds *in vitro* to trimethylated lysine 27 of a Histone H3 tail peptide (H3K27me3) (Fischle et al. 2003). This mark is deposited in the chromatin by the PRC2 complex. Additional domains also found in PcG proteins confer these proteins enzymatic activities that are important for silencing. The CHRASCH complex (Chromatin-associated silencing complex), a PRC1-type complex purified from *Drosophila* culture cells, contains histone deacetylase activity, provided by HDAC1 (Figure 1.3). This activity could contribute to condensation of nucleosomes by increasing the positive charge of histones allowing better interaction with negatively charged DNA molecules. In addition, Ring1 protein contains a Ring domain shown to function as E3 ubiquitin-ligase that mono-ubiquitylates lysine 119 of Histone H2A (H2AK119ub1) (Wang et al. 2004a; Cao et al. 2005). This modification is crucial for silencing of *Drosophila* and mouse Hox genes, although the molecular mechanism involved is still unknown.

Other modulators of PRC1 activity might be the orthologues of PcG genes. These could function as alternatives in different tissues, developmental stages or even at different target genes in the same cell. For example, there are two *ph* genes, encoding Polyhomeotic proximal (PHP) and Polyhomeotic distal (PHD) proteins, the functions of which have not yet been clearly differentiated (Dura et al. 1987). Similarly, PSC and Suppressor of *zeste* 2 (Su(Z)2) are closely related and are thought to have partially overlapping functions (Wu and Howe 1995; Beuchle et al. 2001). Pleiohomeotic (PHO) and Pleiohomeotic-like (PHOL) (Brown et al. 2003) and Extra sex combs (ESC) and Extra sex combs like (ESCL) (Wang et al. 2006) are two other pairs of PcG genes with partially overlapping functions. In the same way, the mammalian PRC1 can be composed of alternative orthologues to the core proteins. Purified complexes include

HPC1, 2 and 3, HPH1, 2 and 3, RING1A and RING1B, BMI and potentially its homologue MEL18. No orthologues of Zeste and PSQ are found associated with mammalian complexes (Levine et al. 2002; Lavigne et al. 2004; Wang et al. 2004a).

The PRC2 complex

The core components of the PRC2 complex in *Drosophila* are Enhancer of *zeste* (E(Z)), Extra Sex Combs (ESC), Suppressor 12 of *zeste* and Nurf55 (Figure 1.3). The key element of the complex is the SET domain of E(Z), responsible for the histone methyltransferase (HTMase) activity of the complex. E(Z) alone has no methyl-transferase activity, but when assembled in the complex it trimethylates H3K27 (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002). This methyl mark is characteristic of PcG target genes (Cao et al. 2002; Schwartz et al. 2006). E(Z) is also responsible for wide spread of mono and dimethylation of more than 50% of H3K27 in the *D. melanogaster* genome (Ebert et al. 2004) and it can also methylate H3K9 *in vitro*, but the roles of these marks remain unknown.

The non-catalytic units of PRC2, Su(Z)12 and Nurf55, are essential for nucleosome binding and ESC is crucial for enhancing the enzymatic activity of E(Z) (Ketel et al. 2005; Nekrasov et al. 2005). In mammals Su(Z)12 is also needed for the HMTase activity (Cao and Zhang 2004; Pasini et al. 2004). Different isoforms of EED, the vertebrate homologue of ESC, have been found associated with the mammalian E(Z) resulting in several PRC2-type complexes with different enzymatic specificities. The mammalian PRC3 complex methylates H3K27 while PRC2 and PRC4 have preference for Histone H1K26 (Kuzmichev et al. 2004; Kuzmichev et al. 2005).

In flies, different approaches and purification schemes have resulted in purification of protein complexes with slightly different compositions. They all share the four core components and have HTMase activity, but they differ in molecular weight and in the presence of some additional proteins (Figure 1.3). The most prevalent complex is the 600KDa PRC2 purified from *Drosophila* embryos (Ng et al. 2000; Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). A 1MDa complex found in early embryos contains PCL, a protein required for Pc silencing of homeotic genes and found at Pc sites on polytene chromosomes (Tie et al. 2003; Papp and Muller 2006). Other larger complex appears at later developmental stages and contains SIR2, the homologue of yeast SIR2 Nad+ dependent Histone deacethylase (Furuyama et al. 2003; Furuyama et al. 2004). This complex is in the 4 MDa range and contains the

members of PRC2 and other additional, still uncharacterized proteins. Curiously this complex does not contain ESC. RPD3 protein, also a histone deacetylase, has been reported to co-purify with some of the larger complexes (Figure 1.3)(Czermin et al. 2002; Tie et al. 2003; Furuyama et al. 2004).

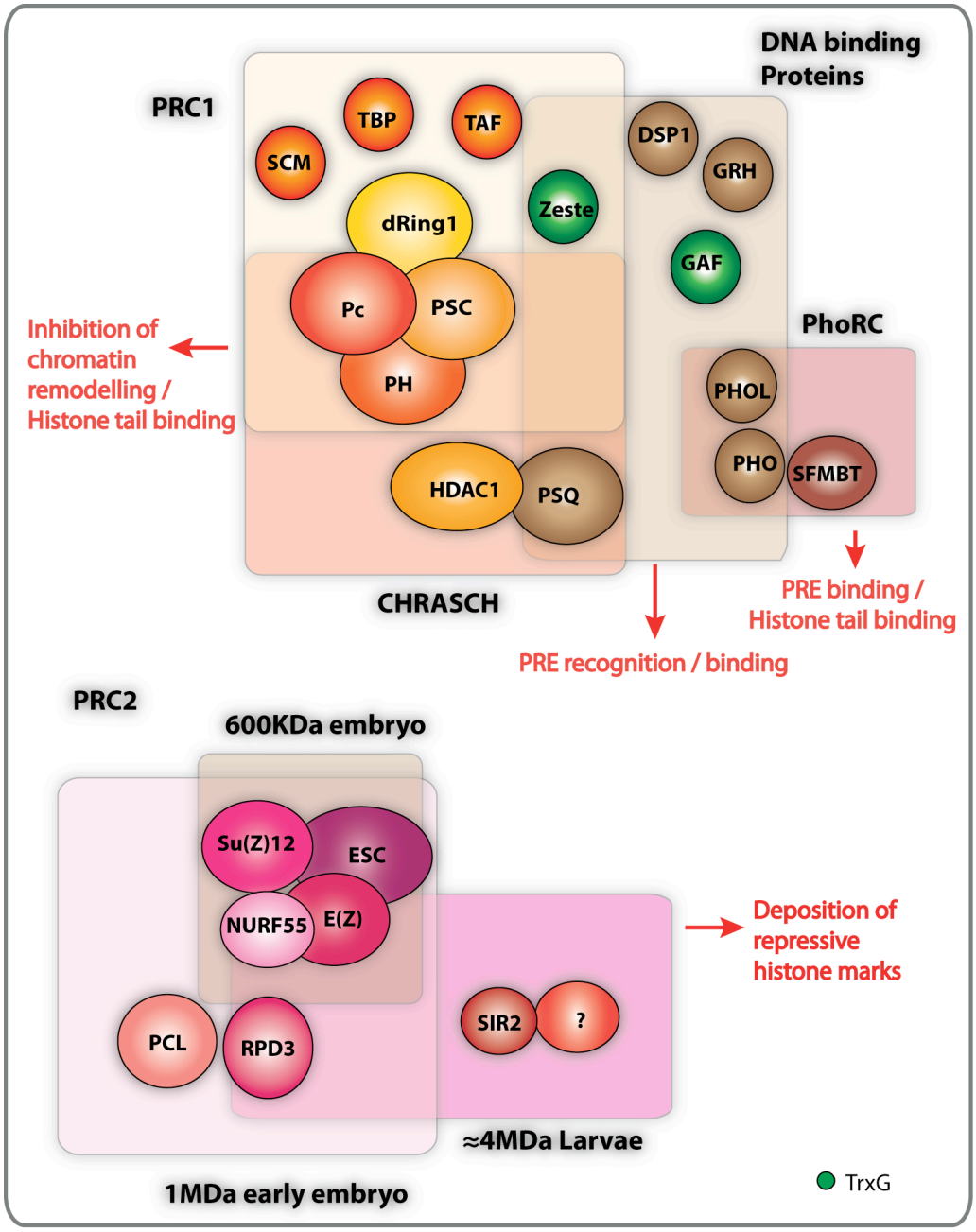

Figure 1.3. Polycomb Repressive Complexes and associated proteins. Three distinct repressive complexes have been purified in *Drosophila*. PRC1, PRC2 and PhoRC. In different tissues other PcG and non-PcG proteins are found associated with the complexes; among them many specific DNA binding proteins with binding sites on PREs. The main enzymatic activities for each complex and their roles in repression are described in the main text (question marks represent unidentified components of the complexes.

Association of RPD3 with PRC2 is disputed (Cao et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002), however in mammals the PRC2 complex was shown to contain HDAC activity (van der Vlag and Otte 1999). Furthermore, mutations in RPD3 enhance PcG mutant phenotypes (Chang et al. 2001) and disrupt PcG mediated silencing of a PRE-containing transgene. SIR2 and RPD3 might aid the action of TrxG activating complexes through their HDAC activities.

An important protein also found to be transiently associated to PRC2 is PHO. This interaction is mediated by the Set domain of E(Z) and might play an important role in the recruitment of the complex to the PREs (Poux et al. 2001; Satijn et al. 2001; Wang et al. 2004b).

Pho-RC complex

There are only a few members of the PcG that can bind directly to DNA. Pleiohomeotic (PHO) and Pleiohomeotic-like (PHOL) have a DNA-binding domain that can bind a specific DNA motif present on PREs. Although PHO has been found to associate transiently with PRC1 and PRC2 by immunoprecipitation experiments and GST pull-downs (Poux et al. 2001; Mohd-Sarip et al. 2002; Wang et al. 2004b), it is not present among the core components of the repressive complexes 1 and 2 (Ng et al. 2000; Saurin et al. 2001; Muller et al. 2002).

Recently, two distinct PHO complexes have been purified from *Drosophila* embryos (Klymenko et al. 2006). The Pho-dINO80 complex that contains the INO80 nucleosome remodelling complex and the Pho-RC complex composed of the *Drosophila Scm-related gene containing four mbt domains* (dSFMBT) protein (Figure 1.3). These complexes can be separated by glycerol gradient sedimentation showing that they exist *in vivo* as separate entities. In *Drosophila*, PHO and PHOL function redundantly to maintain Hox genes silencing (Brown et al. 2003), in agreement with this, both proteins are found to co-purified with both complexes indistinctly.

Surprisingly, the dPho-INO80 complex is not found at *Drosophila* PREs (Klymenko et al. 2006), suggesting that PHO has an additional function independent of PcG silencing. In addition, PHO has been described to occupy extended chromatin domains over the coding region of transcriptionally active genes. Immunostainings on polytene chromosomes revealed that PHO might play a general role in gene regulation, probably helping to re-repress induced genes (Beisel et al. 2007).

The dSFMBT protein present in the Pho-RC complex, binds to PREs and its binding is greatly diminished in PHO mutant background. In addition, removal of dSFMBT in imaginal discs clones causes wide miss-expression of Ubx and Scr (Klymenko et al. 2006). This phenotype is as severe as the one caused by other PcG mutants (Beuchle et al. 2001). Even more, tethering of dSFMBT to DNA as a fusion protein that contains a DNA binding domain, produces transcriptional repression of a reporter gene.

The Pho-RC complex can also bind selectively histone tails. Four MBT domains present in dSFMBT are responsible for the recognition of H3 tail peptides that are mono- or di-methylated at K9 and H4 tails mono- or di-methylated at K20 (Klymenko et al. 2006). Thus, it combines a sequence-specific DNA binding activity through the PHO subunit with a methylated histone binding activity through dSFMBT. Importantly, Pho-RC is specifically targeted to PREs in a manner that depends the presence of PHO binding sites on the DNA, suggesting that binding of dSFMBT to methylated histones is not required for Pho-RC targeting but is needed for repression.

B. Trithorax group complexes

All PcG target genes that have been studied are also known to be positively regulated by Trithorax group proteins. Contrary to PcG, the proteins present in TrxG complexes have enzymatic activities that help to activate transcription by modifying the chromatin at target sites. Many of these proteins are not only involved in TrxG regulation but also in general transcription processes.

At least four different TrxG complexes have been linked to Trithorax function. A 2MDa BRM complex composed of Brahma (BRM), Moira (MOR), OSA and SNR1 (Papoulas et al. 1998) (Figure 1.4), functions as nucleosome remodelling complex powered by the ATPase activity of BRM (SWI2/SNF2 in yeast). *In vitro*, BRM remodelling complex facilitates transcription from nucleosomal templates (Kal et al. 2000).

The 2MDa and 500KDa complexes, containing ASH1 and ASH2 (absent, small and homeotic) proteins respectively, which contain histone methyltransferase activity required for the maintenance of Hox gene expression. ASH proteins have SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domains that thrimethylate lysine residues 4 and 9 of histone H3, and lysine 20 of histone H4 (Beisel et al. 2002; Byrd and Shearn 2003; Klymenko and Muller 2004).

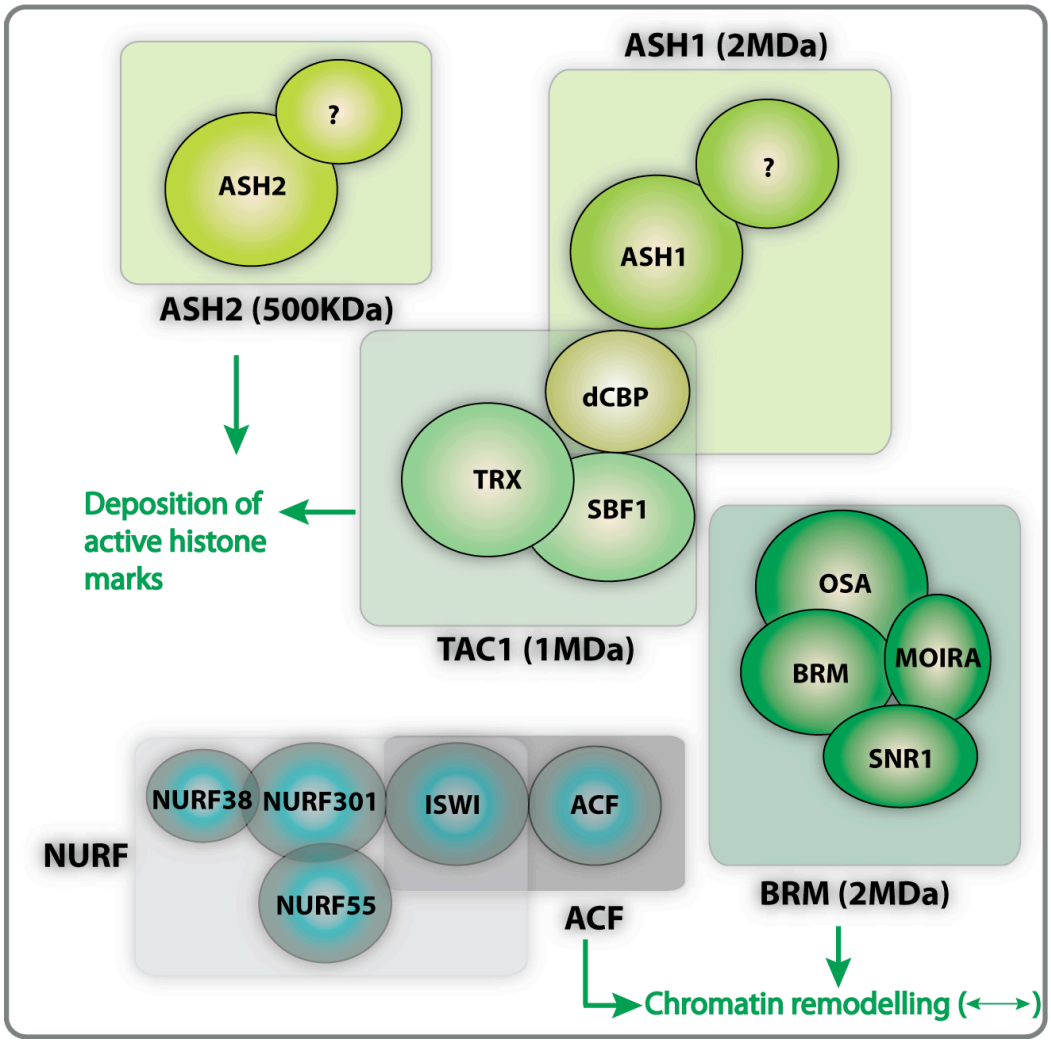

Figure 1.4. Trithorax Group complexes and associated complexes involved in transcriptional activation. All the components of TrxG complexes, ASH1, ASH2, TAC1 and BRM, and their enzymatic activities involved in transcriptional activation. The enzymatic activities and their roles in activation are described in the main text. Other non-TrxG complexes, like NURF and ACF, are involved in chromatin remodelling and might assist TrxG in their function (question marks represent unidentified components of the complexes).

The TAC1 complex (Trithorax Acetylation Complex) of 1MDa contains Trithorax (TRX) protein, which contains histone methyltransferase activity specific for lysine 4 of histone H3. In addition, CREB binding protein (dCBP) and antiphosphatase Sbf1 (Petruk et al. 2001; Smith et al. 2004) have been also described to be part of TAC1 (Figure 1.4). dCBP is a histone acetyltransferase that has also been associated to ASH1. The interaction with ASH1 together with its presence in TAC1 suggests that these two complexes act in concert to maintain active transcriptional states. Both complexes may

act by combining the histone acetylation function of dCBP with H3K4 trimethylation activity of TRX and ASH.

In addition to BRM, two other general chromatin remodelling complexes might contribute to Trx function. Both complexes contain ISWI protein as ATP dependent chromatin remodelling engine. In one complex ISWI is associated with several NURF proteins (Badenhorst et al. 2002) and in the other with ACF protein (Figure 1.4) (Fyodorov and Kadonaga 2002; Fyodorov et al. 2004). Interestingly, in some assays this complex also shows PcG-type functions, suggesting same complex might act as a cofactor of TrxG and PcG (Fyodorov et al. 2004).

There are many other proteins that have been classified as Trithorax group based on genetic behavior and are not found as components of the TrxG complexes. Two factors greatly involved in PcG/TrxG regulation are GAGA (GAF) and Zeste (Z). Although classified as TrxG proteins, these factors are also associated with silencing. GAF is required for PRE dependent repression of reporter genes (Hodgson et al. 2001) and it can stimulate PRC1 nucleosomal remodelling blocking (Mulholland et al. 2003). The same holds true for Zeste, which has been found to interact with PRC1 in addition to recruiting Brahma (Kal et al. 2000; Dejardin and Cavalli 2004). Both proteins seem to have also a more general role in transcription; the DNA binding sequences of GAF and Z are also found in the promoters of many non-polycomb target genes (Pirrotta and Brockl 1984; Pirrotta et al. 1985; Bevilacqua et al. 2000; Ringrose et al. 2003).

1.2.2 PREs: DNA sequences with memory

Polycomb group response elements (PREs) are cis-regulatory DNA elements necessary for recruitment of PcG and TrxG complexes to their target genes and for the inheritance of the determined transcriptional state through mitosis.

Many PREs have been identified in *Drosophila melanogaster*, but most of what is known about their function is through the characterization of a few specific PREs belonging to the well-studied homeotic genes (Figure 1.2). For instance, the Bithorax complex (BX-C) PREs were the first ones to be discovered and characterized (Simon et al. 1990; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Christen and Bienz 1994; Busturia et al. 1997). The BX-C is composed of three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abdA*) and *Abdominal-B* (*AbdB*) (Lewis 1978; Maeda and Karch 2006). These genes are expressed in a specific pattern along anterior posterior axis of the *Drosophila* embryo defining the identities of PS 5-14 (Figure 1.2).

As mentioned before, the homeotic genes are controlled in early development by the products of the gap and the pair rule genes through binding to cis-regulatory elements in the DNA. Several enhancers present in the BX-C are responsible for the specific expression patters of the homeotic genes of this complex. In this way, *abx/bx* enhancer controles *Ubx* expression, *iab2-4* regulate *abdA* and *iab5-9, AbdB* (Mihaly et al. 1998). After the gap and pair rule proteins disappear, the expression patterns of the BX-C genes are maintained by the cellular memory system. The PcG and TrxG proteins exert their function through binding to *bx, bxd, iab2, iab3, Mcp, iab6, Fab-7* and *iab8* PREs and also to a putative PRE in the *iab9* region (Figure 1.2) (Gyurkovics et al. 1990; Busturia and Bienz 1993; Chan et al. 1994; Christen and Bienz 1994; Chiang et al. 1995; Busturia et al. 1997; Barges et al. 2000; Beisel et al. 2007). These PRE are essential for the maintenance of the original transcriptional state of the BX-C genes.

Other PREs have been also identified at various homeotic and non-homeotic loci, including the *engrailed* (Kassis 1994), *polyhomeotic* (Bloyer et al. 2003), *hedgehog* (Maurange and Paro 2002) and even the *Cyclin A* (Martinez et al. 2006) loci. In addition to the genetically identified PREs, the binding pattern of PcG and TrxG proteins on polytene chromosomes reveals the existence of many, yet uncharacterized, PREs.

PREs are compound elements of several hundred base pairs. Many PREs contain clusters of GAGAG motifs, which bind GAF and PSQ and can also contain binding sites for PHO, PHOL and Zeste (Rastelli et al. 1993; Hagstrom et al. 1997; Strutt et al. 1997; Fritsch et al. 1999; Mishra et al. 2001; Huang et al. 2002); these binding motifs were found to be highly clustered. An algorithm based on these motifs allowed the in silico prediction of potential PREs on a genome-wide scale (Ringrose et al. 2003). This bioinformatics approach, in combination with experimental verification, identified many new target sites of PcG and TrxG proteins. The identified genes for the predicted PREs covered a wide rage of cellular functions, including regulation of cell cycle progression, cell fate determination and development. Recently, genome-wide mapping of PcG distribution using ChIP (Chromatin IP) or DamID approach and analysis on genomic tiling microarrays, revealed many more PcG target sites (Negre et al. 2006; Schwartz et al. 2006; Tolhuis et al. 2006). These new sites, missed by the prediction, could reveal the existence of still uncharacterized features in PREs, like motifs for other DNA binding proteins. For instance, DSP1 (dorsal switch protein 1) has been found to be involved in the function of Fab-7 and engrailed PREs (Dejardin et al. 2005). DSP1 protein binds to a

broad range of DNA motifs (Brickman et al. 1999) and it extensively co-localizes with PcG on polytene chromosomes. A dual function has been also attributed to DSP1since it has been shown to behave as TrxG protein at other homeotic PREs (Decoville et al. 2001; Rappailles et al. 2005; Salvaing et al. 2006). Like DSP1, other additional DNA binding proteins might exist that regulate alternative PREs, giving specificity to Pc/TrxG function.

No mammalian PRE has been identified to date, although it is clear that mammalian homeotic genes and many other loci are under PcG/TrxG control (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). One possibility is that mammalian PREs are more extensive or diffuse, and therefore more difficult to identify. A second possibility is that mammalian PREs do not exist and PcG proteins are recruited to their target genes by a different mechanism. The lack of vertebrate homologues for Zeste, GAGA and PSQ points into this direction (Levine et al. 2002; Lavigne et al. 2004; Ringrose and Paro 2004; Wang et al. 2004a).

1.3 Chromatin marks linked to silence and activation

The epigenetic regulation by PcG/TrxG of proteins leaves a track on the chromatin. The enzymatic subunits of the complexes deposit histone marks that are indicative for silencing or activation. The PRC2-type complexes posses H3K27-specific trimethylase activity (Cao and Zhang 2004) and several TrxG complexes have H3K4 trimethylase activity (Byrd and Shearn 2003; Dou et al. 2005; Wysocka et al. 2005).

Recent genome-wide analyses of the distribution of H2K27 and H3K4 trimethylation has help to understand the relationship between these marks, the binding of PcG/TrxG proteins, and the transcriptional state of the targeted genes. The components of the PRC2 complex in flies, mouse and human are found to colocalize with regions that are trimethylated at H3K27 (Boyer et al. 2006; Lee et al. 2006; Schwartz et al. 2006; Tolhuis et al. 2006). In contrast, H3K4 methylation is present at most active promoters in the genome. Other epigenetic mark associated with transcriptionally active euchromatin is histone acetylation, whereas inactive heterochromatin domains are consistently hypoacetylated (Jenuwein and Allis 2001).

A report using quantitative ChIP analysis of the inactive *Ubx* gene showed that while PcG and TrxG proteins bind a more constrained area at the PRE, the H3K27me3 mark is present throughout an extended 100 kb domain that spans the whole inactive gene (Papp

and Muller 2006). More over, H3K9me3 and H4K20me3 were also found to be present at the inactive *Ubx* gene (Ringrose et al. 2004; Papp and Muller 2006). Interestingly, no specific enrichment of H3K27-me3 was found at the PRE, rather a reduction of H3K27-me3 signal consistent with the reduced H3 signals detected at these sites. A similar pattern of H3K27me3 distribution was observed in separate genome wide study on Drosophila culture cells (Schwartz et al. 2006).

In the active *Ubx* instead, presence of this methyl mark is restricted to the upstream control region only, at the PRE. The absence of H3K27me3 in part of the gene correlates with the binding of Ash1 immediately downstream to the promoter, which induces trimethylation of H3K4 that spreads through the coding region of the gene. PcG remains bound at the PRE also in the active state.

The PRC1-type complexes also contain an evolutionary conserved histone modification activity leading to ubiquitylation of lysine 119 of histone H2A (de Napoles et al. 2004; Wang et al. 2004a), which is required for PcG-mediated silencing of *Drosophila* Ubx gene (Wang et al. 2004a). A putative "reader" of this histone mark remains to be identified.

Although no direct link to PcG and TrxG complexes has been found so far, increasing evidence suggests also a role for histone variants in marking the active and silent states. Histone H3.3 is found prominently at sites of abundant RNA pol II and methylated H3K4 throughout the *Drosophila* genome. Interestingly, this mark was also found far upstream and downstream of transcribed regions, probably due to intergenic transcription since the mechanism of H3 replacement by H3.3 is coupled to the transcriptional process it self (Ahmad and Henikoff 2002; Mito et al. 2005; Schwartz and Ahmad 2005). Moreover, a recent work in *Drosophila* SL2 cells revealed enrichment of H3.3 at the functional boundaries of several proximal-to-distal cis-regulatory domains that regulate *abd-A* and *Abd-B* (Mito et al. 2007). Histone H2A might also be involved in marking specific chromatin states, however, it is not clear whether it is silence or in activation (Allis et al. 1980; Meneghini et al. 2003; Rangasamy et al. 2003; Fan et al. 2004). Finally, histone MacroH2A is found on the inactive X chromosome in mammals (Costanzi and Pehrson 1998).

1.4 Molecular mechanisms of the cellular memory

What are the roles of all these histone modifications and are they sufficient to explain PcG-mediated silencing and Trx-mediated activation? Trx TAC1 and ASH1 complexes

methylate histone H3. This H3K4me3 mark is recognized by the PHD finger domain of the Nurf-301 protein (Li et al. 2006; Wysocka et al. 2006). The NURF complex tethered to TrxG responsive promoters might facilitate the recruitment of the transcriptional machinery via ATP-dependent nucleosome remodelling (Figure 1.5). The BRM complex can also move nucleosomes along the DNA. Via their activities NURF and BRM may be involved in the formation of an "open" chromatin structure that might help activators and transcription factors in reaching their target sites. H3K4me3 might also stimulate transcriptional elongation. In particular, H3K4me3 and ASH1 are found downstream of the *Ubx* promoter when the gene is active (Papp and Muller 2006). TRX has also been shown to facilitate transcriptional elongation at heat shock genes (Smith et al. 2004) and more recently, the *Drosophila* TAC1 complex has been proposed to play a global role in transcriptional elongation (Petruk et al. 2006) (Figure 1.5). Consistent with this, the mammalian TRX homologue MLL is associated with the promoters of expressed genes (Guenther et al. 2005). The MLL complex is closely related to the yeast COMPASS complex, which contains SET1 and binds to RNA POL II during transcriptional elongation.

In addition, proteins such as dCBP, part of TAC1 complex, have histone acethylase activity. This activity may help destabilize higher order chromatin structures and recruit bromodomain-containing transcription factors, such as TAFII250, that recognize acetylated lysines (Dhalluin et al. 1999; Jacobson et al. 2000) (Figure 1.5). Although ASH1 and TRX are both histone methyltransferases, it seems that they are not redundant in function. Loss of either protein leads to dramatic loss of *Ubx* expression, both in embryo and in larval imaginal discs, due to silencing by PcG complexes (Klymenko and Muller 2004). This shows that PcG silencing can be re-established at any stage of development if ASH1 or TRX are absent. Furthermore, in PcG/TrxG double mutant embryos, *Ubx* is missexpressed outside of its usual domain, demonstrating that ASH1 and TRX are not required for *Ubx* de novo activation. All together, this results support the idea that TrxG function as anti-repressors, rather than as transcriptional co-activators, and their presence is essential to counteract PcG silencing throughout development.

The enzymatic activity of E(Z) is required for silencing (Muller et al. 2002), but what role does the H3K27m3 mark exactly play? A possibility is that the PRC1 complex is responsible for the repression activity and that the methyl mark merely recruits this complex to the target sites via the chromodomain of Pc protein (Fischle et al. 2003;

Lachner et al. 2003). However, since Pc is constitutively bound to PREs in early embryogenesis, such a model requires that PRC2 would be recruited to all PREs before Pc. There are several lines of evidence that indicate that this is not the case (Muller and Kassis 2006; Ringrose and Paro 2007), and that rather the methylation is part, cause or mere consequence, of the system directly responsible for the silencing or a downstream event after PcG recruitment (Mutskov and Felsenfeld 2004). First, quantitative ChIP analysis suggests that PREs are in fact depleted of nucleosomes (Papp and Muller 2006; Schwartz et al. 2006), and then the relative amounts of H3K27m3 at this sites are actually reduced. Second, it was shown with in vitro assays that Pho and PRC1 can be co-assembled on naked PRE DNA templates in the absence of nucleosomes (Mohd-Sarip et al. 2002); and finally, chromodomain swapping experiments have shown that the chromodomain itself is not sufficient to direct a heterologous protein bearing the chromodomain to the sites where its target histone modification is enriched *in vivo,* strongly suggesting that targeting of PcG and TrxG is not dependent on histone tails modifications (Platero et al. 1995; Ringrose et al. 2004).

An alternative option is that H3K27me3 represses transcription directly, for instance by inhibiting some step involved in transcriptional activation or by preventing the deposition of histone marks associated with gene activation, such as acetylation, ubiquitylation of histone H2B or trimethylation of H3K4. The action of the methyl mark could be reinforced by PRC1 repression ATP-dependent nucleosome remodelling (Shao et al. 1999). Moreover, TBP-associated factors have been found to interact with PRC1 (Breiling et al. 2001; Saurin et al. 2001), suggesting that PcG proteins might contact promoters. In this way, PRE-mediated silencing does not necessarily prohibit recruitment of RNA POLII, but may interfere with transcription initiation or elongation (Figure 1.5) (Dellino et al. 2004).

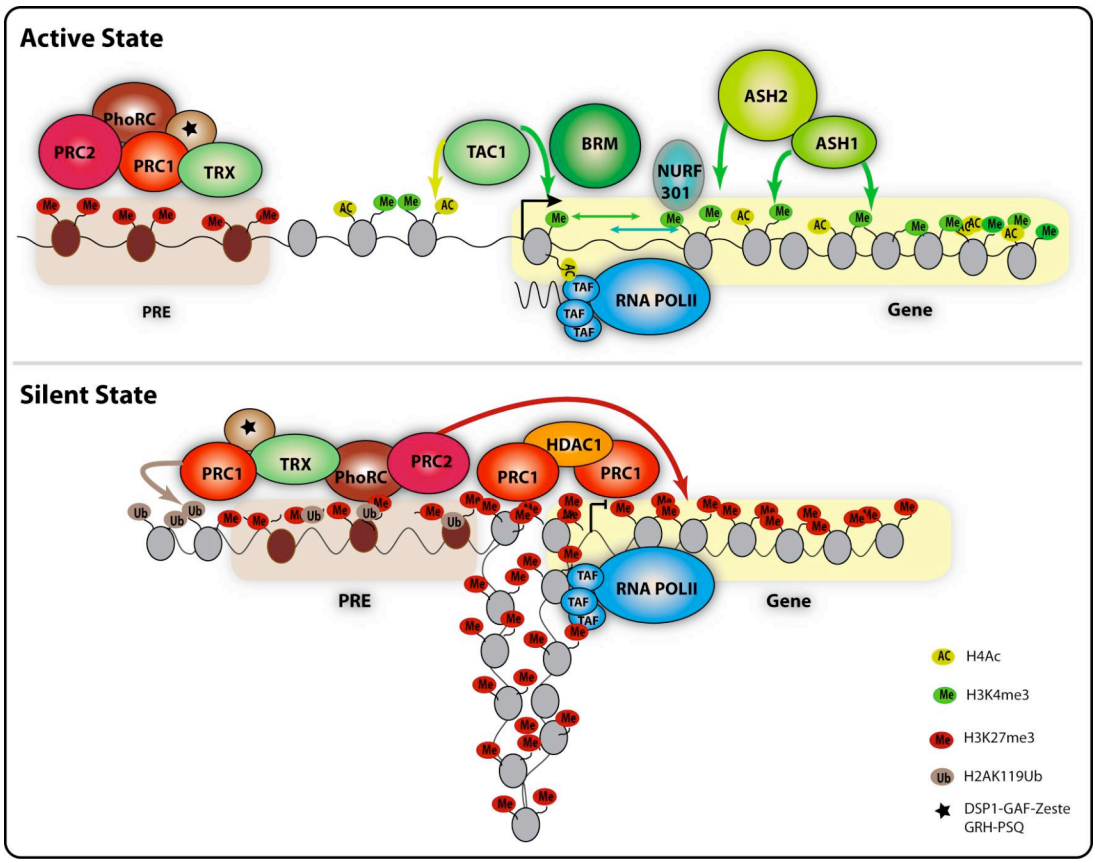

Figure 1.5. PcG and TrxG mechanisms. PcG and TrxG act in protein complexes, which contain enzymatic activities involved in silencing and activation. In the active state, PcG and TrxG are both found at the PRE and so is H3K27me3 mark. TrxG complexes are also found on the promoter and at distinct peaks in the gene. H3K4me3 active mark is spread and nucleosome remodelling complexes facilitate transcription. In the silent state TrxG proteins are still found at PRE. The repressive H3K27me3 mark imposed by PRC2 spreads in the gene body. Looping of chromatin by PRC1 might facilitate spreading. RNA PolII is found at promoter but there is no transcription elongation. Other repressive marks like H2AK119Ub are also found at the chromatin.

If the role of H3K27me3 is not the recruitment of PRC1 then the function of the chromodomain of Pc could be to facilitate transient, long range interactions between the PRE and their target promoters, mediated by the looping of PREs (Wang et al. 2004a; Comet et al. 2006; Muller and Kassis 2006; Lanzuolo et al. 2007; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). This kind of interaction could be responsible for the wide spreading of H3K27me3 mark. Consistent with this, Pc it self is usually found to be spread over wider regions than other PRC1 and PRC2 components (Papp and Muller 2006; Beisel et al. 2007), this could be due to transient interactions during PRE looping that are "fixed" during ChIP experiments. In addition, PRE looping could allow PcG

proteins bound at the PRE to establish specific contacts with promoter-bound components of the transcription machinery (Figure 1.5).

Finally, the PhoRC complex could also be involved in PRE looping since the dSFMBT protein has binding preference for mono and dimethylated H3K9 and H4K20. This complex could sense the surrounding chromatin for this marks and then help bring the chromatin into contact with the HMT of the PRC2 (Klymenko et al. 2006). Such a function could also be important during DNA replication, where newly incorporated nucleosomes must be modified accordingly to maintain the silent chromatin environment

Histone deacetylation by HDACs (Kouzarides 1999) associated to PcG complexes could also contribute to erase the activation mark imposed by the TrxG proteins on the target genes. In an opposite way, histone demethylases could contribute to erase the silencing mark. Recently, the first histone demethylating activity was described (Shi et al. 2004) and since then, more demethylases have been characterized. In the last year the activity of some of these enzymes has been linked to PcG silencing (Sanchez et al. 2007; Swigut and Wysocka 2007) suggesting that they might also be important for the cellular memory.

Noncoding RNAs (ncRNAs) might also be involved in PcG and TrxG regulation, but different studies have produced contrasting results. Early work has shown that ncRNAs produced from the regulatory regions of Hox genes may counteract PcG dependent silencing. Non-coding transcripts in the BX-C of *Drosophila* expressed in patterns that correspond to the domains of activation of homeotic genes were observed (Bender and Fitzgerald 2002; Hogga and Karch 2002; Rank et al. 2002). This non-coding transcription was shown to come from PREs and it was suggested that they could contribute in switching PREs to the active state (Schmitt et al. 2005; Sanchez-Elsner et al. 2006). Furthermore, *bxd* transcripts were found to recruit Ash1 to *Ubx*, inducing transcription of the *Ubx* gene in larval tissues (Sanchez-Elsner et al. 2006). However, these results contrast with recent work that shows that in embryos, *Ubx* is not transcribed in the same cells as *bxd*, and that embryonic *bxd* transcripts may participate in PcG-mediated silencing rather than activation of *Ubx* (Petruk et al. 2006). Examination of this conflicting results by taking developmental timing into account, contributed to clarify some of the contradictions (Lempradl and Ringrose 2008). In summary, ncRNAs are likely to play a role in regulating PcG silencing at a subset of the target genes, but

more work is required in order to clarify their function and understand their molecular mechanisms of action.

1.5 Genome-Wide distribution and biological functions of PcG/TrxG proteins

Genome-wide distributions of PcG proteins and histone methylation marks have been mapped recently in mouse and human cells and in *Drosophila* (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006; Negre et al. 2006; Schwartz et al. 2006; Tolhuis et al. 2006). Although the comparison is not straightforward because different cell types and PcG proteins were analyzed, and also different mapping methods were used (ChIP and DamID), these studies clearly indicate important similarities as well as differences between vertebrates and flies. In all species, binding of PcG proteins seems to be highly correlated with H3K27me3, which can be found sometimes in domains that are hundreds of kilobases in size. However, there are remarkable difference in the binding pattern of PcG proteins between vertebrates and flies. Most of mammalian PcG proteins binding sites are located close to proximal gene promoter elements, within 1 kb of transcription start site (Boyer et al. 2006; Lee et al. 2006), while in *Drosophila* the binding sites can be located near the promoter or more frequently, many several of kb away (Negre et al. 2006). In addition, in vertebrates PcG proteins are found to bind restricted peaks at the genes promoters but also, in some genes including the Hox clusters, they spread over larger regions covering the hole transcription unit (Bracken et al. 2006; Lee et al. 2006). In *Drosophila* there is discrepancy on whether these proteins binding patters correspond to sharp peaks at the PREs or to large domains. Differences between studies are very likely explained by the methodology employed in the analysis. Pc is though to mediate long-range interactions via binding to methylate H3 tails. It is probable that such transient interactions are reflected in the broader binding domains detected by the DamID technique (Tolhuis et al. 2006), which are not detected by ChIP assays (Negre et al. 2006; Ringrose 2007; Schwartz and Pirrotta 2007).

As for the target of the Polycomb group proteins, there is only 30 % of overlap in the genes identified in different studies (Ringrose 2007). These differences might reflect differential regulation of PcG target genes in different tissues and developmental times. In spite of these differences, a remarkable similarity is found in all species, PcG proteins preferentially regulate genes encoding transcription factors, including many

homeodomain-containing genes. Many of these TFs are involved in developmental patterning, morphogenesis and organogenesis, supporting the idea than PcG mediated epigenetic mechanisms play a global role to coordinate many pathways necessary for the development of a multicelullar adult organism.

Finally, although most of the PcG targets are found to be silent, there is minority of 10-20% that is transcriptionally active (Bracken et al. 2006; Schwartz et al. 2006; Ringrose 2007). These genes might represent targets that are to become silenced upon appropriate cell signals; additional proteins from the TrxG might be found at the promoter and coding regions of these genes facilitating transcription (Papp and Muller 2006). Curiously, the binding pattern of TrxG proteins has not yet been studied thoroughly on genome wide scale. In Drosophila Hox genes, TRX is found at PREs even when the target gene is silent (Papp and Muller 2006; Beisel et al. 2007). It would be interesting to analyze on a more global scale, how the binding of these proteins relates to PcG and to the methylation patterns.

1.6 Gene regulation by PcG/TrxG, other players come to action

A simple indication that PcG complex composition might vary at different loci comes from studies in the binding pattern of these proteins on *Drosophila* polytene chromosomes. Although PSC, Pc and Ph are core components of the PRC1 complex (Ringrose and Paro 2004), it has been observed that some loci are only bound by PSC protein whereas other loci only by Pc and Ph (Rastelli et al. 1993). Purification of PcG complexes from different sources and different times in development has resulted in the identification of many interacting factors, which are not always associated with the complex and might be related to PcG function only at certain times or tissues. For instance, multiple TBP associated factors (TAFs) have been co-purified with this complex (Breiling et al. 2001; Saurin et al. 2001). However, it is very unlikely that these general transcription machinery factors that are involved in many aspects of gene regulation, are an integral part of PRC1 (Otte and Kwaks 2003). Another example is the DNA binding protein Zeste that was purified as part of PRC1 from *Drosophila* embryos (Saurin et al. 2001); however the reconstituted PRC core complex inhibits chromatin remodelling without the need of Zeste protein, arguing that this protein might not be part of the core complex but might constitute an associated factor needed for directing PcG complexes to certain targets. As Zeste, other DNA binding proteins, such as GAGA, PSQ, DSP1 and GRH that interact, either genetically or in co immunoprecipitation

assays, with PcG/TrxG proteins (Sato and Denell 1985; Poux et al. 2001; Mahmoudi et al. 2003; Huang and Chang 2004; Dejardin et al. 2005; Blastyak et al. 2006) might also be responsible for helping recruiting Polycomb repressive complexes to their targets. In addition to these proteins other, not yet identified, factors might contribute to the dynamics of the binding during developmental processes.

In the case of PRC2, the composition of the purified complex varies according to the developmental stage used for the purification or the purification protocol. Two different PRC2 complexes one of 600kDa and another of 1MDa have been purified from embryos. The 1MDa complex contains in addition to the core components the histone deacethylase RPD3 (Tie et al. 2003). In larvae, a 4MDa complex is found, which contains also a histone deacethylase, the SIR2 protein (Furuyama et al. 2003). However, it is hardly understandable that the ubiquitous HDAC proteins that participate in many transcriptional processes belong to the core PRCs (Otte and Kwaks 2003).

In addition to tissue and developmental differences in purified complex composition, large variation has also been observed in the binding patterns of PcG/TrxG proteins to their targets at different times in development (Negre et al. 2006), suggesting high degree of dynamics in the gene regulation by cellular memory mechanisms. In their work, Negre and collaborators studied the distribution of three PRE associated proteins, Pc, PH and GAF on *Drosophila* chromosomes 2 and X by ChIP on chip comparing embryo, with pupae stages and adults males with females. The comparison showed that while many Pc and PH binding sites are maintained throughout development, many present major variations. Some new binding sites appear only at late stages, while some of them, present in embryos, disappear during late development. In the case of GAF, the developmental profiles vary little from one stage to the next, indicating different behavior of different proteins involved in the epigenetic regulation. Furthermore, in mammals the expression levels of PcG/TrxG proteins vary from one cell line to another. These differences between cell types indicate existing differences among the tissues from where these cells originate. More evidence supporting this idea comes from recent reports of genomic scale profiling of PcG targets performed on different cell types (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006), where only limited agreement on the mapped target sites is found. Moreover, Human tissues stained for different PcG proteins, showed that within a specific organ, the composition of PcG complexes differs extensively in different cell types (Gunster et al. 2001). Remarkably, in various

developmental stages of the fetal kidney PcG members were found differentially expressed with some components of the PRC1 core complex missing at some points in development. In *Drosophila* some core components the repressive complexes are also expressed in different levels at different stages (Paro and Zink 1993; Hodgson et al. 1997; Furuyama et al. 2003).

Altogether, sufficient evidence has accumulated to support the idea that the complex composition changes at different cell types and developmental times (Otte and Kwaks 2003). In this respect, it is highly probable that different interacting factors, and proteins modifications fine-tune the cellular memory mechanisms at the level of individual loci.

1.7 Aims of the thesis

In *Drosophila*, three distinct PcG complexes and four TrxG complexes have been identified (for review see Ringrose and Paro 2004; Grimaud et al. 2006; Schwartz and Pirrotta 2007). However, many more proteins have been recognized that somehow interact with PcG and TrxG complexes, but for which it remains unclear how they fit into the picture. In addition, there is considerable discrepancy between different studies regarding the proteins found associated to the different complexes. One reason for this, is that the biochemical isolations of the complexes were performed using whole *Drosophila* embryos, or cells. Thus, the isolated complexes represent an average of all complexes at all target genes, and any tissue-gene-specific differences are lost in the purification procedure. So far, little has been done to establish procedures for the differential isolation and characterization of tissue specific and developmental time specific complexes. Furthermore, the biochemical purifications of nuclear complexes do no distinguish between complexes found at the chromatin and those that might have a function somewhere else in the nucleus (Chen et al. 2005).

In addition to this, there is ample evidence from *in vivo* studies that the composition of the PcG is different at different target genes and in different tissues (Zink and Paro 1989; Rastelli et al. 1993; Strutt and Paro 1997; Otte and Kwaks 2003; Negre et al. 2006). However, despite many insights from ChIP and correlative studies, we still do not understand even for a single locus, how activation and silencing works, nor how it is passed from one generation to the next. A prerequisite for this is to characterize the complexes and chromatin modifications at a single locus. Such an approach is vital to understand the molecular mechanisms of epigenetic regulation.

The aim of this thesis was to develop a transgenic system for the un-biased purification of PcG/TrxG complexes bound to a specific locus, in tissue and developmental time specific manner. This thesis describes the fundaments of system design and the development and characterization of the system.

In the first part, the mechanism of the system is illustrated. The second part of this work describes the generation of the transgenic system, production of transgenic flies, characterization of the expression of the different transgenic proteins and generation of proper control lines for protein purification. In the third part, the performance of the system is characterized by chromatin and protein purifications and the fourth part is dedicated to test the system efficiency at different steps involved in the experimental procedure, which influence the system performance. Finally, the last sections are dedicated to the optimization of the different steps and testing of the system performance after troubleshooting.

Results

2. Results

Polycomb (PcG) and Trithorax (TrxG) group of proteins take part in epigenetic cellular memory mechanisms that ensure that their target genes maintain their activation or silent state over many cell divisions. The Polycomb group response elements (PREs) are the regulatory elements through which the PcG and TrxG proteins exert their function. The silencing/activating complexes are recruited to their target genes via binding to their PREs.

Many developmentally important genes, which are expressed differentially in different tissues and at particular times in development, have been found to be PcG and TrxG regulated; but until now only few distinct PcG complexes have been biochemically purified. However there is ample evidence that the composition of the PcG and TrxG complexes varies from gene to gene and in different tissues (Breiling et al. 2007). This raises the question of whether a particular PRE might be regulated by alternative complexes at different situations or if a particular complex might regulate different groups of PREs. In this scenario there might be also different, yet unknown factors that determine which complex is to be found where and when.

A direct way to assess these questions is to generate a system that allows examination of the protein complex composition in a locus specific manner. Avoiding the "mixing" of the complexes produced by classical biochemical purification of soluble complexes from a tissue or a mix of tissues.

2.1 A transgenic reporter system to study the PcG and TrxG composition at different PREs

A system composed of four transgenic elements was design to purify proteins (PcG and TrxG) that bind to a specific transgenic PRE by means of a protein bait that binds in the vicinity of the PRE. The four-element system was composed of:

-A PRE element and the Tet Operator (TetO) sequence. The TetO constitutes a specific binding site for the TetR protein, which constitutes the systems bait. Both elements, TetO and PRE, are placed nearby in the same transgenic construct.

-A bait protein, the TetR, which carries a biotinylation tag (Biotag) recognized by the *E. coli* BirA ligase.

-The BirA ligase, enzyme that specifically biotinylates the Biotag.

-A Gal4 Driver that induces the expression of the transgenic proteins, TetR and BirA.

Figure 2.1 shows how the four component system functions. The expression of TetR and BirA proteins is controlled by Gal4 (1), which can be induced with different drivers. In this way, expression of TetR and BirA proteins is restricted to the tissues and time where Gal4 is induced (Fischer et al. 1988; Brand and Perrimon 1993; Rorth 1998). Thousand of drivers have been reported in the literature for expression of Gal4 allowing the expression the transgenic proteins in many different developmental profiles (Duffy 2002). Upon expression, the TetR is biotinylated *in vivo* by the BirA ligase in the cytoplasm of the cell (2). The biotinylated TetR translocates to the nucleus where it binds its target sequence, the TetO site, in the vicinity of the PRE (3). Proteins and DNA are cross-linked *in vivo* (4) and the chromatin purified and sheared in smaller fragments (5). Streptavidin is used to pull-down the chromatin fractions via the interaction with the biotinylated TetR (6), and after reversal of cross-links the chromatin components, DNA and associated proteins, can be analyzed (7).

In this manner, the system is design to analyze PcG, TrxG proteins and factors that might interact with them at the level of a single locus, allowing tissue and time differential analysis and comparison of different loci.

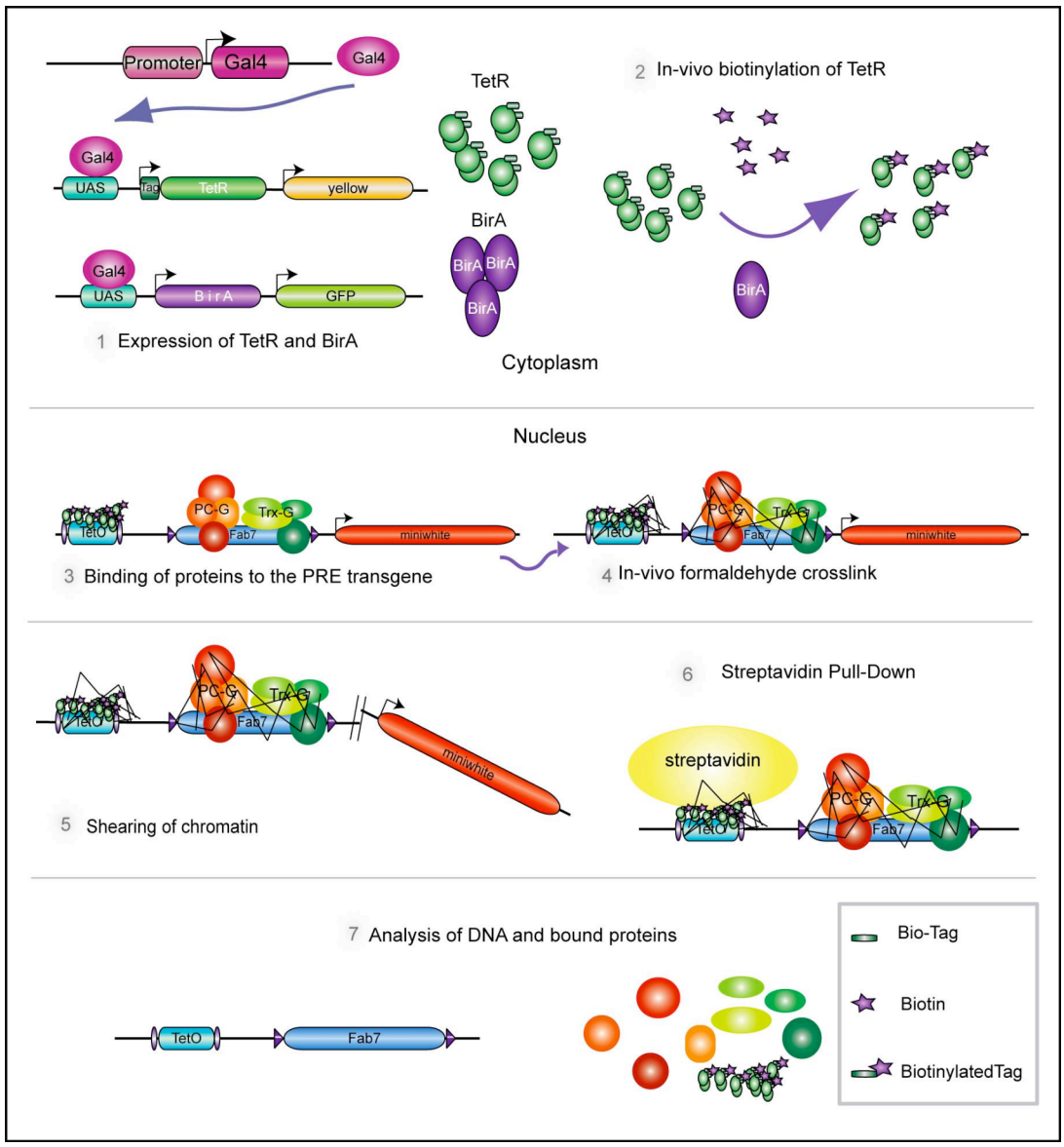

Figure 2.1. Functioning of the transgenic system. (1) TetR and BirA are under control of a UAS region. Transcription of Gal4, controlled by a specific driver, induces the expression of TetR and BirA proteins. **(2)** BirA enzyme biotinylates the BioTag on TetR protein. **(3)** Biotinylated TetR translocates to the nucleus, recognizes and binds its target site, TetO, on the transgene. **(4)** Protein and DNA are covalently cross-linked by *in vivo* formaldehyde treatment. **(5)** Chromatin is extracted and sheared into smaller fragments. **(6)** Biotinylated TetR bound to TetO site is used as bait for Streptavidin pull-down of the transgenic locus. **(7)** After reverting the cross-link protein and DNA are recovered for analysis.

2.2 Establishing the transgenic system

2.2.1 DNA Constructs

A. Fab-7 and vestigial PRE constructs

For the present work two different Polycomb Response Elements were selected. The well-characterized PRE *Frontoabdominal-7 (Fab-7)*, which is part of the homeotic bithorax complex and serves as a role model for PRE function (Zink and Paro 1995; Cavalli and Paro 1998); and a predicted PRE, *vestigial* (Ringrose et al. 2003) subsequently confirmed as a bona fide PRE element in the laboratory of Prof. Renato Paro (Lee et al. 2005).

The PRE constructs were integrated into a pCasper4 vector. This element carries as transformation marker the *miniwhite* gene; this gene is composed of the *white* gene coding region but with a minimal version of the *white* promoter. The activity of the *miniwhite* gene confers the flies the typical red eye phenotype. A feature of a PRE is that when is placed in the vicinity of the reporter gene it can silence its expression (Fauvarque and Dura 1993). In this way when a PRE is placed near to the *miniwhite* gene, in a *white* (-) background, the *miniwhite* gene is silenced and the eyes of the fly turn light orange or *white*. This constitutes a powerful tool that allows the activity of the PRE to be followed phenotypically.

The degree of *miniwhite* expression and PRE mediated silencing is highly influenced by the genomic location of the inserted transgene, for this reason is necessary that in all control lines, the transgene element is placed in the same genomic location. For this, LoxP sites were included flanking the PRE sequence, so that a ΔPRE control line can be generated by crossing this flies to flies expressing the Cre recombinase.

Finally, seven repeats of the TetR binding site, TetO, were included in the construct, to use TetR as bait in the pull-down. FRT sites were included flanking the TetO site in order to *in vivo* excise the region by using FLP recombinase to generate a ΔTetO control line.

Four different versions of the PRE construct were generated for *Fab7* and two for *vestigial* PRE. The TetO site was placed in the generated constructs in different positions, up-stream or downstream, and at different distances of the PRE. The aim was to create from the beginning different constructs in case that binding of TetR to the TetO would interfere greatly with the PRE function. Figure 2.2 shows all generated constructs.

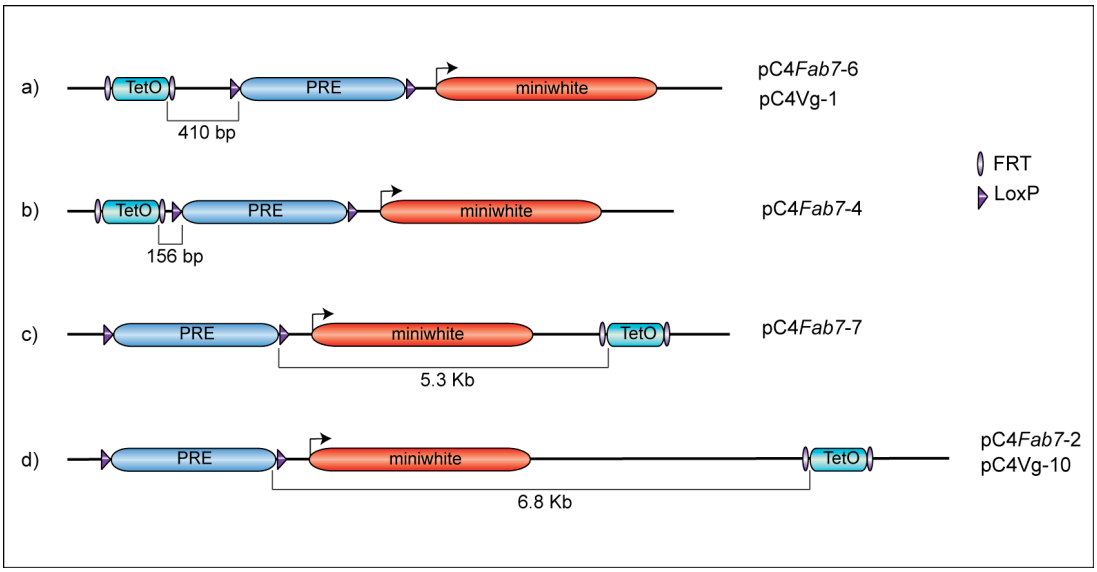

Figure 2.2. Scheme of generated *Fab7* and *vestigial* PRE constructs. Four different constructs were generated differing in the position of the TetO site and the distance to the PRE. In all cases, the TetO is flanked by FRT sites and the PRE by LoxP sites. A *miniwhite* reporter gene was placed immediately downstream of PRE. a) pC4*Fab7*-6 is the construct present in flies used for pull-down experiments.

Many fly lines were generated for *Fab7* as well as for *vestigial* PRE, which differ in the insertion site of the transgene. A small number of fly lines were chosen based on PRE behavior; meaning that they showed at least one of the following characteristics: silencing of *miniwhite*, paring sensitivity or variegated eye phenotype (Fauvarque and Dura 1993; Kassis 1994; Tillib et al. 1999). The *Fab7* construct where the TetO site is 410bp upstream of the PRE was first selected for testing the system.

B. TetR constructs

The coding sequence of TetR protein was amplified by PCR from pUHD142- 1 (kindly provided by Prof. Hermman Bujard, ZMBH) and cloned in the pUASTy vector, which contains the *yellow* gene as a transformation marker. The cloned TetR contains a Nuclear Localization Sequence (NLS) for the transport of the expressed protein into the nucleus. A biotinylation tag, 5'gga gcc gcc ggc gtt cga gcg cca ctc cat ctt ctg gga atc cag gat ctg gcg cag cga gga ggc 3, was added to the 3' or the 5' end of the TetR. This biotinylation tag (BioTag) codes for a 22 aminoacids peptide that is specifically recognized and

biotinylated by the BirA ligase enzyme (de Boer et al. 2003). Figure 2.3 shows a diagram of the resulting vectors.

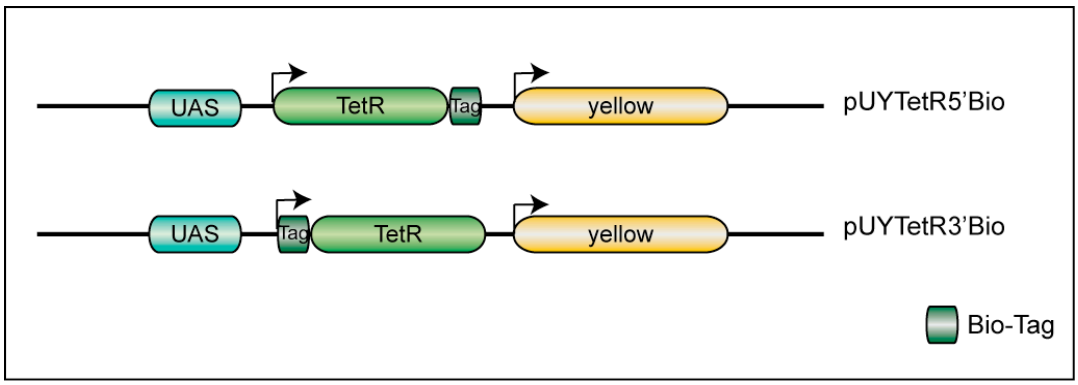

Figure 2.3. Scheme of generated TetR constructs. Two constructs were generated. In both constructs TetR is tagged with a biotinylation tag (BioTag), pUYTetR5'Bio has the tag in the amino-termini and pUYTetR3'Bio in the carboxyl-termini of the protein. pUYTetR5'Bio is the construct present in the flies used for pull-down experiments.

As mentioned above the expression of the TetR protein in this system is inducible and under the control of a Upstream Activating Sequences (UAS) element (Brand and Perrimon 1993). The transcription of the TetR gene depends on the presence of the Gal4 factor and it can then be targeted in a temporal and spatial fashion. This feature is intended to allow the purification of the transgenic locus from a specific tissue or at specific time in development.

C. BirA construct

Flies carrying the BirA construct were previous generated in the Paro laboratory by Christian Popp (de Boer et al. 2003). In this construct the BirA expression is also under the control of the UAS region and thus controlled by binding of Gal4.

2.2.2 *TetR expression and in vivo biotinylation*

A. TetR is expressed and in vivo biotinylated in Drosophila SL2 cells

In order to test the protein expressing constructs before embryo injection and to verify the *in vivo* biotinylation of the TetR protein by the BirA enzyme, *Drosophila* SL2 cells were transfected with the TetR constructs. pUYTetR3'Bio or pUTYTetR5'Bio were transfected alone or in combination with the BirA construct. A Cu^{+2} inducible Gal4 expressing vector was co-transfected to drive the expression of both TetR and BirA

proteins that were under the control of an UAS element. Three days after transfection the $CuSO_4$ was added to the media and 48 h later the cells were harvested. The expression of TetR was analyzed by Western Blot using TetR monoclonal mix antibody (MoBiTec) and the biotinylation was followed using Streptavidin-HRP. As shown in figure 2.4 TetR protein is expressed and *in vivo* biotinylated in SL2 cells (lanes 3). Transfection with TetR vectors and/or BirA vectors alone without Gal4 showed no leaky expression of these constructs (lanes 2 and 5 N-Tagged and 4 and 5 C-Tagged). The expression of TetR protein in the TetR3'BioTag transfected cells (lane 2 in C-Tagged) was very low compared to the TetR3'BioTag / BirA (lane 3) transfected cells probably due to a low transfection efficiency in this well. TetR3'BioTag expressing cells showed the expected 28KDa band and in addition a smaller specific band that was also biotinylated. The TetR5'BioTag construct generated only the expected 28KDa band.

Expression of BirA alone did not produce a detectable increase in the amount of endogenous biotinylated proteins.

This result demonstrate that TetR protein is expressed in SL2 cells and that it is specifically *in vivo* biotinylated by BirA ligase in *Drosophila* cells. Nor the overexpression of TetR neither of BirA have a toxic effect on the cells.

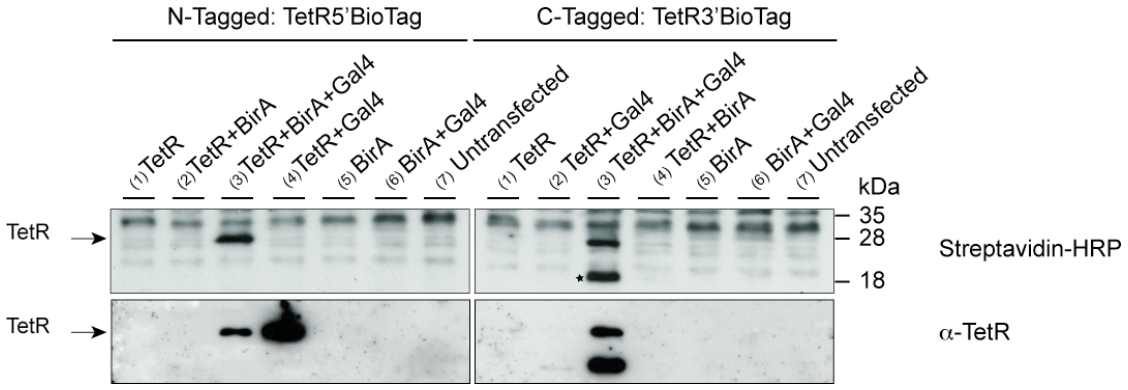

Figure 2.4. *In vivo* biotinylation of TetR in SL2 cells. Total lysates from cells co-transfected with TetR, BirA and inducible Gal4 plasmids analyzed by SDS-PAGE and Western Blot with α-TetR, to detect TetR expression and Streptavidin-HRP to detect biotinylation. N- and C- tagged constructs are compared. TetR protein is indicated by an arrow. (*)Smaller specific band detected in the carboxyl-tagged protein.

B. TetR is expressed and in vivo biotinylated in flies

The tested constructs, pUYTetR3'Bio or pUTYTetR5'Bio, showed efficient expression and biotinylation of TetR in SL2 cells so they were next injected in *Drosophila yw* embryos. The resulting TetR transgenic fly lines were detected by their transformation marker, *yellow*, and crossed to obtain homozygotes. In order to test the expression of the TetR, these flies were crossed to GlassGal4 driver flies, which drives the expression of Gal4 in the eyes of the fly, and the expression of the TetR protein was analyzed directly in fly head extracts by Western Blot. Flies of the *yw* line were used as negative control. (Figure 2.5).

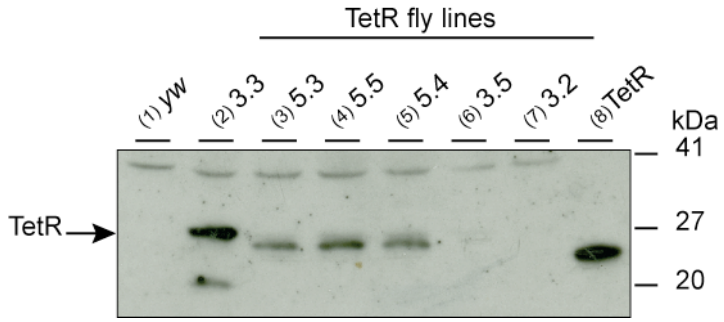

Figure 2.5. TetR is expressed in flies. TetR transgenic fly lines crossed to Glass-Gal4 driver line at 25°C. Protein extract was isolated from 10 fly heads from each cross and subjected to SDS-PAGE and Western Blot. Fly lines 3.3, 3.5 and 3.2 (lanes 2, 6 and 7) constitute different insertion sites of the pUTetR3'Bio construct carrying the Bio-Tag on the carboxyl termini of the protein. Fly lines 5.3, 5.5 and 5.4 (lanes 3,4 and 5) constitute different insertion sites of the pUYTetR5'Bio construct carrying the Bio-Tag on the amino termini of TetR. Flies from *yw* line were used as negative control (lane 1). TetR (lane 8) is 10ng of recombinant TetR protein (MoBiTec).

Selected positive TetR expressing lines 5.4 and 3.3 were recombined to BirA lines and crossed to homozygotes. The generated lines, 5.4 (2) and 5.4 (3) that originated from 5.4 and 3.3(3) that originated from 3.3, carrying both TetR and BirA transgenes on the same chromosome were tested for expression of TetR and *in vivo* biotinylation in flies. For this, the TetR-BirA flies were crossed to GlassGal4 driver flies and the expressed proteins were detected from fly heads by Western Blot (Figure 2.6).

Flies carrying the TetR and BirA constructs but not the Gal4 driver were used as negative control. As shown in figure 2.6 the general levels of expression of TetR and biotinylation seemed to be lower in flies than in cells (compare to fig. 2.4). TetR protein expression was observed in all three generated lines (lanes 3, 5 and 7) but biotinylation levels appear to be higher on line 5.4(2) and 3.3(3) (lanes 3 and 7) than in 5.4(2) (lane 5). As observed in SL2 cells, the expression of the carboxyl tagged protein (TetR3') in flies generated a specific smaller band that was also biotinylated as detected by TetR antibody

and streptavidin-HRP (lane 7). Altogether this results show that TetR protein is expressed and in vivo biotinylated in *Drosophila* flies.

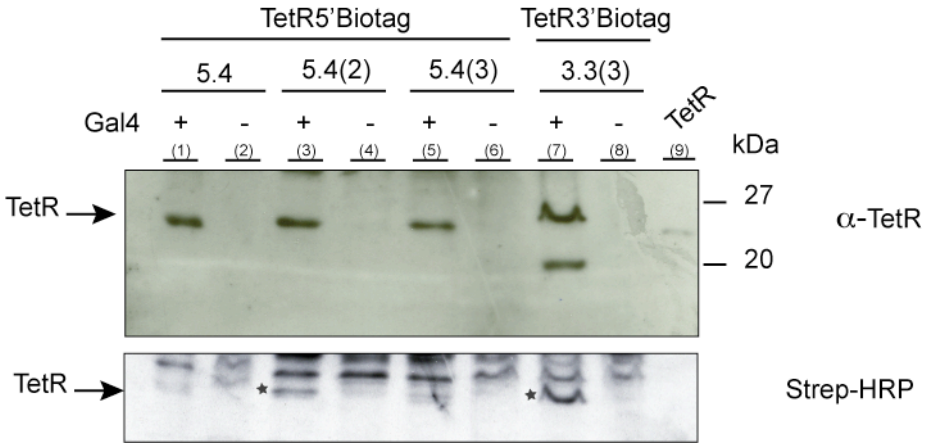

Figure 2.6. TetR is biotinylated in flies. Fly lines expressing TetR (5.4 TetR5'Bio-tag and 3.3 TetR3'Bio-Tag) were recombined to BirA flies. The generated lines, were crossed with a Glass-Gal4 driver line at 25°C. For each cross, protein extract was isolated form 10 fly heads and subjected to SDS-PAGE and Western Blot. The 5.4 line was used as control for (+) TetR expression and (–) Biotinylation (lanes 1 and 2). Recombination of 5.4 TetR5'Bio-Tag with BirA originated 2 lines with different recombination points, 5.4 (2) (lanes 3 and 4) and 5.4 (3) (lanes 5 and 6). Recombination of 3.3 line generated 3.3 (3) line (lanes 7 and 8). Recombinant flies with no Gal4 driver were used as control. 10 ng of TetR recombinant protein (MoBiTec) was loaded as positive control (lane 9).

2.2.3 Mapping of the PRE transgenes

Generation of transgenic flies by P-element mediated germ line transformation of *Drosophila melanogaster* may result in the insertion of several copies of the transgenic construct in the genome. Since the generated PRE lines were to be used for purification of chromatin bound proteins in a specific locus, it was necessary to obtain lines carrying only one transgene copy.

To check the number of inserted elements, the generated PRE lines were subjected to Southern Blot. A *miniwhite* probe was generated by EcoRI digestion of pC4*Fab7*-6 plasmid and purification of the 4.6 kb fragment containing the *miniwhite* gene-coding region. This probe can also hybridize with part of the endogenous *white* gene still present in the *yw* flies used for the injection of the transgenes, giving a background band.

DNA from the different *Fab7* and *vestigial* lines was digested with EcoRI and subjected to Southern Blot. As shown in figure 2.7 the 6.1 *Fab7* line (lane 2) and the 10.7 *vestigial*

line (lane 3) have only one insertion site while the 10.25 *vestigial* line (lane 4) contains 3 insertion sites, indicated by the three additional bands. *Fab7* PRE, line 6.1 and vestigial PRE, line 10.7 were selected from all tested lines (data not showed) for further experiments.

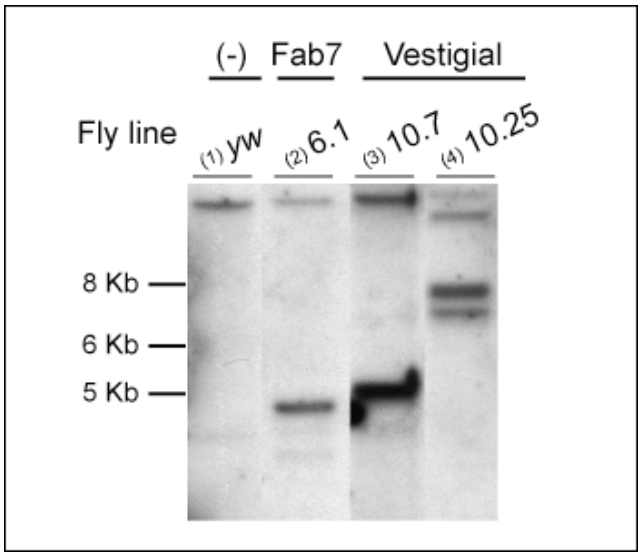

Figure 2.7. Number of insertion sites of PRE lines. Southern Blot of fly lines carrying *Fab7* and *vestigial* PRE to determine the number of transgene insertions. DNA extracted from PRE lines was digested with EcoRI. A probe expanding the *miniwhite* gene was used for hybridization of the Blot, this probe can also recognize the endogenous *white* gene resulting in the presence of a background band (upper band in all lines). *yw* flies, were used as (-) control (lane 1) . 6.1 *Fab7* line and 10.7 and 10.25 *vg* lines were analyzed (lanes 2-4). The number of hybridizing bands shows the number of transgene copies inserted in the genome.

The site of the transgene insertion of the 6.1 *Fab7* line, used in system optimization, was further mapped by DNA-FISH on polytene chromosomes of larval salivary glands. For this, a 2.5 kb DNA probe that hybridizes with the *Fab7* PRE region was generated. Figure 2.8 shows that the pC4*Fab7*-6 transgene was located to chromosome 2L, more specifically at position 25F.

With this, one *Fab7* and one *vg* PRE lines, 6.1 and 10.7 respectably, carrying a single copy of the transgene in the genome were selected from all obtained transgenic lines, for further crosses.

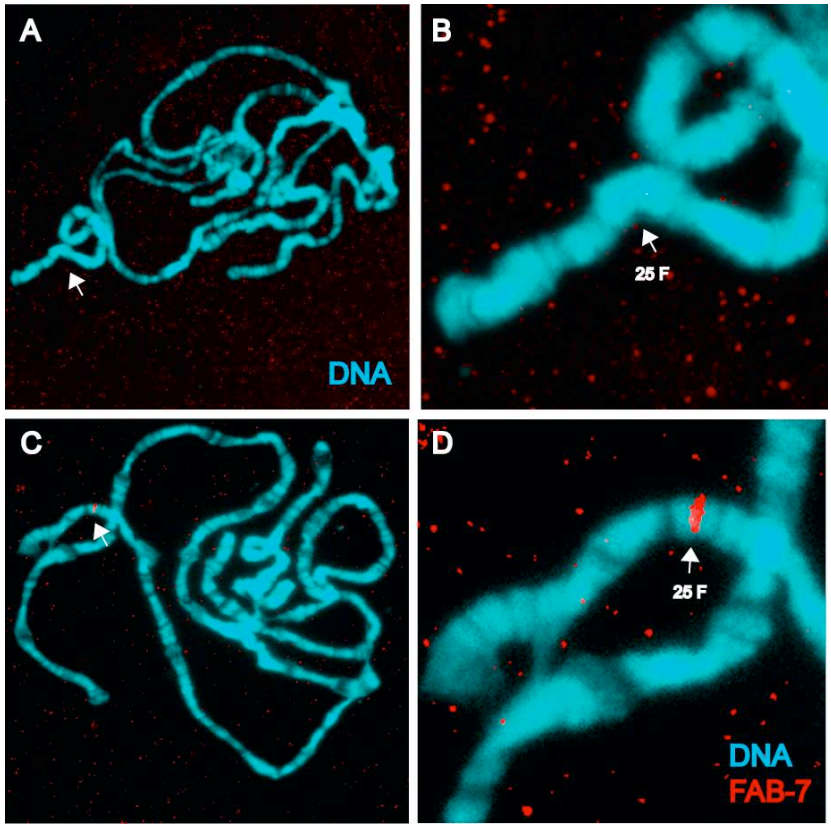

Figure 2.8. Mapping of pC4*Fab7*-6 transgene insertion site on polytene chromosomes. DNA FISH using a probe specifically hybridizing with the Fab-7 fragment resulted in a distinct band on chromosome arm 2L (C) absent in the *yw* control (A). Insertion site of pC4*Fab7*-6 mapped to position 25F (B and D). DNA: blue; Fab-7 DNA FISH: red.

2.2.4 *Generating the correct controls for the 4 elements system*

As mentioned above, a PRE silencing capability is influenced by the genomic location where the transgene is inserted. If the PRE lands in heterochromatin the silencing of the reporter gene is greater than if it lands close to a highly transcribed region. This effect is probably caused by factors already present in the chromatin that influence the silencing by the PRE by activating or repressing transcription and may also be affected by other PREs nearby (DeVido et al. 2008). The aim of this work was to purify protein complexes and associated factors bound to chromatin whose presence depends on the presence of a PRE. For this it was absolutely necessary to design a strategy to determine which are the background proteins that are bound in the locus were the transgene landed regardless of the presence of the PRE. At the time of design of these experiments, the simplest way to

generate such a proper control for a ΔPRE line was to excise the PRE from the locus *in vivo* once the transgene was inserted in the genome.

The PRE removed from the transgene *in vivo* by Cre recombination as described in 5.5.7. For this, transgenic males were crossed to virgins expressing the Cre recombinase; males hatching in the first generation, bearing the transgene (P (*w*+)) but not the *CyO* marked *Cre* recombinase were selected and crossed to a balancer line.

Males hatching in the second generation back-crossed to the balancer flies in single pair mating (fig. 5.3). In the third generation, successful PRE excision was confirmed by single fly PCR (figure 2.9 B). For the PCR analysis three primers were used; as showed for the control line 6.1 if the PRE was still present, no recombination, the primer pair 1-3 generated a PCR band of 660bp. If the recombination was successful the primer pair 1-2 generated then a PCR band of 410bp. The efficiency obtained for Cre recombination was very high, over 90%, as indicated by the proportion of PRE excised lines vs. total analyzed lines. Line 6.1 (14) was chosen for further crosses.

Excision of the PRE by Cre recombinase activity reverted the silencing of the reporter gene. As expected, the generated flies showed a darker eye phenotype (figure 2.10) resulted from de-repression of the *miniwhite* gene.

To control for background proteins during the purification procedure a ΔTetO line was generated by FLP recombination (see 5.5.6 and fig. 5.2). Because in this line the TetR binding sites, TetO, are missing, no TetR binding is expected, therefore all proteins purified from this line should correspond to unspecific proteins that bind to the beads, e.g. endogenous biotinylated proteins.

FLP recombination was not as efficient as Cre, resulting in about 40% positive lines. As showed in figure 2.9 (C), PCR with primer pair 4-5 resulted in a 415bp band in the lines where the excision was positive and, because PCR through TetO is not efficient under the conditions set for the experiment, no amplification band was obtained in the control 6.1 and in the FLP negative lines. The line 6.1(55) was chosen for further experiments.

TetO excision was confirmed by Southern Blot (figure 2.9 D). For this, genomic DNA was digested with PstI, separated by electrophoresis and hybridized to a probe generated by PCR amplification with CreFw and *Fab7*Rv (Pst1) primers using pC4*Fab7*-6 as template. This probes hybridized partially to the endogenous *Fab7*, present in all lines (see lane 1). In the 6.1 original line containing TetO, the probe hybridized with a 3kb

fragment containing TetO and *Fab7* (lane 2), while in the 6.1(55) ΔTetO line this fragment was smaller, 2.7 kb (lane 3), confirming the TetO excision.

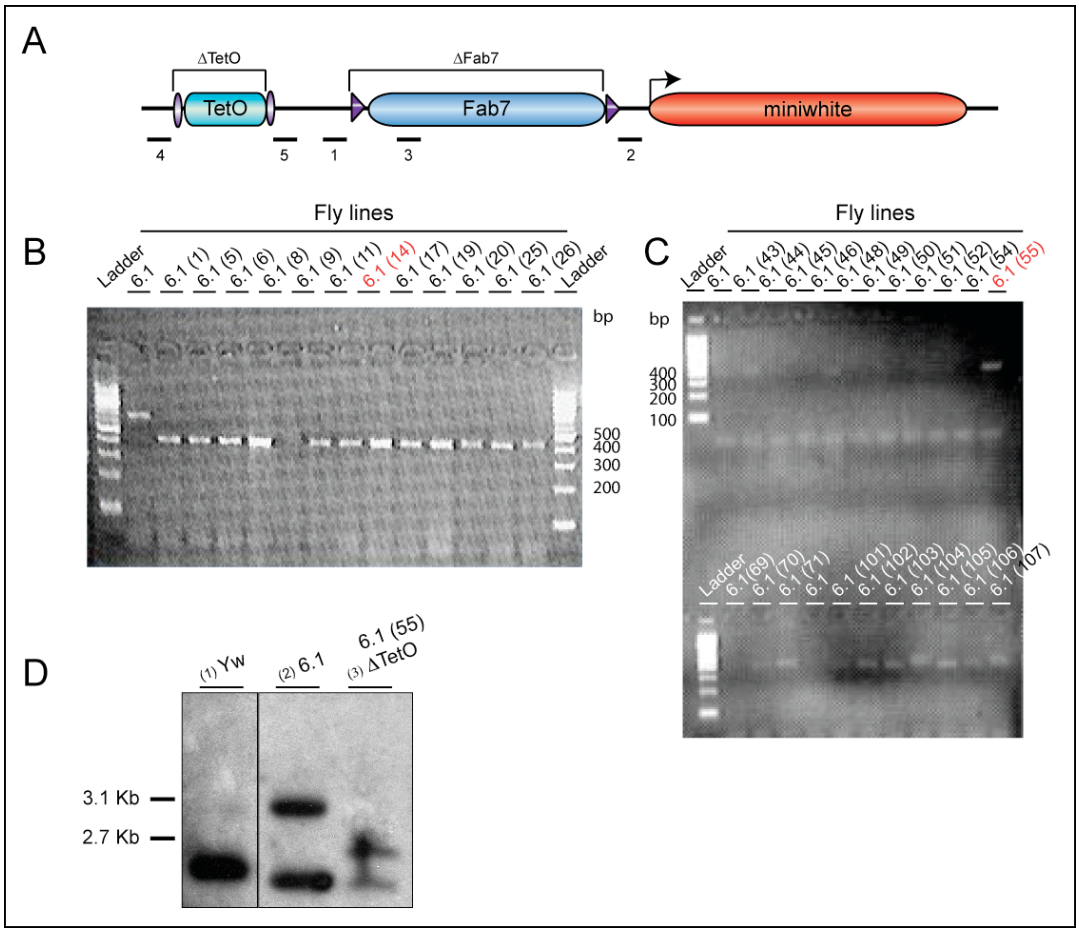

Figure 2.9. Cre-mediated and FLP-mediated excision of *Fab7* and TetO. A) Scheme of the pC4*Fab7*-6 construct with the positions of the primers used in the PCRs indicated by the black lines underneath. **B)** *CRE recombination test:* 6.1 control flies, (-) recombination, yielded the expected 660bp product in the PCR using primers 1 (PreCreTest Fw), 2 (PreCreTest Rv) and 3 (*Fab7*X up). In (+) Cre recombination lines (Δ*Fab7*), the PCR yielded the expected product of 410bp. **C)** *FLP recombination test:* 6.1 control flies, (-) recombination, PCR using primers 3 and 4 (TetOflptest) yielded no amplification product. In (+) FLP recombination flies (ΔTetO) the PCR yielded the expected 415bp product. **D)** Southern blot with a *Fab7* probe to confirm the excision of TetO. *yw* control line showed the endogenous *Fab7* band. 6.1 flies, (-) recombination, showed an additional specific band of 3kb and 6.1(55) flies, (+) recombination, showed a band of 2.7kb, the expected size after TetO excision. In red, lines selected for further experiments.

Elimination of TetO site showed no effect on the expression of the *miniwhite* gene in flies where no TetR protein was expressed (Figure 2.10 A). However, when the TetR construct was brought in the ΔTetO-*Fab7* flies and the protein was expressed, a slightly higher repression of *miniwhite* was observed in the ΔTetO-*Fab7* line than in the TetO-

Fab7 indicating that the presence of the TetR in the chromatin has a mild activating effect (Figure 2.10 B).

The same procedure was followed to obtain the controls lines for *Fab7* 6.25 line and for *vestigial* 10.7 transgenic line (data not shown).

The generated PRE fly lines were crossed to flies carrying TetR and BirA transgenes, and the offspring screened for recombinants in which the three transgenic elements were recombined in the same chromosome. Once the flies were made homozygous they were crossed to DaGal4 flies, to drive the expression of the TetR and BirA proteins (See 7.2). This line was chosen because it drives ubiquitous expression of Gal4 in the *Drosophila* embryo and could constitute a good starting material for system optimization since embryos are relatively easy to collect and protein purification protocols for embryos are already available. By crossing to DaGal4 the following lines were generated:

-6.1 *Fab7*-TB-DaGal, 6.1.14.3Δ*Fab7*-TB-DaGal, 6.1.55.2ΔTetO-TB- DaGal

-6.25 *Fab7*-TB-DaGal, 6.25Δ*Fab7*-TB-DaGal, 6.25ΔTetO-TB- DaGal

-10.7 *vg*-TB-DaGal, 10.7 Δ*vg*-TB-DaGal, 10.7 ΔTetO-TB- DaGal

Generation of these fly lines took several fly generations, in the mean time, for some experiments the FBT line was used. This line was generated through a simple cross between *Fab7* 6.1 and TetR-BirA 5.4 lines (3) and it is heterozygous for all elements (*yw*; TetO-*Fab7*/ TetRBirA).

6.1 *Fab7* line and its corresponding control lines were selected for the optimization of the system. Genomic DNA from these lines was used as template for PCR with different pairs of primers to confirm the presence of the 4 transgenes. Figure 2.11 shows the obtained results for the PCRs in different fly lines. The expected results are described in table 2.1

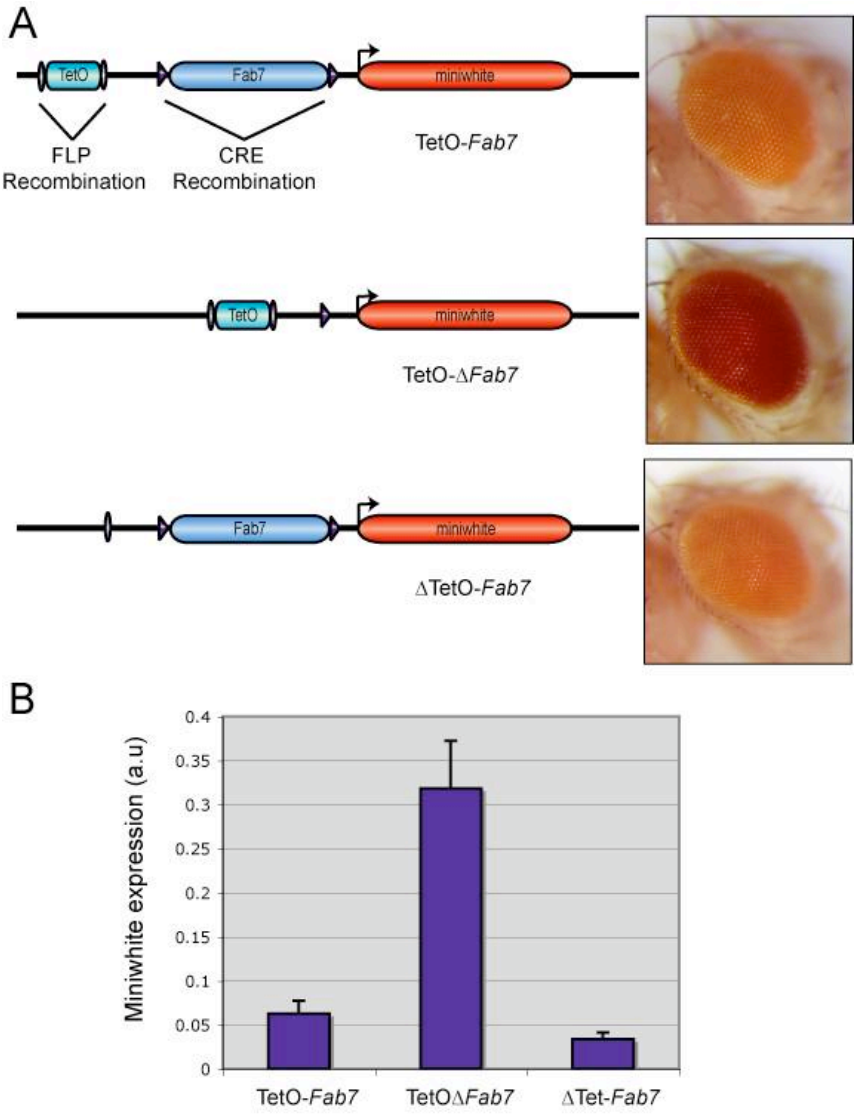

Figure 2.10. Generation of Δ*Fab7* and ΔTetO controls. A) Left: Scheme of the pC4*Fab7*-6 transgenic construct, and the resulting constructs after Cre or FLP recombination. Right: Eye colour before and after excision of *Fab7* or TetO in fly lines that do not express TetR. **B)** *miniwhite* expression levels determined by photometric pigment measurements in fly lines recombined to TetR/BirA constructs and expressing Gal4. In these lines the TetR protein is expressed and can bind to the TetO site in the chromatin.

Altogether, control lines for the purifications were generated by excision of TetO and PRE sites by *in vivo* recombination. The generated lines were recombined to fly lines carrying the other system components, TetR, BirA and Gal4, generating fly lines that contain all four components of the system.

heterozygous line crossed to DaGal4 flies to drive the expression of proteins in the embryos.

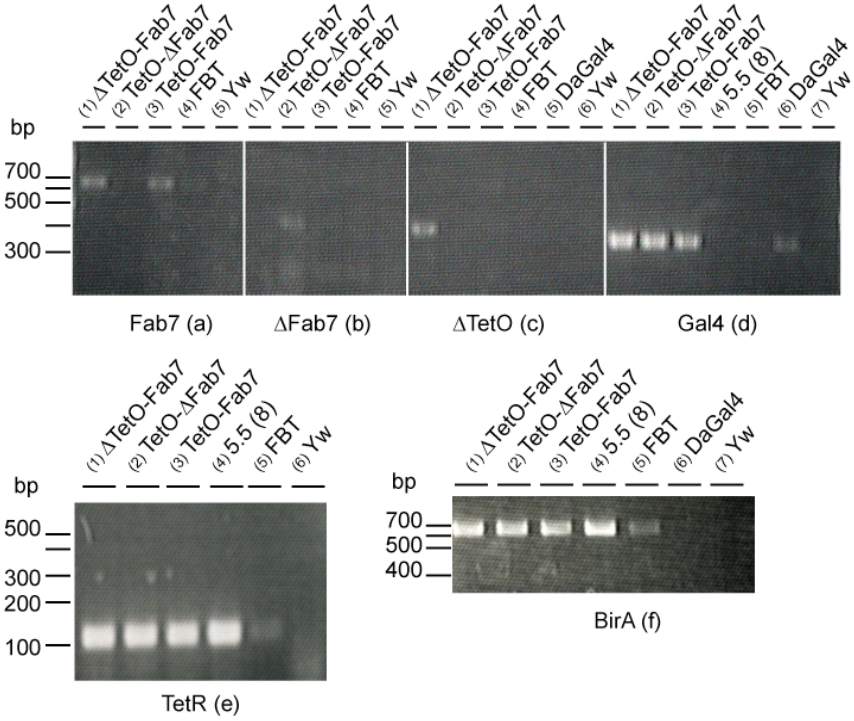

Figure 2.11. Fly lines containing all four system elements. PCR to confirm the presence of all the proper constructs in the final fly lines. **a)** PCR with primers PreCreTest Fw and *Fab7*X up yielded the expected 660bp product only in PRE + lines (lanes 1 and 3). FBT (+) control line showed weaker band as expected for a heterozygous line (lane 4). **b)** PCR with primers PreCreTest yielded the expected 410bp band for the only in the Δ*Fab7* line (lane 2). **c)** PCR with primers TetOflptest yielded the expected 420bp product in the ΔTetO line (lane 1) while the others showed no product. **d-f)** PCR with Gal4-1, TetR5' and BirA primers yielded the expected 340bp, 130bp and 700bp products in all generated lines (lanes 1-3). 5.5(8)TetR-BirA, the heterozygous FBT and DaGal4 flies were used as (+) controls and *yw* flies as (-) controls.

Element tested / Fly line	*Fab7* (a)	Δ*Fab7* (b)	ΔTetO (c)	Gal4 (d)	TetR (f)	BirA (g)
ΔTetO-*Fab7* 6.1.55.2	+ (lane 1)	– (lane 1)	+ (lane 1)	+ (lane 1)	+ (lane 1)	+ (lane 1)
TetO-Δ*Fab7* 6.1.14.3	– (lane 2)	+ (lane 2)	– (lane 2)	+ (lane 2)	+ (lane 2)	+ (lane 2)
TetO-*Fab7* 6.1	+ (lane 3)	– (lane 3)	– (lane 3)	+ (lane 3)	+ (lane 3)	+ (lane 3)
FBT	+ (lane 4)	– (lane 4)	– (lane 4)	– (lane 4)	+ (lane 5)	+ (lane 5)
5.5 (8) TetR-BirA				– (lane 5)	+ (lane 4)	– (lane 4)
DaGal4			– (lane 5)	+ (lane 6)		– (lane 6)
yw	– (lane 5)	–	– (lane 6)	– (lane 7)	– (lane 6)	– (lane 7)

Table 2.1. Expected PCR products in generated lines. Generated fly lines containing all four system elements were tested by PCR for the presence of the transgenes. The expected PCR results for each line tested in figure 2.11 are shown.

2.3 Testing the system's performance

2.3.1 Biotinylated TetR is located to the nucleus of fly embryos

Recombination of all transgenes in one line takes several fly generations. During this time, expression and biotinylation of TetR in fly embryos was tested using the FBT

To determine whether the biotinylated TetR was translocated to the nucleus, protein nuclear extracts from the FBT-DaGal4 line were compared with total head lysates of flies carrying TetR and BirA crossed to GlassGal4 (see fig. 2.6). For this 0-12 h embryos from FBT-DaGal4 line were analyzed by SDS-PAGE and Western blot.

Figure 2.12 shows that TetR protein was also expressed under the control of DaGal4 driver in embryos (lane 1, α-TetR blot) and the protein was *in vivo* biotinylated and translocated to the nucleus of the cell (lane 1, Streptavidin-HRP). This result shows that biotinylated TetR is found in the same cellular compartment than the transgene with the TetO binding site, giving the bases for bait dependent pull-down of a chromatin fragment.

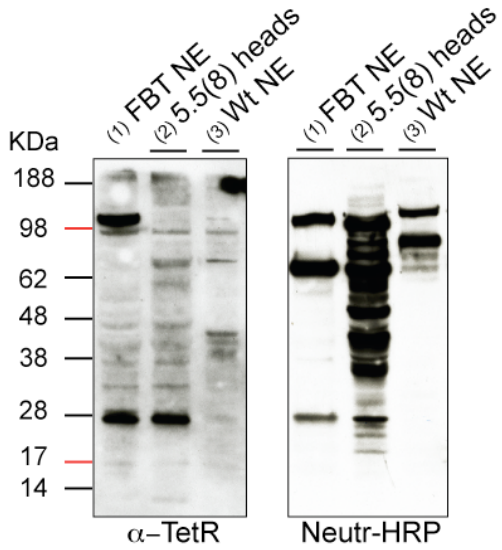

Figure 2.12 Biotinylated TetR is present in the nucleus. FBT fly line was crossed to DaGal4 to drive expression of TetR and BirA in embryos. Nuclear extract was prepared form 0-12 h embryos and the presence of biotinylated TetR was analyzed (lane 1). For (+) control fly heads extract from 5.5(8) (TetR-BirA x GlassGal4) was used (lane 2). NE from *wt* embryos was included as (-) control (lane 3).

2.3.2 Efficient Chromatin IP using TetR as bait

ChIP experiments were conducted to determine whether TetR is bound to the TetO and if the PRE locus could be pulled-down using the biotinylated TetR as bait.

The principle of this technique is to precipitate formaldehyde cross-linked and sheared chromatin with antibodies specifically recognizing a protein of interest, in this case

Streptavidin binding to biotinylated protein. Subsequently, the precipitated DNA is purified and quantified.

For this, protein-chromatin nuclear extract (pc-NE) was prepared from formaldehyde-cross-linked embryos from transgenic fly lines carrying the *Fab7* PRE and its corresponding controls; TetO-*Fab7*, TetO-Δ*Fab7* and ΔTetO-*Fab7* (6.1 *Fab7*-TB-DaGal, 6.1.14.3Δ*Fab7*-TB-DaGal, 6.1.55.2ΔTetO-TB- DaGal).

After precipitation of chromatin with streptavidin beads or antibodies, the cross-link was reversed and the DNA recovered and used as template for semi-quantitative PCR. A fragment within the *bxd* locus served as positive control for a Pc regulated locus in embryonic material (Orlando et al. 1997), and fragment within the *g6pdh* gene was used as negative control, both for Pc (Roustan-Espinosa 2005) and Streptavidin binding. Primer pairs PR4 (Chip2Fw-Chip1Rv) and PR5 (Chip3Fw-Chip3 Rv) were used to amplify specifically the transgene (Figure 2.13A). The PCR products were quantified using AIDA software from an agarose gel.

The ChIP experiments confirmed the presence of the TetR at the TetO site. The transgene was enriched in the streptavidin pull-down in those lines where the TetO site is present. In the ΔTetO line there was no enrichment of the transgene, confirming that the pull-down is specific for the locus where the TetR binding site is located (Figure 2.13A).

As expected, Pc was bound to the transgenic PRE in both lines carrying *Fab7* but not in the ΔPRE line, showing that PcG binding is PRE mediated.

The absence of error bars in the graphic is due to the permanent lost of one of the transgenic fly lines so that the experiment as such could not be repeated.

ChIP experiments were repeated with TetO-*Fab7* and ΔTetO-*Fab7* lines and the results are shown in figure 2.13 B. In this case ChIP with anti Trithorax antibody was also included. The results obtained were consistent with those of the first experiments. In case of Trithorax protein, the enrichment at the transgene seems to be less significant than for Polycomb.

The results obtained by ChIP experiments show that pulling of a locus using an external bait is possible indicating that the system is biologically functional.

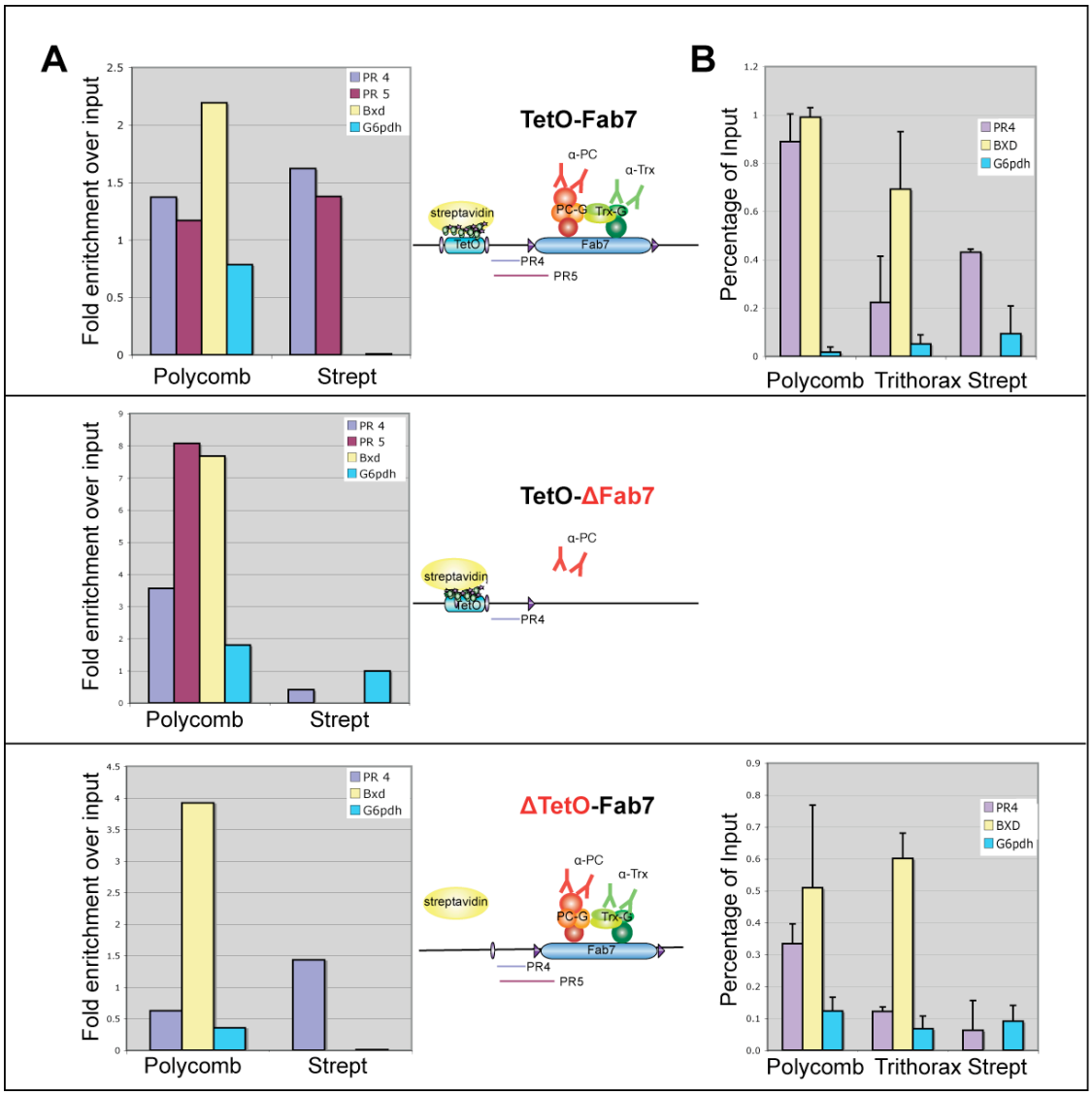

Figure 2.13. Locus specific pull-down using TetR-BioTag as a Bait. ChIP analysis on protein-chromatin nuclear extracts with Stretpavidin, α-Pc and α-Trithorax antibodies. A) Left: quantification of immunoprecipitated material after PCR amplification. Right: scheme of locus with expected binding proteins and position of the PCR amplification products for each of the three fly lines. **B)** Quantification of immunoprecipitated material after PCR amplification in recovered lines. Results are expressed as percentage of input. Enrichment on *BXD* locus was used as (+) control for Pc and Trx ChIP and *g6pdh* gene as (-) control; PR4 amplifies a specific region of the transgene between TetO and *Fab7*, which is present in all 3 fly lines; PR5 amplifies a region that includes *Fab7* PRE, absent in the Δ*Fab7* line. Results are expressed fold enrichment over input (A) when a linker-mediated PCR amplification step was performed and as percentage of input (B) when enrichment was evaluated directly without a pre- amplification step.

2.3.3 Protein pull-down from protein-chromatin nuclear extract

A protocol for protein pull-down of a biotinylated protein and its interacting partners from *Drosophila* nuclear extract has been optimized in the Paro laboratory (Gero Strübbe, unpublished results). This protocol, with some modifications, was followed with the aim of purifying PcG and TrxG proteins bound to the transgenic *Fab7* PRE using biotinylated TetR as bait. For this purpose, 5mg of protein of a protein-chromatin nuclear extract (pc-NE), prepared by sonication of *Drosophila* embryo nuclei (see 5.2.2), was incubated over night with Streptavidin Beads as described in 5.3.9. After several washes the bound proteins were recovered by incubating the samples for 30 min in SDS-sample buffer and analyzed by SDS-PAGE and Western Blot. ΔPRE and ΔTetO lines were used as controls; in these lines TetR enrichment was expected, since the protein is expressed, but no PcG-TrxG proteins should be co-purified since the PRE or the bait binding sites were missing.

Blotting with Neutravidin-HRP showed that TetR protein was nicely enriched by the pull-down, indicating that the pull-down of the biotinylated bait from *Drosophila* pc-NE was possible (figure 2.14, upper panel).

Previous ChIP experiments (figure 2.13) have shown enrichment of Pc in the *Fab7* transgenic locus. To determine whether this protein was co-precipitated with TetR, the membrane was probed with α-Pc antibody (fig. 2.14, middle panel). Pc protein was not enriched in the pull-down. One possibility could be that the generated chromatin fragments were in average not big enough to contain the TetO and the PRE in the same fragment. If this would be the case then histones from nucleosomes should still be present in the same fragments with TetO-TetR if the chromatin is at least 150 bp long. For this reason the membrane was stripped and incubated with α-Histone H3 antibody (fig. 2.24, lower panel). There was also no enrichment of histones in the pulled-down sample.

This experiment shows that the protocol for streptavidin pull-down was functional and TetR protein purification via the biotinylated tag possible. The fact that Pc protein was not co-purified with TetR suggests that the system might be saturated by soluble non-chromatin bound TetR that binds to the streptavidin beads and dilutes out the chromatin-bound fraction decreasing the amount of Pc that can be co-purified.

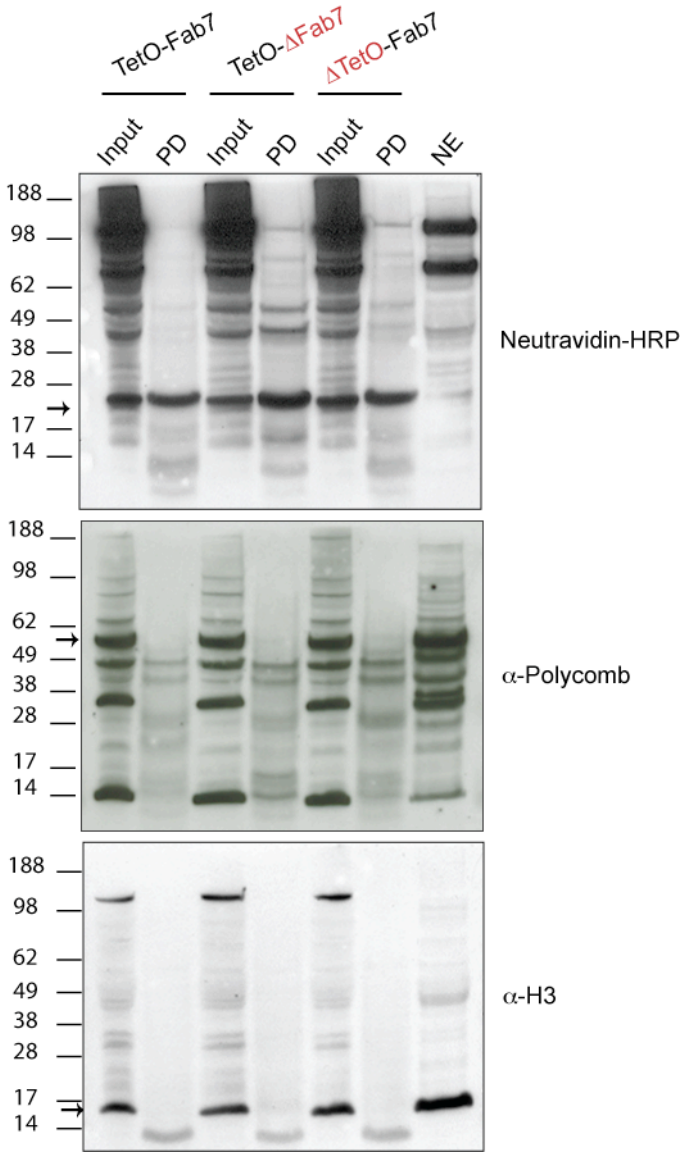

Figure 2.14. Protein pull-down using streptavidin. Cross-linked pc-NE from transgenic lines, TetO-*Fab7*, TetOΔ*Fab7* and ΔTetO-*Fab7*, were used for Streptavidin pull-down. Inputs and pull-down proteins were reversed cross-link by heating at 95°C and analyzed by SDS-PAGE and Western Blot using Streptavidin-HRP, α-Pc and α-H3 antibodies. The expected specific bands are pointed by an arrow.

2.4 Testing the efficiency of each step

2.4.1 Formaldehyde cross-link, is it really reversible? Can the efficiency be improved?

Formaldehyde is a high resolution (2 Å) cross-linking agent capable of producing protein-DNA, protein-RNA, and protein-protein cross-links *in vivo* (Varshavsky et al. 1974; Solomon and Varshavsky 1985). Addition of formaldehyde to living cells results, within minutes, in the formation of cross-linked networks of biopolymers and prevents the redistribution of cellular components. Formaldehyde cross-linking has proved to be a very useful tool to study the distribution of proteins at high resolution over extended

chromosomal regions by ChIP (Orlando et al. 1993). In this kind of approach mild cross-linking conditions are applied and the cross-link is easily reverted by incubation at 65°C and complete digestion of chromatin-bound proteins by Proteinase K allowing the recover of the molecule of interest, the DNA. However, little is known about the efficiency of the reversal of the cross-link when no protease is used and when the bound proteins must be preserved for further analysis. Previous work (Jackson 1978) describes two different protocols that allow selective reversal of either histone-DNA or histone-histone cross-links. Mild conditions are used for protein-DNA complexes that are incubated for 2 days at 37°C in 1% SDS, resulting in recovery of some histones monomers but also higher molecular weight histone dimers. If histones are directly cross-linked to each other, this protocol seems to be inefficient for the complete reversal of protein-protein cross-links. For this, samples are adjusted to 0.5 M 2-mercaptoethanol and heated at 95°C for up to 60 min. In order to determine if similar experimental conditions would be suitable for reversal of formaldehyde cross-link of chromatin preparations from *Drosophila* embryos a reversal of cross-link experiment was performed. For this, *Drosophila* embryos were *in vivo* cross-linked as described in 5.2.1. With the aim of increasing protein cross-link to be able to detect larger differences between cross-linked and uncross-linked samples, different cross-link times were performed (15, 30 and 60 minutes). Subsequently, an aliquot of each sample was reversed cross-link in a similar way than described for ChIP samples, at 65°C for 5 h with 1% SDS, or not reversed (un-treated sample). SDS-sample buffer containing ß-Mercaptoethanol was added to all samples. Of each sample, reversed and no reversed, half was incubated at 95°C previous to loading on an SDS gel and the other half of the sample was loaded directly on the gel with no incubation. In this way for each cross-link time four samples were compared, reversed cross-link (65°C 5h) + 10 min at 95°C, no reversed cross-link + 10 min at 95°C, reversed cross-link (65°C 5h) + no further incubation and no reversed cross-link + no further incubation; this last sample was never exposed to high temperatures. Embryo extracts from non cross-linked embryos were used as control.

Figure 2.15 (A) shows that all samples treated at 95°C showed the same pattern on a coomassie stained gel (see chromatin reversed vs. no reversed samples). On the contrary when samples were not treated at 95°C, differences between samples heated at 65°C and non treated samples were detected (B). In the no reversed samples, all proteins of high

molecular weigh were missing from the gel. Comparison between figures 2.15 A and B indicated that incubation of samples 10 min at 95°C was sufficient to reverse formaldehyde cross-links and retrieve proteins to the soluble fraction. In addition, there were no notorious differences between samples incubated for different times with formaldehyde suggesting that the chosen cross-link conditions were rather mild.

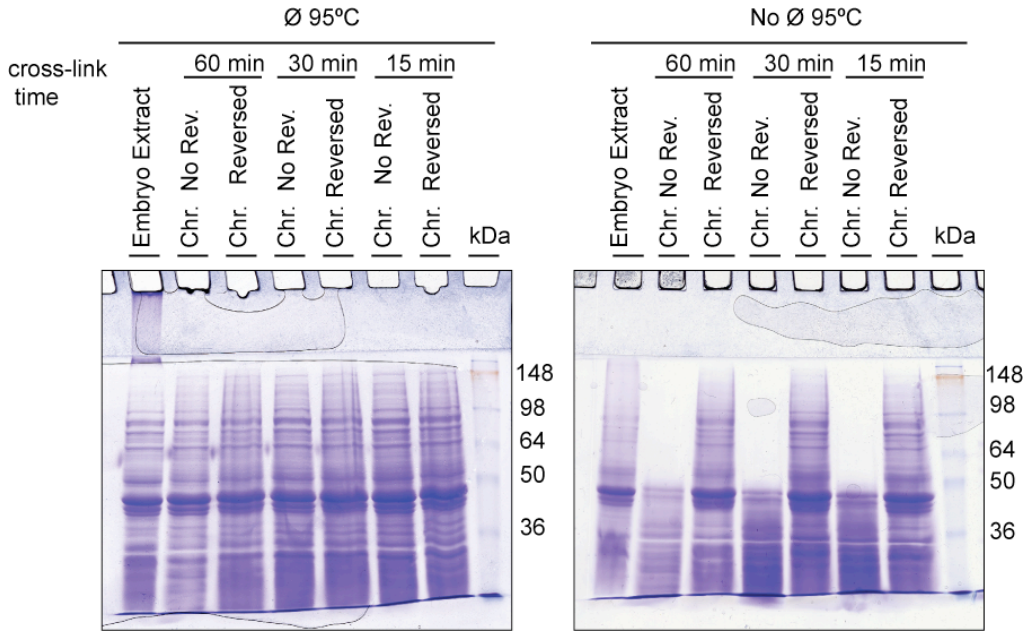

Figure 2.15. Reversal of cross-link by incubation at 95°C. Embryos were cross-linked for 15, 30 or 60 min. Protein-chromatin nuclear extracts were prepared from all samples. Each sample was divided in two and reversal of cross-link was carried out only on one of the samples by incubation at 65°C for 5 h in presence of 1% SDS. Both samples, reversed cross-link (Chr. Reversed) and no reversed cross-link (Chr. No Rev.) were further divided into two samples, and only one was heated 10min at 95°C previous loading on a gel, while the other was loaded directly. For each cross-link time 4 samples were generated, Chr. Reversed: 5 h at 65°C + 10 min at 95°C; Chr. No Rev: 10 min at 95°C (gel A); Chr. Reversed: 5 h at 65°C; Chr. No Rev: no heating (gel B). Total Embryo extract from un-cross-linked samples was used as control. All samples were subjected to SDS-PAGE and Coomassie Blue staining.

To determine whether the TetR protein can be cross-linked with formaldehyde and if this cross-link can be reversed; purified TetR protein (MoBiTec) was incubated with formaldehyde for 30 min at RT. Reversal of cross-link was carried out for 2, 5 or 10 min at 95°C or 5 h at 65°C in presence of SDS-sample buffer. Figure 2.16 shows that TetR protein was efficiently cross-linked into dimer, tetramer and higher molecular weight species. This could be reversed by incubation at 95°C, in 10 minutes the reversal was not

yet completed. Longer incubation time at 65°C resulted in protein loss probably due to precipitation or degradation.

Altogether, these experiments confirm that protein-protein interactions can be stabilized using formaldehyde and that the cross-link can be reversed by incubation in the presence of sample buffer, containing SDS and β-mercaptoethanol, for times longer than 10 minutes.

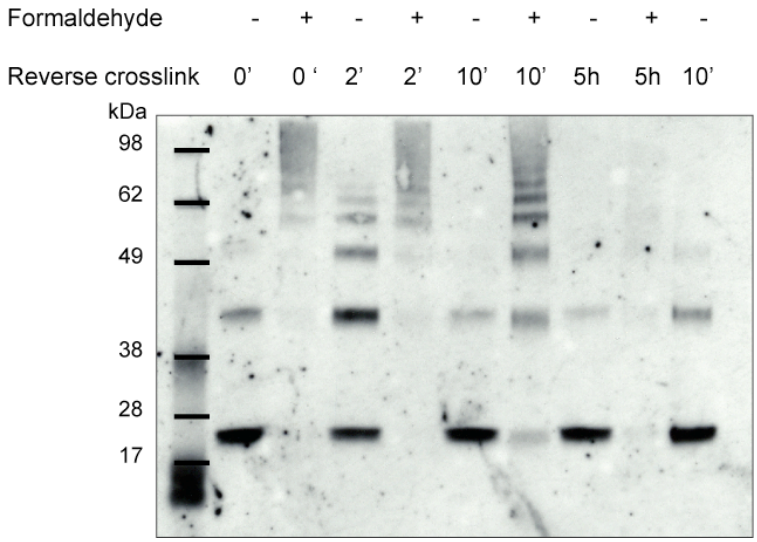

Figure 2.16. TetR is reversibly cross-linked by formaldehyde. 10 ng of TetR recombinant protein was cross-linked with formaldehyde and the reversal of cross-link was carried out for 2, 2, 5 and 10 min by incubation at 95°C in SDS-sample buffer. 10 min incubation at 95°C partially retrieved the cross-linked protein. 5 hour at 65°C incubation resulted in lost of protein due to precipitation.

2.4.2 In vivo biotinylation of TetR: How efficient is it in Drosophila?

It has previously been shown that biotinylation of a transcription factor carrying a small (<23aa) artificial tag by *E. coli* BirA ligase is an efficient process; when the enzyme is expressed in mammalian cells an efficiency close to 100% was reported (de Boer et al. 2003). In mice there are very few naturally biotinylated proteins, thus the background is very low. To determine the amount of biotinylated proteins in Drosophila, whole embryos protein extracts (EE), embryo nuclear extracts (NE) and SL2cells extracts were compared using Streptavidin-HRP to develop Western Blots (Figure 2.17 A). In *Drosophila* the amount of endogenous biotinylated proteins was found to be higher than described for mouse. Most of these proteins seem to be cytoplasmic, since the level of background was reduced in the NE preparation. For this reason purifications were performed using protein-chromatin nuclear extract (pc-NE) instead of whole embryos as starting material (see 5.2.2).

In order to analyze the biotinylation efficiency in *Drosophila*, a streptavidin-shift experiment was performed. For this, pc-NE from the TetO-*Fab7* line was subjected to a

streptavidin pull-down as described in 5.3.9 to enrich for biotinylated TetR. Aliquots of Input, pull-down material and unbound fraction were incubated in sample buffer to reverse the cross-link and cooled down to room temperature. Streptavidin was then added to half of each sample (see 5.3.5) and all samples were loaded on an SDS-polyacrilamide gel and analyzed by Western Blotting.

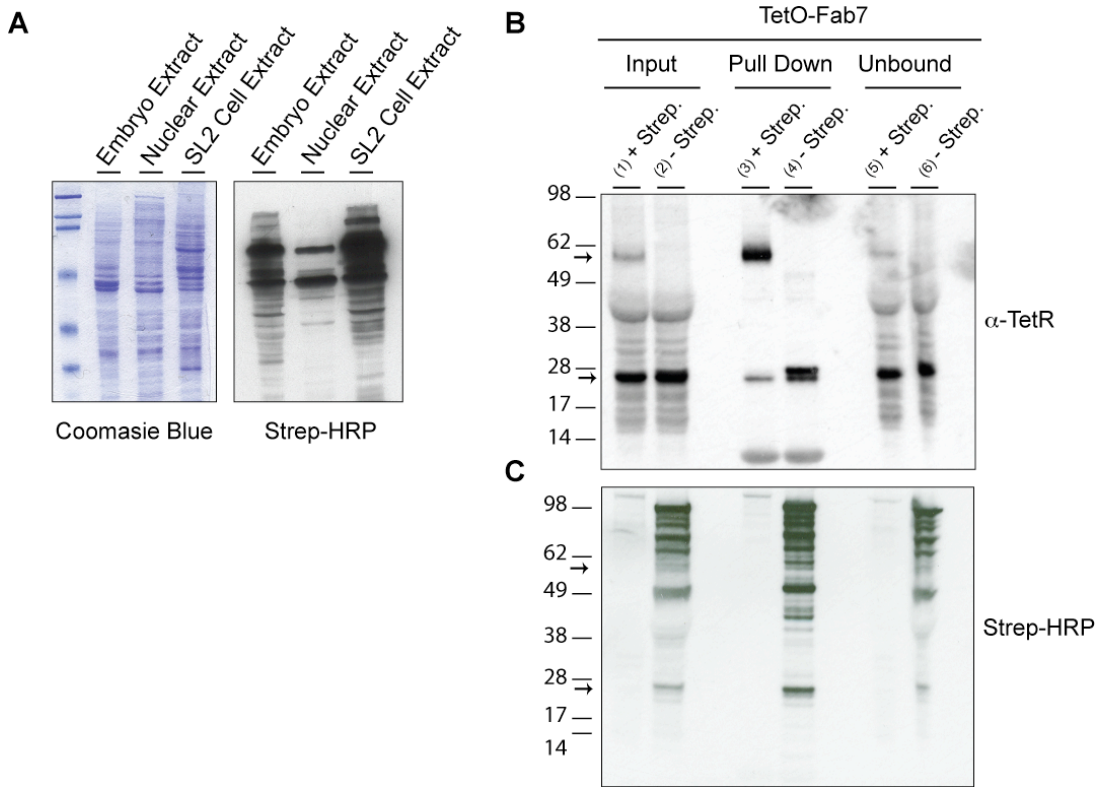

Figure 2.17. Biotinylation in *Drosophila melanogaster*. A) Whole embryo extracts and nuclear extracts from *wt* flies and cell extracts were compared for the amount of endogenous biotinylated protein by coomassie staining and WB with streptavidin-HRP **B and C)** Streptavidin shift experiment with Streptavidin pulled-down material from TetO-*Fab7* line. 8 μg of Streptavidin were added to half of input, Pull-down and unbound samples previous to loading in the gel. The band shifts were detected by Western Blot with α-TetR antibody (B) and Neutravidin-HRP (C).

As shown in figure 2.17 B, lane 2 the TetR protein in the input fraction with no Streptavidin ran as a double band with very small size difference between the two. Addition of streptavidin (lane 1) caused a super shift of the upper band only, indicating that the upper band constituted the biotinylated TetR while the lower one was TetR protein that was not biotinylated. As expected, in the pull-down material most of TetR present in the sample was shifted by addition of streptavidin (lane 3). There was a small

portion of un-biotinylated TetR that also present in the pull-down sample. This un-biotinylated TetR was probably pulled with the streptavidin beads through its interaction with biotinylated TetR molecules due to TetR dimer formation. In the unbound fraction there was still biotinylated TetR protein present (lane 5), indicating that the efficiency of the pull-down was also lower than expected.

Previous experiments had shown that streptavidin shift is very efficient producing the shift of all biotinylated proteins present in the sample. To confirm this, the blot was stripped and incubated with Streptavidin-HRP. In this case, no TetR band or any other biotinylated protein could be detected in the samples where streptavidin was added (fig. 2.17 C, lanes 1, 3 and 5) indicating that all biotinylated proteins had been shifted and that all un-shifted TetR corresponds to the un-biotinylated fraction (fig. 2.17 B, lanes 1, 3 and 5).

Quantification of the bands with Aida software revealed that 23% of Total TetR protein was biotinylated and 9.5% of the biotinylation fraction was precipitated. Of total TetR, biotinylated and non-biotinylated, 2.2% was pulled-down.

2.5 How to improve the efficiency of the system?

All previous experiments performed to test the system revealed that although the system is biologically manageable and in principle the locus specific pull-down via the protein bait viable (see 2.3.2), there are several steps where the system efficiency could be optimized and as a result initial efforts to purify PcG-TrxG proteins bound to the PRE failed (fig. 2.14). Different approaches were implemented to try to optimize these steps and new protocols were included to improve the effectiveness of the purification.

2.5.1 Addition of DSP and DTBP, protein-protein cross-linkers

Cross-link tests have shown no great difference between samples cross-linked for 15 minutes with those of 60 min, (Figure 2.15) this supported the idea that formaldehyde cross-link is relatively mild. Increasing the proportion of cross-linked proteins could be a way to improve the pull-down performance. One method to do is would by including a protein-protein cross-linker to help stabilize protein interactions.

DSP is a water insoluble, homobifunctional N-hydroxysuccimide ester (NHS-ester). It has a spacer arm of 12 A° and its NHS-ester reacts with primary amines to form covalent amide bonds (Carlsoon 1978; Partis 1983). DSP cross-link is reversible under reducing conditions; by simply adding SDS-sample buffer with DTT or ß-Mercapthoethanol to the

sample the proteins can be recovered. It was chosen among many other protein cross-linkers because it has one of the longer spacer arms and is commonly used for mass spec applications.

1.25mM of DSP was added to the cross-link buffer in addition to formaldehyde and these embryos were compared to embryos cross-linked with formaldehyde alone. After cross-link, protein-chromatin nuclear extract was prepared and analyzed by SDS-PAGE. To reverse cross-link the extracts were treated with sample buffer with ß-Mercaptoethanol combined with 30 min at 95°C; and to prevent the reversal and conserve the cross-links, sample buffer with Iodoacetamide was used combined with no heating of the sample. Addition of DSP to the embryos resulted in no obvious improvement of cross-link as detected by Coomassie Blue staining, figure 2.18 (A).

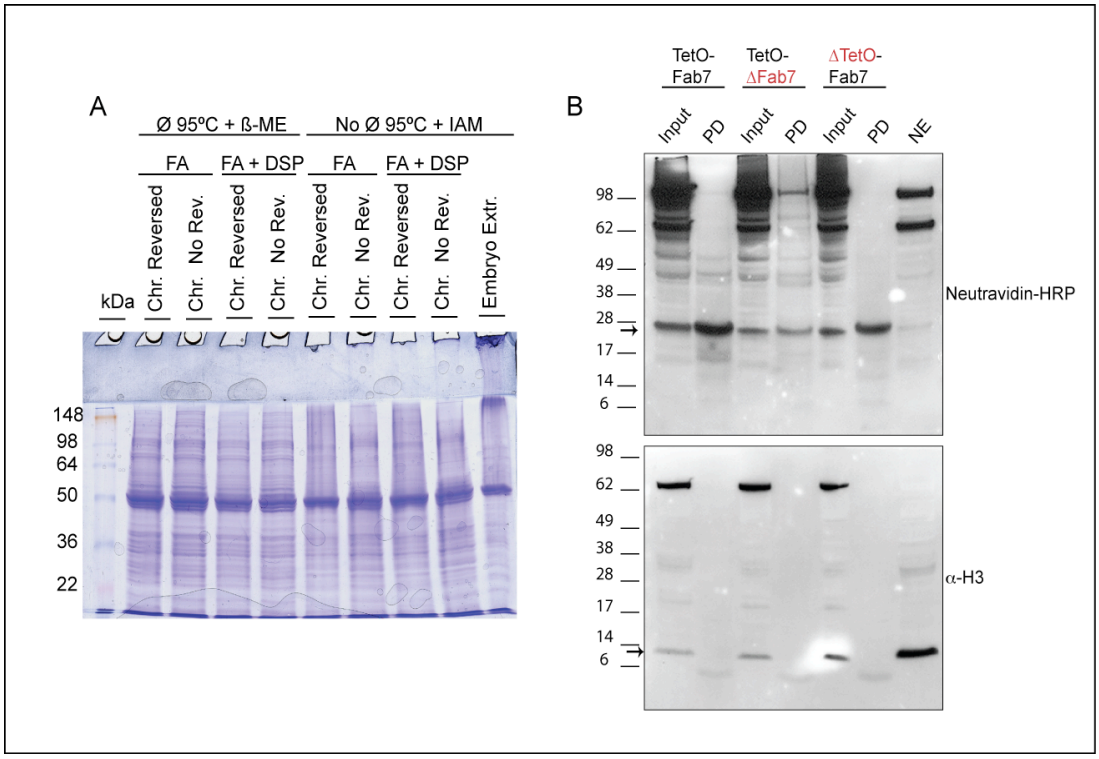

Figure 2.18. DSP and DBTP, protein-protein cross-linkers. A) Coomassie Blue stain of SDS-PAGE of protein-chromatin total extract from embryos cross-linked with formaldehyde alone (FA) or formaldehyde and DSP (FA + DSP). Reversal of cross-link was carried out by addition of ß-Mercaptoethanol sample buffer (ß-ME) and heating at 95°C. For the un-reversed samples, Iodacetamide sample buffer was used and heating was avoided. **B)** Protein pull-down of DTBP treated samples. Nuclei from FA cross-linked embryos were treated with DTBP, shared and the protein-chromatin NE was used in a streptavidin pull-down. The Western Blot was developed with Streptavidin-HRP and α-Histone H3 antibody.

This could be due to lack of diffusion of the cross-linker into the embryos and into the nucleus. For this reason a second experiment was performed in which isolated nuclei from already formaldehyde treated embryos were incubated with a protein cross-linker. For this experiment a second cross-linker was tested; this time water soluble one. DTBP is a membrane permeable, homobifunctional imidoester, also reversible with addition of reducing sample buffer (Hand and Jencks 1962; Mattson et al. 1993). DTBP was added to nuclei at 10 mM concentration and incubated for 2 h at 4°C. After washing, the nuclei were sheared and the protein-chromatin NE was used for Streptavidin Protein pull-down. As shown in figure 2.18 (B), incubation with DTBP did not increase the efficiency of the pull-down, which was equivalent to previous pull-downs using only formaldehyde as cross-linker (figure 2.14).

These experiments show that incubation of embryos or nuclei with protein-protein cross-linkers had no effect in the performance of the system, probably due to poor penetration of the cross-linking reagents in the nuclei of the cell. Another possibility is that formaldehyde efficiency is already at a level that could not be further increased by an additional cross-linker.

2.5.2 Shearing the chromatin at the right size

The chromatin fragment size could have a large influence on the performance of the purification. Since the system relies on the purification of proteins bound to a PRE via a bait, and the connection between the bait and the PRE is the DNA fragment between the two, it is necessary to shear the chromatin in fragments of about 3 kb, which are large enough to contain both TetO and PRE.

To investigate whether the chromatin could be digested with restriction enzymes to generate a chromatin fragment of the correct size, purified nuclei from cross-linked embryos were re-suspended in restriction enzyme and sonicated for three short cycles (10 sec, medium power with a microtip sonifier) to break the nuclei open without shearing the chromatin (see 5.2.5). The protein-chromatin NE was then incubated with NotI and SalI that have restriction sites at the transgene producing a 4.3 kb fragment (figure 2.19).

Previous experiments had shown that incubation of a chromatin sample at 37°C resulted in partial digestion of the sample by endogenous endonuclease (data not shown). For this reason, to determine the extent of the endonuclease activity, the extract was incubated with restriction enzymes (figure 2.19, lane 1) and without restriction enzymes (lanes 2

and 3); in presence of EDTA to inhibit endogenous endonuclease activity (lane 2) or without EDTA (lanes 1 and 3). For negative control an aliquot of extract was kept frozen (lane 4). As shown in figure 2.19, incubation of the extract at 37°C without addition of restriction enzymes resulted in total shearing of the chromatin to fragments of around 1 kb size (compare lanes 3 and 4). Addition of EDTA to the sample inhibited the endonuclease activity to some extent, however shearing of the chromatin was still observed (lane 2). Incubation with restriction endonucleases had no visible effect due to sample shearing by the endogenous nucleases.

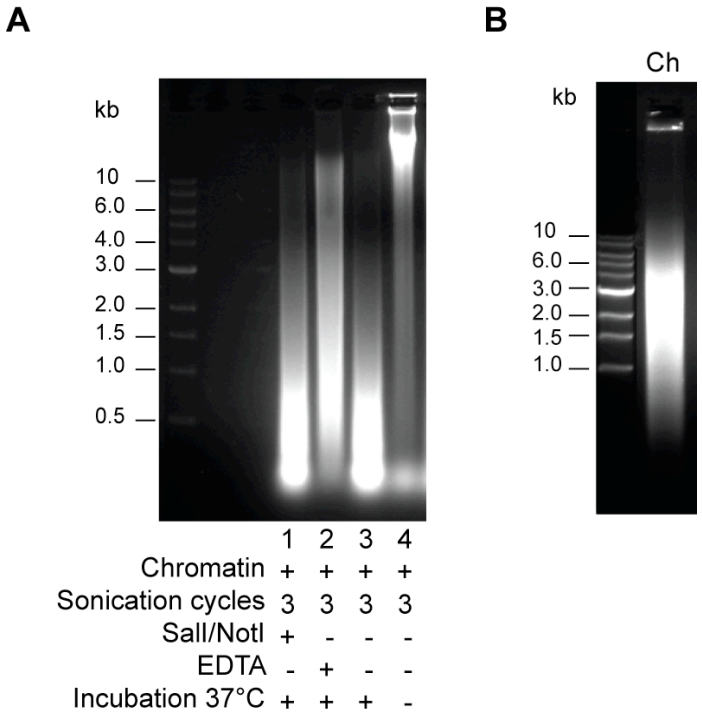

Figure 2.19. Chromatin digestion with Restriction Enzymes. A) Agarose gel of pc-NE incubated in different conditions. Lane 1, SalI-NotI digestion at 37°C; lane 2, incubation at 37°C with EDTA to inhibit Endonuclease activity and no RE; lane 3, incubation at 37°C without EDTA and no RE; lane 4, starting sample used in different treatments, aliquot kept at -20°C to avoid any degradation. **B)** Chromatin obtained after shearing by sonication as described in 5.2.2. After treatments all samples were reversed cross-link and loaded on agarose gel.

Southern blot analysis using an specific probe to detect the fragment containing the transgene revealed a slight enrichment of the desired fragment on the restriction enzyme treated sample (data not shown), indicating that restriction enzymes are active on cross-linked samples and cable of digesting the sample to some degree.

In summary, these experiments indicates that restriction nuclease digest is not feasible due to the presence of endogenous endonucleases that shear the chromatin unespecifically resulting in fragments of small size.
Subsequently, in order to produce a sample enriched for chromatin fragments of around 3 kb size needed for pull-down experiments, a sonication procedure was optimized to obtain the desired fragments. Figure 2.19 B shows an example of a sonicated chromatin sample using the optimized protocol (see 5.2.2 for description of sonication conditions).

2.5.3 In vitro-biotinylation using recombinant BirA ligase

As described in section 2.4.2, *in vivo* biotinylation of TetR in *Drosophila* resulted less efficient than expected. Poor biotinylation might affect the efficiency of the pull-down since transgenic locus carrying the majority of un-biotinylated TetR bound to TetO might fail to bind the streptavidin beads and thus be lost during purification.
In order to improve the biotinylation of TetR, protein-chromatin nuclear extracts from cross-linked *Drosophila* embryos were incubated in the presence of recombinant BirA produced in *E. coli* (kindly provided by Dr. Christian Beisel). The incubation was carried out at 30°C for 1h in the presence of Biotin and ATP. As observed by Western Blot developed with Neutravidin-HRP, incubation of the protein-chromatin NE with BirA resulted in an increase of the degree of biotinylation of proteins (figure 2.20A); this increase was proportional to the amount of biotin used in the assay. The recombinant BirA, self –biotinylated appeared in the Western Blot as a prominent band of approx. 37 kDa. Since this incubation increases also the background, this approach would be useful to improve the amount of biotinylated TetR only in combination with an efficient method to separate chromatin.
A Chromatin IP experiment was performed to test whether pre-incubation with exogenous BirA would increase the efficiency of the system. For this, protein-chromatin NE from the TetO-*Fab7* transgenic line (6.1.4 *Fab7*-TB-DaGal) was incubated for 1h with BirA in the presence of 1 mM biotin as described above. In this case the concentration of BirA added was approximately 20x lower than in the previous experiment to test conditions feasible with large amounts of extracts needed for protein purification. As control an extract was incubated at 30°C for 1h without addition of BirA and biotin. After the incubation the extracts were subjected to CsCl gradient purification to eliminate biotinylated proteins, soluble TetR and added BirA. The obtained chromatin was used for ChIP. Both extracts, (+) BirA and (-) BirA, were used for Streptavidin

ChIP, and a third ChIP with α-TetR antibody on the (-) BirA chromatin and was included to compare efficiency between TetR antibody and streptavidin. The ChIP samples were analyzed by PCR and quantified using Aida software. The transgenic line 6.1.4 (TetO-*Fab7*) used in the experiment contains all the 4 elements of the system so biotinylation also occurs *in vivo*, and the locus should be pulled-down with streptavidin. Primers against the *g6pdh* gene were used as (-) control and primer pairs PR4 (Chip2Fw-Chip1Rv) and PR5 (Chip3Fw-Chip3 Rv) (see fig. 2.13) were used to evaluate the enrichment of the transgenic locus.

As shown in figure 2.20 B pre-incubation with BirA and Biotin had no positive effect on the efficiency of the pull-down, in contrast, the amount of immunoprecipitated chromatin was about half of the one from the sample that did not contain exogenous BirA.

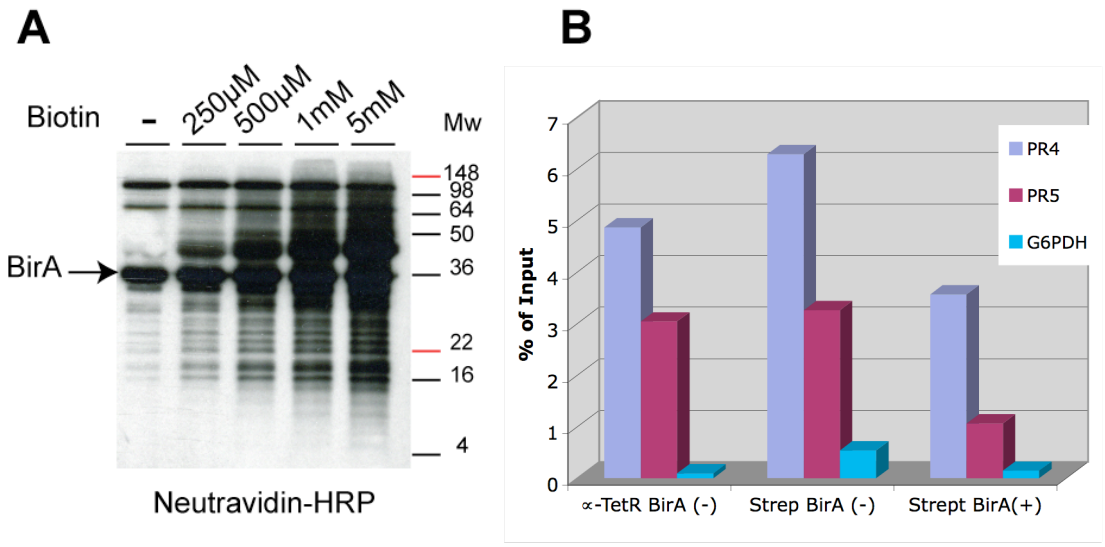

Figure 2.20. ***In vitro*** **biotinylation of Drosophila extracts. A**) pc-NE from TetO-*Fab7* was incubated in the presence of 10μg of BirA and increasing concentrations of Biotin. After reversal of cross-link the samples were separated on a 15% polyacrilamide gel and analyzed by Western Blot with Neutravidin-HRP. **B)** Pc-NE from 6.1.4 (TetO-*Fab7*) was incubated in the presence or absence of BirA and Biotin. Next, the chromatin was purified by CsCl gradient and used in a ChIP experiment with Stretpavidin and α-Tet antibody. The graphic shows the quantification of immunoprecipitated material after PCR amplification. The *g6phd* locus was used as (-) control. PR4 and PR5 pairs were used to specifically amplify the transgene. PR4 amplifies the region between TetO and *Fab7* while PR5 expands also a fraction of the *Fab7* PRE (see fig. 2.13)

The enrichment of the transgenic locus obtained with the α-TetR antibody was also lower than the Streptavidin ChIP on the (-) BirA sample. These results suggest that although there is an increase in the degree of biotinylation proteins when smalls amounts

of extracts are incubated in presence of large amounts of BirA, this effect is not detectable in ChIP experiments where larger samples are used and the concentration of BirA is reduced.

2.6 Chromatin purification, separating the DNA bound proteins from the unbound

The expressed TetR protein has a NLS that allows the protein to be located at the nucleus to be able to bind the TetO site in the chromatin. However, since there are only seven TetO sites per transgene only a few molecules of TetR can bind per nucleus, seven dimers per chromosome, fourteen per nuclei. On the other hand, the protein is over-expressed, therefore there is a high amount of unbound free nuclear TetR. In addition to the free TetR, there are in the nucleus some endogenous biotinylated proteins that are more abundant than the amount of TetR that is bound to TetO. Altogether, these soluble biotinylated proteins can compete with the chromatin-bound TetR for the interaction with streptavidin beads resulting in a great dilution of the pulled chromatin. Thus, the efficiency may be improved by including a chromatin pre-purification step to separate this fraction from nuclear soluble proteins.

2.6.1 Separation of nuclear extract and nuclear pellet with salt extraction

Chromatin is a large molecule; although in the nuclei it is soluble, slightly changing the buffer conditions in a test tube might result in its precipitation. This phenomenon is used routinely when preparing nuclear extract; high concentrations of salt are used to extract the chromatin bound proteins and precipitate the DNA separating it from the proteins that remain soluble.

This principle was used to separate chromatin with its cross-linked proteins from free nuclear proteins. The aim of this experiment was to precipitate the chromatin with salt separating it from the soluble proteins to then recover the purified chromatin by bringing it back in solution with a buffer containing low salt concentrations. Precipitation of chromatin with this method would allow elimination of soluble TetR, biotinylated proteins and in addition endogenous endonucleases, allowing further incubation of the recovered chromatin with specific restriction enzymes to generate chromatin fragments of the adequate size. Alternatively, sonication could also be employed for fragmentation of the chromatin after the purification.

For this, nuclei from cross-linked embryos were prepared and treated as described in 5.3.10. 400 mM of NaCl were added to un-sheared chromatin (total nuclear content) at 4°C. As expected, addition of salt to the sample was sufficient to precipitate the chromatin. After centrifugation, the supernatant was separated and the chromatin pellet was washed several times, one short (10 sec) sonication pulse was used to resuspend the pellet in the wash buffer. After several washes, the washed chromatin pellet was resuspended in a low salt buffer with a short pulse of sonication. After this, the sample was centrifuged to analyze what proportion of the sample was successfully brought in solution and what proportion remained un-soluble. All fractions were reversed cross-link and analyzed by agarose gel electrophoreses; some of these fractions are shown in figure 2.21. Lane 1 shows an aliquot of the input chromatin, showing that most of the starting material was of high molecular weigh, some degree shearing was observed produced probably during mild sonication to break the nuclei open. Lane 2 represents the soluble fraction after the first wash, as shown in this lane some chromatin was brought into solution during washes consisting mainly of chromatin sheared to fragments smaller than 2 kb. Lane 3 represents the pellet fraction after the first wash. The chromatin pellet was washed two additional times and it was finally sonicated for resuspension. Lines 4 and 5 show the soluble fraction and what remains un-soluble after the final sonication. In the soluble fraction (line 4) only fragments smaller than 1 kb are found while the un-sheared chromatin (line 5), which should constitute the input material for restriction enzyme digest, remains un-soluble. This experiment indicates that in order to resuspend the precipitated chromatin, it would have to be sheared by sonication to fragments of about 1 kb.

The pull-down of the proteins bound to a PRE using the TetO as bait depends on both elements remaining in the same chromatin fragment. The tested protocol for separation of chromatin from soluble proteins resulted in chromatin fragments that are too small to contain both elements; therefore this approach resulted not applicable to the system.

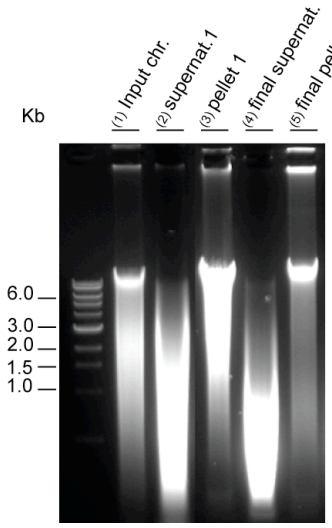

Figure 2.21. Chromatin separation by salt extraction. Chromatin was precipitated from pc-NE prepared from cross-linked embryos by addition of 400mM NaCl. The pellet was washed several times by short sonication pulses. Input material (lane 1), supernatant after salt precipitation (lane 2), first wash (lane 3) and final soluble and pellet fraction (lanes 4 and 5 respectively) were reverse cross-link and loaded on agarose gel.

2.6.2 Vivaspin ultrafiltration spin columns for chromatin separation

Since chromatin has a high molecular weight, another possibility was to separate it from soluble nuclear components by means of its size. Vivaspin ultrafiltration spin columns are designed for rapid concentration and/or purification of biological samples. They are suitable for sample volumes up to 20 ml and are available with a choice of membranes with a rage of different pore sizes. Two different MWCO (molecular weight cutoff) were selected, 300 and 1000 (the biggest available). Protein-chromatin NE from cross-linked *Drosophila* embryos from the ΔTetO-*Fab7* line were diluted and centrifuged in Vivaspin centrifugation devices (see 5.3.11) with the aim of filtering through the membrane all molecules small enough to diffuse through pores and retain the chromatin and those nuclear protein complexes that are larger.

Since TetR is only 28KDa it was expected that this molecule would be able to pass through the membrane pores and thus, a reduction of the total TetR protein present in the pc-NE after centrifugation would be observed. As shown in figure 2.22 the amount of TetR in the sample before and after centrifugation was the same. There was no obvious depletion of TetR in the centrifuged samples even with the 1000 MKCO device. In addition, comparison of DNA and protein concentration in all the samples showed that there is extensive chromatin and protein precipitation during centrifugation probably due to fast concentration of the samples. The first minutes of centrifugation produced also a film of precipitated materials on the surface of the membrane impairing the flow through the pores. Thus, ultrafiltration devices are not suitable for samples containing chromatin probably due to the instability of this macromolecule in solution, where small

perturbations in the sample caused by concentration of solutes or volume reduction results in chromatin to precipitate.

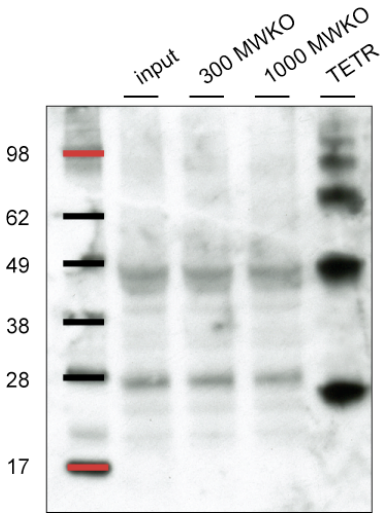

Figure 2.22. Ultrafiltration of chromatin. Protein-chromatin NE was centrifuged on ultrafiltration spin columns. 300KDa and 1000KDa MWCO membranes were compared. After centrifugation samples were reversed cross-link and equal protein amounts analyzed by SDS-PAGE and Western Blot using α-TetR antibody. TetR recombinant protein (MoBiTec) was used as control for blotting and antibody detection procedure.

2.6.3 High molecular weight cutoff dialysis

Contrary to centrifugal devices used in 2.6.2, the changes of sample volume and salt concentration during dialysis are very gradual. This could help avoid sample precipitation and blocking of the membrane pores observed during ultracentrifugation.

To test in which conditions precipitation could be avoided or minimized, protein-chromatin NE aliquots from *Drosophila* cross-linked embryos from TetR and BirA expressing flies, were subjected to dialysis using a Spectra/Por Cellulose Ester membrane with a MWCO of 300 KDa, the largest commercially available (see 5.3.12). An aliquot of input material was saved for further analysis. One sample was dialyzed for 6 h with a starting NaCl concentration of 1 M that was decreased over 3 buffers changes to 250 mM. A second sample was dialyzed over night with no addition of salt and a third sample was dialyzed over night with starting 1M NaCl concentration and diluted to 125mM after 4 buffer changes. After dialysis, the samples were centrifuged and supernatant and pellet analyzed after reversal of cross-link. To determine if the chromatin was retained inside the dialysis membrane and how much of it remained soluble, the samples were analyzed by agarose gel electrophoresis. As showed in figure 2.23 (A) the chromatin fraction was retained by the dialysis membrane and in the shorter incubation time in the presence of salt very little chromatin precipitated (lanes 1-3). Overnight incubation with NaCl produced more precipitation and no addition of NaCl resulted in high chromatin precipitation (lanes 4-7).

Although incubation of the samples in the dialysis tube in the presence of NaCl resulted in some degree of sample precipitation (figure 2.23 A) a second experiment was performed in the same way, in which dialysis was carried out for a longer period, 24 h, in the presence of salt and with 2 extra changes of dialysis tubing during the incubation. The tubing changes were included to avoid the blockage of the membrane pores by precipitating material as occurred during ultracentrifugation (see 2.6.2).

After dialysis the sample was recovered and centrifuged to separate precipitated material. Figure 2.23 (B and C) shows analysis of aliquots of input (lane1), soluble material after dialysis (lanes 2 and 3) and precipitated material (lane 4) after SDS-PAGE and Western Blot with neutravidin-HRP and α-TetR antibody. Although precise quantification of the proteins bands present in 2.23 B is difficult because some of the lower bands in lane 1 have suffer some distortion, comparison between the intensity of the higher protein bands (high MW) and the lower ones (low MW) reveals that low molecular weigh proteins were depleted at some extent, from the chromatin sample (fig. 2.23 D). However, a considerable fraction of TetR and biotinylated proteins were retained inside the dialysis tubing. In addition, quantification of protein concentrations in total input, soluble after dialysis and pellet fractions revealed that at least 1/3 of the protein precipitated while 1/3 was retained soluble inside the tubing and the rest had presumably diffused through the membrane into the dialysis buffer.

Altogether these results indicate that the dialysis technique was effective in some extent to separate the chromatin from soluble nuclear proteins. However, the efficiency in the separation achieved by this protocol is not sufficient to enrich the chromatin fraction to the level needed for the functioning of the system. Furthermore, higher molecular weight biotinylated proteins are not depleted from the extract.

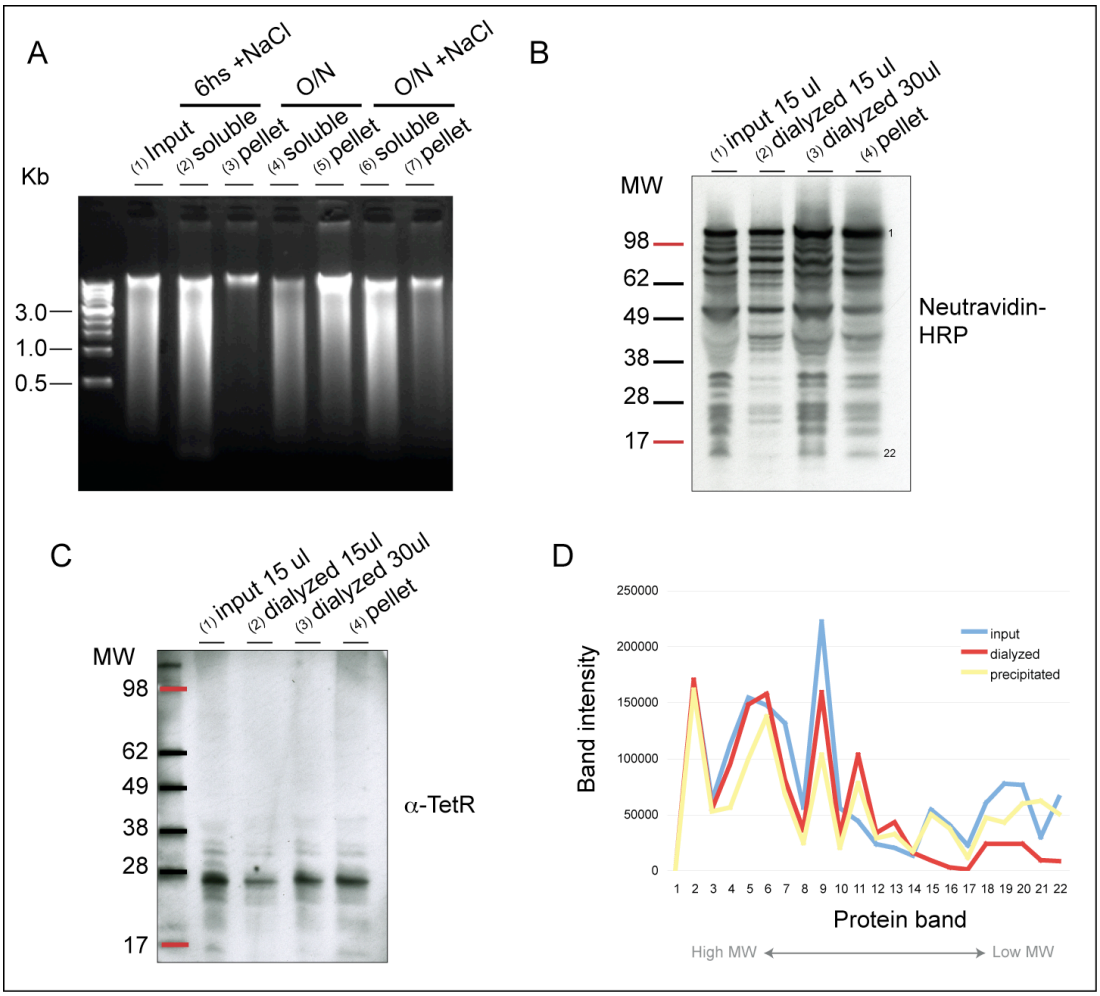

Figure 2.23. Dialysis of chromatin. Pc-NE was dialyzed using a 300 KDa MWCO tubing. **A)** Samples were dialyzed for different times (6 hs vs O/N) in presence or absence of 1M NaCl. After dialysis the samples were centrifuged and pellet and soluble fraction were analyzed by agarose gel electrophoresis after reversal of cross-link and DNA recovery. **B-C)** Pc-NE was dialyzed for 24 h in presence of NaCl with two changes of dialysis tubing. After dialysis the sample was centrifuged and soluble fraction and pellet analyzed by Western Blot using Neutravidin-HRP (B) and α-TetR antibody (C). Input 15µl=52µg protein (lane 1); soluble fraction after dialysis, dialyzed 15µl=25.5µg protein (lane 2), dialyzed 30µl=51µg protein (lane 3); precipitated fraction after dialysis 50µg of protein (lane 4). **D)** Quantification using Aida software of protein bands present in B, input (lane 1), dialyzed (lane2) and precipitated (lane 4).

2.6.4 TetO DNA beads to deplete extracts from soluble TetR

Previous experiments to pre-purify chromatin based on the physical and chemical properties of this molecule failed to deplete the chromatin fraction from TetR and biotinylated proteins. For this reason, a different approach was tested based on the high affinity, specific interaction between TetR and its binding site TetO.

In order to deplete pc-NE from over-expressed soluble TetR, beads carrying TetO sites were produced. For this, a double stranded DNA oligo carrying a single TetO site was coupled to SulfoLink® coupling gel (Pierce) as described in 5.3.13. Approximately 30.000 pMol of DNA were used in the coupling reaction with 2 ml (50% slurry) of SulfoLink beads. Efficient coupling was determined by measuring DNA concentration in input, supernatant and bead washes after coupling reaction. Approximately 80% of the input DNA was coupled to the beads. The generated TetO beads were then used for depletion experiments.

The TetR-TetO interaction has been previously exhaustively studied as inducible tetracycline expression systems were designed (Hillen et al. 1982; Kleinschmidt et al. 1988; Wissmann et al. 1988; Berens et al. 1992; Gossen and Bujard 1992; Deuschle et al. 1995; Orth et al. 2000). Hillen and collaborators have described the ideal conditions for the TetR-TetO interaction (Hillen et al. 1982; Kleinschmidt et al. 1988; Wissmann et al. 1988); these conditions were used in the binding experiments.

First, binding of pure commercial TetR to the TetO beads was tested (Figure 2.24 A). For this, 12 ng of TetR protein (0.44 pmol) were incubated in the presence of Mg^{+2} with 50 µl of TetO beads (1200 pmol TetO). As control 12 ng of TetR protein, input material, were incubated in the same condition with beads buffer. After 30 min incubation at 30°C the beads were centrifuged, and the unbound fraction was separated (lane 2). The beads were then washed several times with buffer, resuspended in sample buffer and boiled for 10 min to elute the bound fraction (lane 3). All samples were analyzed by SDS-PAGE. As shown in figure 2.24 (A) TetR protein was successfully bound to the TetO beads since no TetR remained in the unbound fraction (lane 2). However comparison between input material (lane 1) and bound fraction eluted from the beads (lane 3) indicates that there was some TetR eluted from the beads during washes or, more unlikely, that TetR-TetO interaction was not disrupted completely via incubation of the beads for 10 min at 95°C in presence of SDS-sample buffer.

Subsequently, the same experiment was performed using pc-NE from TetO-*Fab7* cross-linked embryos. In this case two aliquots of 1.75 mg of pc-NE containing between 150-300 ng of TetR protein (5-11 pmol) as estimated by Western Blot, were incubated with 100 µl of TetO beads (2400 pMol of TetO) as described in 5.3.13. One aliquot was used to check whether the chromatin remains in the unbound fraction or if it binds un-specifically to the beads during incubation, and the other was used to determine whether

TetR is depleted from the extract and retained bound to the TetO beads. After binding reaction, unbound fraction, washes and beads were analyzed.

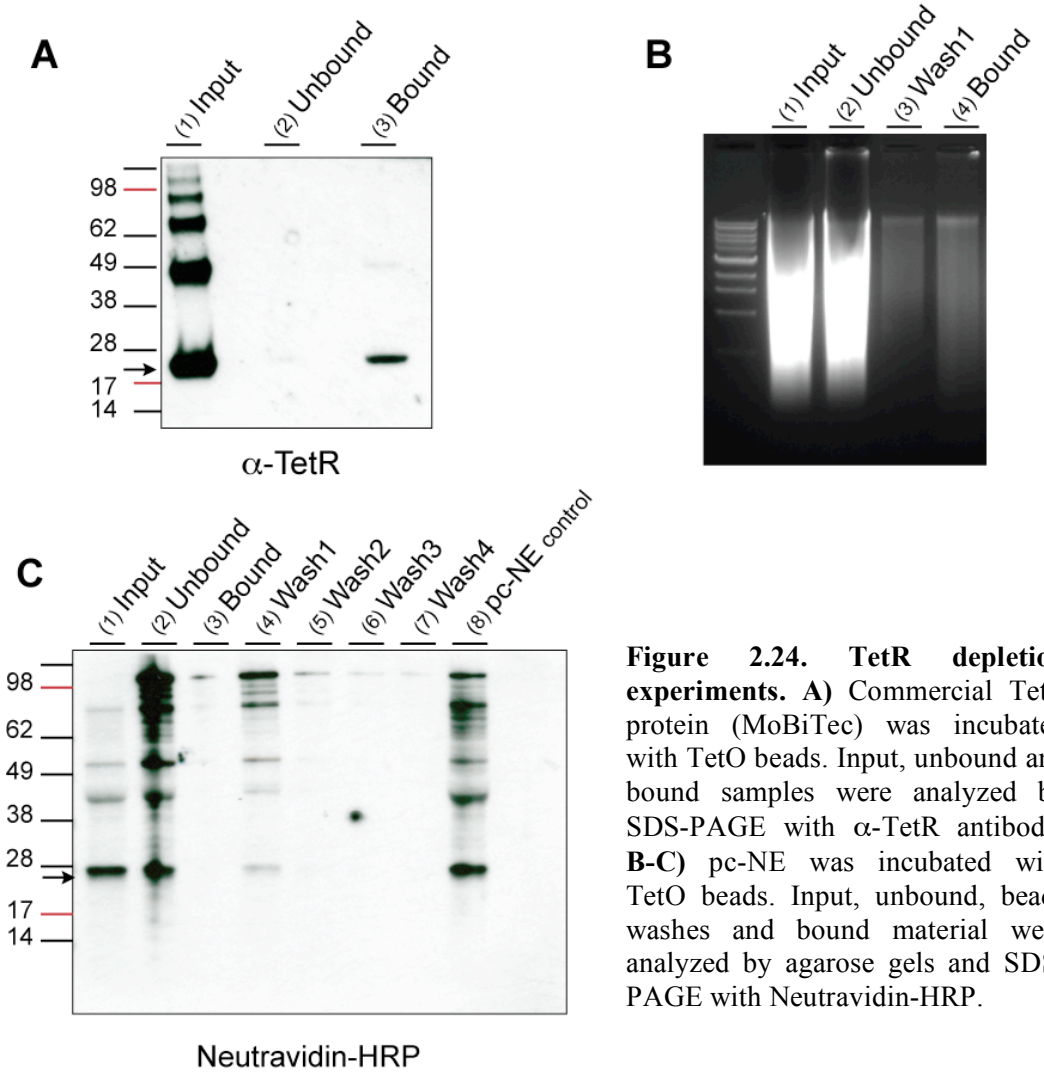

Figure 2.24. TetR depletion experiments. A) Commercial TetR protein (MoBiTec) was incubated with TetO beads. Input, unbound and bound samples were analyzed by SDS-PAGE with α-TetR antibody. **B-C)** pc-NE was incubated with TetO beads. Input, unbound, beads washes and bound material were analyzed by agarose gels and SDS-PAGE with Neutravidin-HRP.

For DNA analysis the samples were reversed cross-link and DNA was purified by phenol-chlorophorm extraction. Figure 2.24 (B) shows that the chromatin fraction present in the samples remained soluble after incubation (compare input, lane 1, with, unbound fraction, lane 2) and did not bind to the sulfolink beads (lanes 3 and 4).

For protein analysis the samples were incubated with sample buffer at 95°C for 40min for reversal of cross-link and analyzed by SDS-PAGE. Contrary to commercial TetR, no fraction of the TetR protein present in the pc-NE remained bound to the TetO beads (Figure 2.24 C, lane 3) and the majority of TetR protein was found in the unbound fraction (lane 2). Although in this case, the ratio TetO molecules/TetR molecules was around 6-12 times smaller than for pure TetR, it was expected that a fraction of TetR

would be retained. These results suggest that the presence of chromatin or cross-linked molecules in the sample somehow interferes with the interaction between TetR and TetO site and thus it is not possible to deplete the pc-NE sample from soluble TetR by using TetO beads.

2.6.5 Cesium Chloride density gradients partially solve the chromatin separation problem

Cesium Chloride (CsCl) density gradients have been commonly used in the past for purification of DNA. CsCl has a remarkable characteristic. If a solution of CsCl is centrifuged long enough at a sufficiently high speed, a higher concentration of the heavy salt collects at the bottom of the tube and the density continuously decreases to the top of the tube so that a gradient is formed. Because proteins, DNA and RNA can be distinguished by means of their densities, these gradients were used for DNA purification. However, for effective separation, centrifugation times of several hours are required; for this reason this technique has been replaced in present-days for simpler and faster ones.

Unlike the current methods that separate naked DNA, CsCl gradient centrifugation allows the separation of chromatin (DNA and associated proteins) from non-chromatin bound (soluble) proteins, naked DNA and RNA (Orlando et al. 1997). For this reason this method was applied to protein-chromatin nuclear extracts. For this, CsCl was added to pc-NE and the sample was loaded in a centrifuge tube as previously described (Orlando et al. 1997). After 40 h of centrifugation, fractions were collected from the bottom of the tube using a peristaltic pump. An aliquot of each fraction was desalted using Micro Bio-Spin P-30 columns (BioRad), reversed cross-link and analyzed by agarose gel electrophoresis to determine which were the fractions containing the chromatin. Figure 2.25 (A) shows the typical distribution of chromatin among the fractions obtained after a gradient.

Chromatin containing fractions were pooled and dialyzed over night to eliminate the CsCl, aliquotted and frozen. Protein and DNA concentration measurements of the chromatin fraction obtained by CsCl gradient showed that extract had been depleted from great part of nuclear proteins (data not shown). However the chromatin was greatly diluted and much material was lost.

In spite of the drawbacks of this method, it constituted the most effective approach to purify chromatin, for this reason CsCl purified material was used in proteins pull-downs.

In addition, this methodology also provided a powerful tool to verify the efficiency of the formaldehyde cross-link of the pc-NE samples. Chromatin (pc-NE) from embryos cross-linked with half the amount of formaldehyde (1.8%) normally used in this work (Figure 2.25 B) resulted in poor separation of the chromatin fraction, as evidenced by even distribution of the DNA between fractions and presence of high amounts of uncross-linked material (fractions 1 and 2). Altogether, comparison between a normal sample and a poorly cross-linked one, provided evidence of efficient cross-link of the working samples.

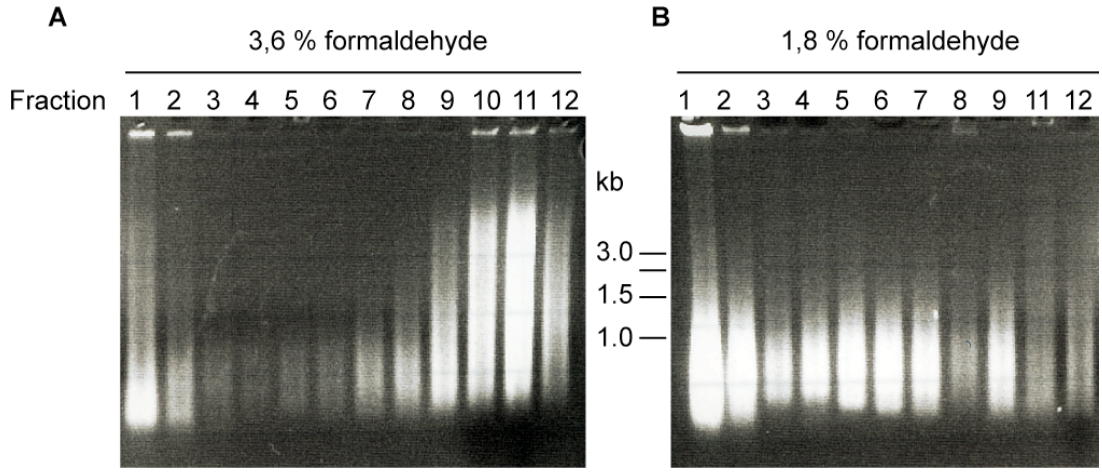

Figure 2.25. CsCl gradient profile of cross-linked chromatin. Protein-chromatin NE, shared by sonication, was fractioned by a cesium chloride gradient. Twelve gradient fractions were collected starting from the bottom of the tube and desalted. The DNA was purified after reversal of cross-link and loaded on agarose gel. **A)** Gradient fractions from pc-NE from embryos cross-linked with 3.6% of formaldehyde. **B)** Gradient fractions from pc-NE from embryos cross-linked with 1.8% of formaldehyde.

2.7 Protein pull-down with optimized conditions

After testing several different protocols to try to optimize the system's efficiency and the purification procedure, the optimal conditions were selected for a final purification protocol. For this, formaldehyde cross-linked, sonication-sheared chromatin was pre-purified by a CsCl gradient.

An approximate estimation, considering number of TetR binding sites per cell, number of cells per embryo, number of TetR molecules per ng of protein, indicated that 9.5 grams of embryos would be needed to produce 5 ng of TetR bound to chromatin, amount easily detected by the α-TetR antibody

Considering that the calculated efficiency of the system is low (see 2.4.2), more material should be used as input. However, there are other factors that set limits, among others, sample concentration, sample volume, amount of beads, beads concentration and elution volume. Therefore, in a final attempt to purify locus bound complexes, 30 grams of embryos were used as starting material for protein purification.

For this, pc-NE was prepared from TetO-*Fab7* andΔTetO-*Fab7* (6.1 *Fab7*-TB-DaGal and 6.1.55.2ΔTetO-TB- DaGal lines, respectively) as described in 5.2.2, 30 grams of embryos resulted in a total volume of 90 ml of pc-NE which were centrifuged for 40 h in a CsCl gradient to separate the chromatin from soluble proteins. Two rounds of centrifugation per transgenic line were needed to purify the 90 ml of pc-NE. After centrifugation, the chromatin containing fractions of the gradient were pooled and dialyzed overnight to remove the cesium chloride, resulting in a total volume of 65 ml / fly line. The purified chromatin extracts were used for the protein purification. Each sample contained approximately 25mg of total protein.

For the pull-down, 32.5 ml of chromatin extract from each fly line was incubated with 500 μl of streptavidin sepharose beads (50% slurry). After 12 h incubation, the tube was centrifuged to settle the beads and the supernatant (unbound) was replaced by another 32.5 ml of chromatin that was further incubated for 12 h over night. The next day, the second supernatant was removed (unbound fraction), and the beads transferred to an eppendorf tube and washed thoroughly. Finally the pulled-down proteins were eluted from the beads as described in 5.3.14 by incubation with formamide, concentrated by TCA precipitation and analyzed by SDS-PAGE. Aliquots of input and unbound fractions were also precipitated with TCA and included as controls.

Figure 2.26 shows a Western Blot analysis of the generated samples. The pull-down protocol included many complex steps with sample loss, such as elution from beads and TCA precipitation, thus direct quantification of bands on the blots would not be informative. For instance, input samples subjected to TCA precipitation contained equivalent amounts of protein (lanes 1 and 4 in blots A and B), however the recovered amount of protein was higher in the ΔTetO-*Fab7* line (lane 2).

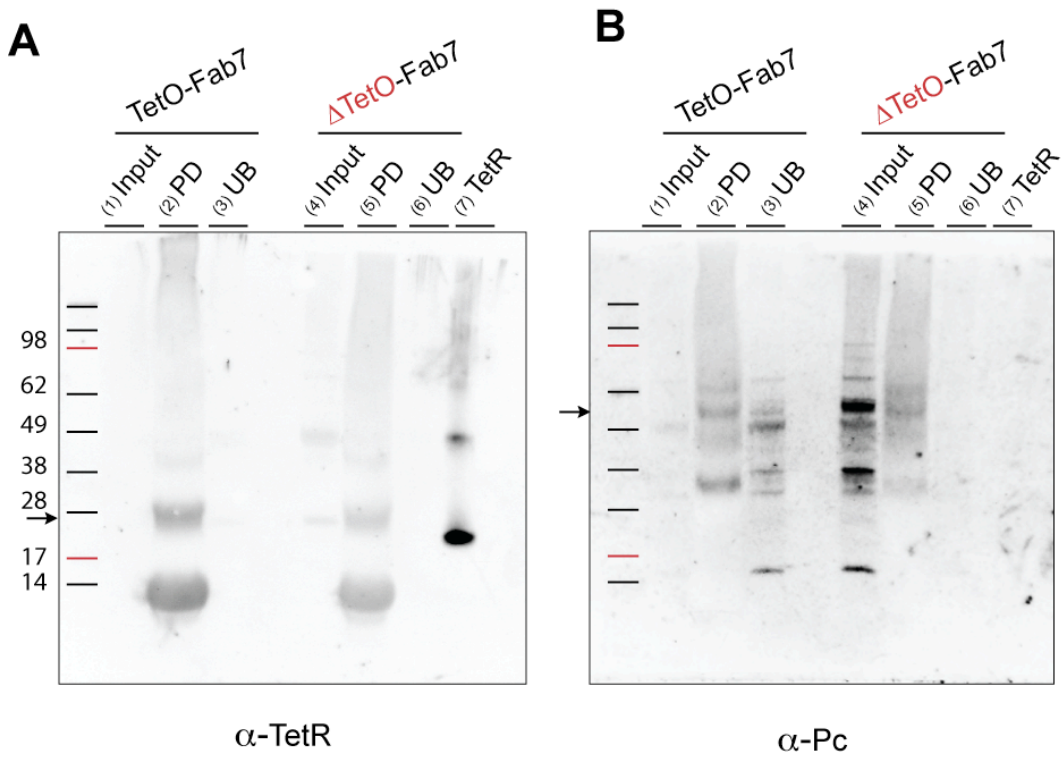

Figure 2.26. Protein pull-down of chromatin complexes. Chromatin purified by CsCl gradient from TetO-*Fab7* and ΔTetO-*Fab7* lines was subjected to Streptavidin pull-down. The pulled material was eluted from beads with formamide; Input, pull-down and unbound material were precipitated with TCA to concentrate the samples, resuspended in SB and analyzed by SDS-PAGE and Western Blot with α-TetR **(A)** and α-Pc **(B)** antibodies to detect the bait protein (TetR) and PcG proteins bound to the *Fab7* PRE.

Qualitative analysis of the blot developed with α-TetR antibody (blot A) reveals that the TetR protein was successfully purified from the chromatin extracts by streptavidin pull-down (lane 2). The amount of TetR protein present and precipitated in the input and unbound fractions (lanes 1 and 4, and lanes 3 and 6 respectively) was smaller than 10 ng, as revealed by comparison with TetR control (lane 7). Contrary to expectations, comparison between TetO-*Fab7* line (lane 2) and ΔTetO-*Fab7* line (lane 4) showed only a small difference in TetR concentration between the two samples, although in the ΔTetO-*Fab7* line there is no TetR binding site in the chromatin. This result indicates that some soluble proteins remained in the sample even after the CsCl gradient purification.

To determine whether PcG proteins were enriched in the TetO-*Fab7* line versus the ΔTetO-*Fab7* control line, the blot was incubated with α-Pc antibody. This protein was chosen as one of the most characterized PcG members normally present at transgenic PREs, and because it was found bound to the transgenic PRE by ChIP (see 2.3.2).

Comparison of TetO-*Fab7* vs. ΔTetO-*Fab7* line gave no clear result; although Pc appeared to be slightly enriched in the TetR-Fab7 line, small differences between samples may have been caused by loss of material during precipitation.

As shown by these experiments, formamide elution and TCA precipitation had a direct effect on the recovery of the samples, suggesting that a more direct approach should be implemented for the analysis of the pulled samples. These two steps, elution and precipitation, were necessary to recover the pulled proteins from the large volume of streptavidin beads (500 μl) used for the precipitation. Using such large volume of beads was necessary because the input material was diluted to 65 ml during CsCl purification, and using a smaller amount of beads would result in loss of beads during purification. The amount of beads determined then the volume of elution, which had to be sufficient to cover the beads bed. Ideally, qualitative and quantitative differences in sample composition could be detected by mass spectrometry analysis. However, in this approach elution of the purified proteins in a large volume resulted in very diluted samples, which were not suitable for analysis with mass spectrometry techniques and had to be then, analyzed by Western Blotting. In order to do so, the samples had to be precipitated with TCA and resuspended in a minimal volume with SDS-sample buffer, which is also incompatible with mass spectrometry. Furthermore, the results obtained in this experiment suggest that even when the proteins bound to the beads could be eluted directly in a small volume of buffer, the sample would not be concentrated enough for mass spectrometry analysis. However, increase of the sensitivity of mass spectrometry techniques in the future might provide a way to analyze the purified samples.

Discussion

3. Discussion

Polycomb and Trithorax group of proteins are part of a memory system that ensures the faithful transmission of cell identities through cell division. They operate as part of multimeric protein complexes. Core components of these complexes have been purified and contain a defined number of PcG/TrxG of proteins. In addition, many other proteins also involved in gene regulation, have been genetically identified or found, by immunoprecipitation or ChIP approaches, to interact with Pc and Trx group proteins (Sato and Denell 1985; Poux et al. 2001; Mahmoudi et al. 2003; Huang and Chang 2004; Dejardin et al. 2005; Blastyak et al. 2006). However, the fact that these interactions do not survive complex purifications procedures suggests that they are weaker that the ones among the core components of the complexes, supporting the idea that they might constitute transient interactions resulting from regulatory functions. During development and cell differentiation, there are dynamic changes in the composition of purified complexes. Moreover, the composition of proteins bound at certain target genes is different in different tissues (Zink and Paro 1989; Rastelli et al. 1993; Strutt and Paro 1997; Otte and Kwaks 2003). Genes that are silenced may become activated and vice versa. Altogether, the memory system appears to be more complicated that the simple static binding of complexes to a PRE. Additional factors that come in to action at certain points in development might exist, helping direct the binding of the complexes to certain genes and not to others. These factors might help differentiate one locus from the others, and one tissue from the rest. In support of this, many PcG proteins have alternative isoforms some of which are expressed in different tissues (Dura et al. 1987; Wu and Howe 1995; Beuchle et al. 2001; Brown et al. 2003; Wang et al. 2006).

So far, the question of the composition of Pc and Trx group complexes has been addressed in a quite general manner. Purification of protein complexes from soluble extracts from whole embryos or cells, may give just an oversimplified view of the real components of the cellular memory.

The aim of this thesis work was to design and test a system to analyze the components of chromatin when still bound to the DNA; in other words, to produce a snapshot of the chromatin composition of a locus at certain tissue and time in development. Such a system should allow the purification of DNA and proteins bound to it in an un-biased way.

During this work the system was developed, all its components were introduced in *D. melanogaster* flies and it was shown that the pull-down of a specific locus using external bait is possible. The performance of the system for protein purification was tested, however many methodological problems were encountered that affected the efficiency of the system at various levels. Each problem was confronted separately and experiments were conducted to find a better solution for each experimental step that would lead to a better output.

Although the system proved to be biologically possible, protein pull-downs after system optimization resulted in no quantitative enrichment of PcG proteins, as revealed by Western Blot techniques. The current development of sophisticated mass spec technology might allow in a near future accurate quantification of the obtained samples without necessity of further improvements; in the mean time direct analysis of the pull-down samples to detect qualitative differences on sample composition, by comparison between the generated control lines, might be possible after modification of some aspects of system design. In this respect, the characterization and system optimization performed during this work, has helped to identify the critical steps for functioning of the system. Alternatives to some system components are discussed in more detail giving an overview of what aspects should be modified if the system were to be re-designed to improve its function.

3.1 Single locus analysis: any other options?

The system was based on the use of a external protein bait, meaning that the pulled-down protein is not involved in the biological process under investigation, and its binding site is also separated from the binding site of the proteins of interest. This gives the possibility to purify all proteins bound to a locus and not just the ones interacting with the bait. Other approaches used for protein purification have included tagging one of the proteins involved in the studied process. The biggest drawback in this kind of method is that it only allows purification of the proteins interacting directly with the tagged bait or strongly with other components of the complex. In the case of chromatin interacting factors, all those proteins that bind chromatin independently from the tagged bait or that have weak interactions with it, are lost. In addition, this kind of approach does not distinguish between chromatin bound and free complexes.

So far, the only approach that allows the analysis of chromatin composition at a particular locus is Chromatin IP (ChIP), which also depends on the antibody used in the

purification. ChIP is a directed search that can answer a Yes or No question: Is this particular protein present at this locus? This method always depends on the previous knowledge of a protein interaction with a particular locus or a protein function, but does not allow identification of new factors. Moreover, when applied to embryo material, ChIP gives an average of binding to a target in all cells, abolishing tissue specific differences.

Although these techniques have brought many insights into PcG/TrxG regulation, they are not able to answer other types of questions that need to be answered in the PcG/TrxG field. Which proteins are bound to a PRE? What are the differences between different silent loci? Is the protein composition of a locus different in different tissues? What happens during cell differentiation? The system was designed to look for answers to these questions.

Introducing an inducible external bait, the TetR protein and its binding site TetO to the system, gives also the possibility to purify proteins from a particular tissue or time in development by differential expression of the bait. Using the Gal4 system the bait expression can be tightly controlled, and there are already many Gal4 drivers available specific for different tissues (Fischer et al. 1988; Brand and Perrimon 1993; Rorth 1998).

The "BirA *in vivo* biotinylation" element was introduced for two reasons. First, to give an extra level of conditionality to the system; the BirA gene was placed under the control of Gal4 so its expression is coupled to the expression of TetR, but eventually it could be uncoupled from the bait by placing it under control of a conditional promoter derived from heat shock inducible genes (Lis et al. 1983), or a binary expression system such as LexA/$(LL)_4$ (Szuts and Bienz 2000) providing another point of control. In this way, the TetR could be expressed only in certain tissues and the BirA only at certain times in development resulting in the restricted presence of biotinylated TetR to a particular tissue in a particular time. The second reason was that the interaction of streptavidin and biotin is very strong ($K_d= 10^{-15}$, several orders of magnitude stronger than that of antibodies), allowing stringent washing conditions, after cross-linking, to eliminate background. In addition, others have been successful in purification of Bio-tagged proteins that are biotinylated in vivo using a single step purification protocol which constitutes also an advantage over other protein purification protocols (de Boer et al. 2003; van Werven and Timmers 2006; Sanchez et al. 2007).

Two Polycomb Response Elements were selected for the analysis, *Fab7* and *vestigial*. The first one was selected because it is one of the best characterized PREs and has been extensively used in the Paro laboratory in transgenic assays (Zink and Paro 1995; Cavalli and Paro 1998; Cavalli and Paro 1999; Rank et al. 2002; Schmitt et al. 2005). The second one, was selected from many predicted PREs (Ringrose et al. 2003) because it has been genetically characterized in the Paro laboratory (Lee et al. 2005) and because it belongs to a gene outside the Antennapedia and bithorax complexes and might represent a different class of PRE that could be used for comparison with *Fab7*.

Only a *Fab7* PRE line and its derived control lines were used to test the functioning of the system. The control lines were generated by Cre and FLP recombination of PRE and TetO sites resulting in TetO-Δ*Fab7* and ΔTetO-*Fab7* lines (fig. 2.9). Excision of *Fab7* resulted, as expected, in activation of the expression of *miniwhite* reporter gene (Brand and Perrimon 1993), confirming the PRE has silencing activity (Zink and Paro 1995). Excision of the TetO had no effect on the *miniwhite* expression in those lines where the TetR protein was missing (fig. 2.10 A). However, expression of TetR protein had a small effect on the PRE silencing. Pigment measurements of all three lines showed a slightly stronger repression of *miniwhite* in the ΔTetO-*Fab7* line compared to the TetO-*Fab7* line (fig. 2.10 B) in flies expressing TetR protein, indicating that binding of TetR to the transgenic locus has a small activating effect. This mild effect was also observed in another *Fab-7* line where the insertion site in the genome is different, and also in a *vg* transgenic line, arguing that the effect is independent of the insertion site and of the identity of the PRE, and probably produced by recruitment of other factors to chromatin. In this case, the additional factors could be identified by comparison between TetO-PRE and TetO-ΔPRE lines. Only those proteins purified in the presence of the PRE and absent in the ΔPRE sample are specific and the others constitute the background proteins recruited to the locus by TetR or by other insertion site dependent factors. Moreover, de-repression in the Δ*Fab7* line in presence of TetR was very strong, showing that the PRE is still functional and exerting repressive activity also in presence of TetR. Altogether, the slight activation effect caused by TetR binding should not give false positive results on the identification of PRE bound factors.

3.2 Pulling from the bait: the proof of principle

Although previous work has shown that *in vivo* biotinylation of a tagged substrate by *E.coli* BirA enzyme is possible (de Boer et al. 2003; van Werven and Timmers 2006; Sanchez et al. 2007), this has not previously been undertaken in flies. Therefore, it was important to test whether the BirA was functional when expressed in *Drosophila melanogaster*. The TetR was found to be biotinylated in *Drosophila* culture cells (fig. 2.4) and also in fly heads when TetR and BirA ligase were co-expressed using a GlassGal4 driver, which drives strong expression of Gal4 in the eye (fig. 2.6), showing that biotinylation also occurs in this species and that the system is also applicable to flies (Popp 2004). Biotinylated TetR was also found in fly embryo nuclear extract (fig. 2.12), showing that the biotinylated protein is correctly located in the nucleus where it can meet its binding site.

The functionality of the system was also verified by ChIP experiments (fig 2.13). Biotinylated TetR protein was found bound at the TetO site, and the *Fab7* PRE was also enriched in the streptavidin pull-down, showing that purification of the PRE via chromatin linkage to the bait is possible. This enrichment was dependent on the presence of biotinylated TetR on the chromatin since there was no PRE enriched in samples from the ΔTetO-*Fab7* line.

Polycomb protein was found at the transgenic *Fab7* and the recruitment of this protein was PRE specific since the binding was abolished in the TetO-Δ*Fab7* line (fig. 2.13). Trithorax protein, known to bind the endogenous *Fab7* in embryos already at cellular blastoderm stage (Orlando et al. 1998), was also found at the transgenic *Fab7*, but in lower amounts than in the *bxd* control locus (fig. 2.13 B). This effect might be caused by absence of additional regulatory factors in the transgene since a moderate binding of Trx was also observed for a transgenic *Fab7* in a similar experimental set up (Schmitt et al. 2005).

3.3 Ineffective protein pull-down: a matter of bait abundance or low efficiencies?

Although this was a very ambitious project the initial quantitative estimations showed it could be possible to purify proteins from a single locus. Taking into account molecular weight of TetR, number of TetR binding sites included per locus, genomic dosage, number of cells per embryos and weight of a *Drosophila* embryo (See table 3.1), it was

calculated that in order to obtain 5 ng of TetR protein bound to chromatin, around 9.5 grams of embryos would be necessary.

TetR size	28KDa = 28.000gr/mol
n° cells x embryo (stage 12 approx.)	10.000 cells
n° copies of transgene x cell	2
n° TetR molecules x locus	14 (7 TetO repeats/transgene, TetR binds as dimer)
n° TetR molecules bound to Chromatin per embryo (approx.)	280000 molecules
n° of TetR molecules in 5 ng	5ng=$1.78x10^{-13}$ mol= $1.075x10^{11}$molecules
n° of embryos needed for 5 ng TetR	383928
Grams of embryos for 383929 embryos (approx.)	9.5 gr

Table 3.1 Calculation of TetR molecules present in *Drosophila* embryos. Number of cells in *Drosophila* embryo estimated based on Ashburner (1989) and Foe (1989) (Ashburner 1989; Foe 1989). Number of embryos per gram from Dr. L Ringrose (personal communication).

Since Streptavidin-Biotin interaction is so strong, it was expected that the pull-down efficiency would be considerably higher than for other methods such as antibody pull-downs. Thus, it was estimated that quantities of embryos containing only 1-5 ng chromatin bound TetR should be sufficient starting material for the purifications.

The first pull-down experiment was performed on material originating from 5 grams of embryos. In this experiment, total nuclear extract containing all soluble proteins and chromatin (pc-NE) was used as input material for the pull-down. The TetR protein bait was purified with streptavidin beads, but no Polycomb protein was co-purified with the bait (fig. 2.14), showing that the overall efficiency of the system was lower than estimated.

ChIP experiments showed that Pc and Trx are bound to the transgenic locus, so if these proteins were not co-purified in the protein streptavidin pull-down it was due to experimental problems. Factors that could affect the protein purification efficiency are:

-Cross-link efficiency, if it is low, the protein-chromatin-protein bridge would be lost and proteins, including TetR, would come out of chromatin during purification. In this case enrichment of non-chromatin bound TetR would still be expected but not of other chromatin bound proteins. The enrichment of *Fab7* transgene in samples subjected to ChIP with streptavidin suggests this is not the case (fig. 2.13), and that, at least to some extent, there are cross-linked molecules in the sample. However, during ChIP experiments there is an amplification step of the precipitated DNA samples, and it could be that the cross-link efficiency is very low but still sufficient to give a positive signal in ChIP but not high enough that the majority of transgene molecules are cross-linked to proteins with the concomitant decrease in pull-down efficiency.

-Reversal of cross-link, in standard reversal of cross-link protocols used for ChIP experiments, total proteins in the sample are digested with Proteinase K and only DNA is recovered. For protein purifications, proteins must be preserved so different conditions for reversal of cross-link must be used. If the reversal of cross-link is not complete, a fraction of the chromatin bound proteins would be lost, resulting in lower efficiency of the purification.

-DNA fragment size, a key point for a successful purification with a bait is that both TetO site and PRE are contained in the same chromatin fragment at the moment of the pull-down. If the chromatin is sheared in fragments that are too small then TetO and *Fab-7* would be in separate fragments and TetR purification would be still successful but not of PRE associated proteins. Although ChIP experiments suggest this is not the case, it could again be the case that a minority of right size fragments are enough to give a positive enrichment after PCR but not on a protein pull-down.

-Biotinylation efficiency, if most of TetR molecules bound to chromatin are not biotinylated then the number of locus molecules that bind the streptavidin beads would be reduced.

-Unbound TetR and other biotinylated proteins, free protein that is not bound to the chromatin can interfere with the purification by saturating the system. If the proportion of free protein to chromatin bound protein is high, then it could be that all streptavidin binding sites on the beads get saturated by binding of free TetR and other biotinylated proteins and the proportion of bound (purified) TetR that is attached to chromatin would be very low. This would prevent a detectable enrichment of the locus of interest. This seemed to be the case, since there was no difference in the amount of TetR pulled down

from the lines containing TetO site in the chromatin with the one that has no binding site (fig 2.14), indicating that most of pulled TetR was free in the nucleus.
Having identified the possible causes for the lack of Pc in the pull-downs, several experiments were conducted to try to improve each step.

3.4 Problems associated to working with lines carrying many transgenes

In order to purify proteins from *Drosophila* a large quantity of embryos had to be collected. To do so, the fly lines generated had to be continually amplified to set up fly cages for embryo collection. Transgenic lines are often less viable than *wt* flies. Having 4 transgenes inserted in the genome seemed to be very disadvantageous; the generated transgenic flies were weak, they did not lay many eggs and they were often smaller than *wt*. After some months of continuous amplification, the transgenic lines ceased to produce TetR protein. PCR analysis of the lines showed that all transgenic elements were still present in the flies and yet these flies expressed no TetR. These flies also gave more progeny and grew faster than the original flies, indicating that they could overgrow the original fly stock almost completely in a very short period of time.
It is not clear how these flies originated. It could be that chromosomal re-arrangements caused the lost of expression without loss of the transgenes elements from the DNA. Alternatively, it is possible that the expression of Gal4 or of TetR protein became epigenetically silenced. In both cases, the flies would lose TetR and become able to out-compete the TetR expressing phenotype. This phenomenon affected all 3 transgenic lines (TetO-*Fab7*, TetO-Δ*Fab7* and ΔTetO-*Fab7*). For two of the lines, flies containing all transgenes and still expressing the transgenic proteins were found mixed with flies that have ceased to express TetR. By separating and crossing these flies, the lines could be rescued and re-amplified. This was not the case for ΔPRE line where the transgenic line could not be recovered, not even from the small stocks kept at 18°C. For this reason, many experiments were carried on with TetO-*Fab7* line and only one control, the ΔTetO-*Fab7* line.

3.5 What could and could not be done to increase efficiency

Several experiments were conducted to measure chromatin shearing, cross-link, reversal of cross-link, biotinylation and chromatin shearing efficiencies and try to increase them.

3.5.1 Cross-link and reversal of cross-link

In *Drosophila* embryos, different cross-link times with formaldehyde resulted in minor changes in running pattern of proteins on SDS-Gels giving the indication that the observed proteins might correspond to the uncross-linked fraction of the sample (fig. 2.16). To increase the proportion of cross-links other protein cross-linkers were tested. Although the only available chemical cross-linker able to produce DNA-Protein cross-links is formaldehyde, the addition of a protein-protein cross-linker could help stabilize protein-DNA complexes by fixing together proteins of a same complex or to histones present in the chromatin. DSP and DTBP, two protein-protein cross-linkers were tested. Addition of DSP directly to embryos had no effect on overall protein cross-link levels (fig. 2.19A). DSP is a widely used cross-link in studies of protein-protein interaction but it has the drawback that it is water insoluble so it is normally used directly on protein extracts. When used for *in vivo* cross-link it could be that the cross-linker did not penetrate the embryo into the cell nuclei. For this reason a similar cross-linker, but water soluble, was tested. Addition of DTBP directly to nuclei had also no effect on the cross-link efficiency as shown in a pull-down experiment (fig. 2.19B).

This approach was tested because it was thought that using a different cross-linker than formaldehyde could help to increase the stability of chromatin via cross-link of proteins to histones. However, no improvement was observed in the pull-down efficiency. It might be the case that cross-linking proteins to histones does not affect the overall efficiency of the pull-down or it might be that the cross-linking is not taking place. Histones and other proteins might simply not be physically close enough to be cross-linked. In addition, there is increasing evidence that PREs are depleted of nucleosomes (Papp and Muller 2006; Schwartz et al. 2006) and this might also be the case at the TetO site.

Although no differences were observed when cross-linking with formaldehyde for different times, this chemical efficiently cross-linked pure TetR protein and the reversal of the cross-link was achieved by incubating the proteins in sample buffer for over 10 min (fig. 1.17). More over, chromatin from formaldehyde crossed-link embryos showed the typical fraction distribution on CsCl gradients for cross-linked chromatin (fig. 2.25). For these reasons, cross-links were continued with formaldehyde.

3.5.2 Chromatin shearing

The chromatin fragment size could have a large influence on the performance of the system. Fragments that are too small (less than 2-3 kb) will not contain TetO and PRE in the same fragment resulting in failure of PRE bound proteins to co-purify with the bait. The ideal method to obtain a homogeneous chromatin population of defined size fragments, which contains both, TetO and PRE, would be to digest the chromatin with restriction enzymes. These enzymes are usually used for digestion of naked DNA, which has been pre-purified and contains no bound proteins. In this case, the material to be digested was cross-linked chromatin, which contains many fixed proteins, so there was the possibility that the restriction sites were not available to the enzymes. When tested, the chromatin was digested, but with low efficiency. In addition, there were endogenous endonucleases that further digested the chromatin un-specifically into fragments around 500bp size (fig. 2.20 A). Normally, adding EDTA to the chromatin buffer inhibits the activity of endonucleases, but restriction enzymes are also inhibited, so EDTA could not be used. Moreover, a restriction enzyme digest of the extracts would be quite complicated because the amount of sample to be digested (around 60 ml), and it would require incubation for several hours at 37°C, which might be deleterious for the proteins, making this approach worth implementing only if the efficiency of the digest would be very high and it would give a clear advantage over sonication, which was not the case. Instead, the sonication procedure, which is much more rapid, with no need of incubation at 37°C was optimized to obtain an average fragment size of 3 kb (fig. 2.20 B).

3.5.3 Biotinylation

Using a biotinylated tag is an attractive approach for protein complex purification due to the very high affinity of avidin/streptavidin for biotinylated templates. Biotinylation can occur either by the cell endogenous protein-biotin ligases or through the co-expression of an exogenous biotin ligase. In mammalian cells or even in mouse embryo the biotinylation of tagged transgenic proteins by a transgenic BirA ligase seems to be a very efficient process (de Boer et al. 2003). In addition, previous work performed in yeast showed that the use of biotin tagged transcriptions factors is an efficient approach to increase the sensitivity of ChIP experiments (van Werven and Timmers 2006). Biotinylation was shown to take place in *Drosophila* (fig. 2.12), but with what efficiency? To answer this question, a streptavidin band shift experiment was performed in the eluted material from a streptavidin pull-down. The reason for using pull-down

material was to first enriched on biotinylated protein, since previous experiments on total nuclear extract have shown no detectable band shift (results not shown), this experiment indicated that the biotinylation was lower than expected. Quantification of TetR protein in the different samples confirmed that the biotinylation efficiency is only around 23%. Previous work in mammalian MEL cells reported a biotinylation efficiency of about 100% (de Boer et al. 2003). In addition the efficiency of the pull-down is also lower than expected, resulting in only 2.2% of total TetR in the extract being purified. This means that in order to pull-down 10ng of TetR bound to chromatin, the starting input material should be around 420 gr of embryos. Using so many embryos was not feasible within the scope of this project since there were other factors that set experimental limits;, for instance embryo collection of unhealthy stock, sample volume, beads amount, beads elution volume, amount of protein that can be loaded in one well in a SDS-PAGE, among others; scaling up to this level would require new optimization of each step and finding other experimental alternatives to cope with large amount of materials.

The TetO site in the transgene is composed of seven TetR binding sites. 23% biotinylation efficiency means that out of fourteen TetR molecules expected to be bound at the TetO site, only 3.2 molecules are expected to be biotinylated. It could be possible that the presence of only three biotinylated TetR molecules out of fourteen bound to the locus would be sufficient to bind the fragment to streptavidin. In this case increasing the biotinylation efficiency would have no effect on the pull-down efficiency. However, there is also the possibility that three molecules is not sufficient and that by increasing biotinylation the pull-down efficiency would also be increased.

In vitro incubation with recombinant BirA did not produce an increase on Streptavidin ChIP efficiency. The BirA enzyme showed little activity and in order to produce noticeable biotinylation it had to be added in large amounts to the pc-NE, which exceeded the amounts available given the kind of volumes of extract that were to be handled. In addition, Biotin also had to be added exogenously. In this case, both BirA and Biotin, had to be eliminated from the pc-NE before the pull-down (ChIP) since they would saturate the system. It was shown by ChIP that addition of an *in vitro* biotinylation step actually reduced the efficiency of the system probably due to remaining enzyme and biotin in the sample after CsCl gradient (fig. 2.21). The degree of biotinylation obtained was probably insufficient to make a difference on the biotinylation levels of the TetR or maybe the chromatin-bound TetR was not available for biotinylation.

3.5.4 Soluble TetR and biotinylated proteins

The expressed TetR protein has a NLS that allows the protein to be located at the nucleus to bind the TetO site in the chromatin. However, since there are only few TetO sites per transgene only a few TetR molecules, seven dimers per homologous chromosome, can bind per nucleus. On the other hand, the TetR protein is over-expressed; therefore there is a high amount of unbound free nuclear TetR. In addition to the free TetR, there are in the nucleus some endogenous biotinylated proteins that are quite abundant if compared to the amount of TetR present in the chromatin. Altogether, these soluble biotinylated proteins can compete with the chromatin-bound TetR for the interaction with streptavidin beads resulting in a great dilution of the pulled-chromatin. Thus, it was expected that the efficiency of the pull-down could be improved by including a chromatin pre-purification step to separate this fraction from nuclear soluble proteins.

Several approaches were tested in order to purify chromatin based on different physical-chemical properties of this macromolecule. Solubility, precipitation with high salt was effective, however the precipitated chromatin could not be re-solubilized in mild conditions (fig.2.20); the only possibility was to shear the chromatin by sonication in very small fragments which were then solubilized. However, this approach was not compatible with the system that requires chromatin fragments of about 3 kb. Molecular weight, chromatin is a macromolecule of a size, which is several orders of magnitude bigger that protein complexes that are present in the nuclei, and certainly much bigger that the 28KDa TetR thus, in principle, it could be separated by size exclusion. Two similar methods were tested, based on the use of semi-permeable membrane with pores of sizes that let molecules up to 300 KDa and 1000 KDa go trough and, in principle, retain chromatin. First Centricom like devices and second dialysis membrane were tested. In both cases the soluble proteins did not diffuse through the pores of the semi-permeable membranes and were retained with the chromatin (fig. 2.21 and 2.22). A similar effect was observed when trying to separate the chromatin by charge using ion exchange resins. In this case all soluble proteins remained in the unbound fraction with chromatin (data not shown). This effect was unexpected since ion exchange matrixes with opposite charges were tested and it could be expected that some proteins bind to at least one of them. However, all biotinylated proteins present in the sample, regardless of their isoelectric point, behaved the same way and remained in the same fraction than chromatin.

A complete different approach was next tested consisting on using the specific interaction between TetR and TetO binding site to separate TetR protein from pc-NE. Although this approach was effective for separation of pure TetR, which was retained in the column via interaction with TetO DNA, it failed to separate the TetR protein from the chromatin fraction when pc-NE samples were tested (fig 2.24); giving the indication that it was something present in the pc-NE sample, which interfered with the interaction.
All together, these negative results clearly show that is the nature of the sample, and not the methods as such that interfere with the different tested purifications. Many could be the reasons that act together to cause this behavior: First, allosteric effects of chromatin intercepting the interaction of the soluble proteins with the different beads and matrixes. Second, a mater of solubility or free diffusion, the pc-NE containing chromatin are quite concentrated; the chromatin is in a very unstable solution, freezing and thawing, centrifugation or changes in salt concentration lead to precipitation of chromatin. It could also be that in this dense solution proteins are not free to diffuse so easily. Moreover, the chromatin is cross-linked, and also are many of the soluble proteins, this could also influence the diffusion of proteins. It might be that cross-links itself, produces aggregates of proteins that cannot fit through the pores of the dialysis membranes and cannot bind charged resins. All these possible factors are linked to the characteristics of the sample, cross-linked chromatin, but it is exactly this type of sample that the system relies on, and cannot be changed or modified. Using native chromatin purification could help to overcome problems caused by the presence of over cross-linked proteins. However, in this case, stringent washes conditions would wash away low affinity interacting proteins. It would only be possible to use native chromatin by reducing the stringency of the pull-down conditions and thus abolishing the main advantage of using the biotin streptavidin interaction. In addition, it might be the nature of chromatin it self and not the cross-link what interferes with the above mentioned methods.
Separation of chromatin by centrifugation on a density gradient was the only method that achieved higher degree of separation. In this kind of gradients the chromatin was separated from the free proteins and also from un-cross-linked DNA (fig. 2.25). The main disadvantage of this approach was that the sample had to be greatly diluted, resulting in volumes that were very difficult to handle. In addition, chromatin material was also lost during the purification procedure. Another disadvantage was that the

method is time consuming and that the extracts had to be centrifuged for two days at RT and dialyzed over night after centrifugation, increasing the risk of protein degradation.
Streptavidin protein pull-down was performed on chromatin samples after CsCl purification (fig. 2.24). For this experiment the maximum possible amount of embryos was used as starting material. The results indicate that the amounts of TetR and biotinylated proteins present in the sample after CsCl gradient separation were still quite high, since no differences were observed between the TetO-*Fab7* line, which contains the TetR binding site on the DNA, and the ΔTetO-*Fab7* line, which does not. However, even in this case, the ratio chromatin-bound / soluble TetR should be much higher than previous to the gradient, so if there would be enough chromatin bound protein in the sample, and the efficiencies of the pull-down would be high enough, it could be expected that chromatin bound proteins were also pulled-down and a difference of enrichment of PRE associated proteins could be detected between sample and control. Theoretically there are about 16ng of TetR bound to chromatin in 30 gr of embryos, but taking into consideration that the efficiency of the pull-down resulted about 2.18%, then very little TetR was expected. It is not know what the stoichiometry of PcG proteins at PREs is, then it was not clear how many molecules of Pc to expect per each TetR molecule. The obtained results show that there was not sufficient protein to see a difference between the two fly lines.

3.6 The future of locus-specific factors identification

The system designed in this work, for locus specific analysis of regulatory proteins showed to be biologically functional, however the identification and quantification of specific proteins proved to be difficult due to several experimental limitations that were not anticipated during the design phase of the system. Substantial effort was put into the detailed analysis of each step involved in the system. This analysis revealed which were the incorrect assumptions made during system design and the important features that should be improved if one is to use this system for locus specific purification of proteins. Altogether, this work has given precious information to answer important questions for the future of "locus specific": What lessons have we learned? What could be done with the system as it is? What could be modified in the system to improve its performance?

3.6.1 What is to be done: other possible applications of the system

As mentioned above ChIP analysis revealed that the system is biologically functional and the locus is pulled-down when using the TetR bait (fig. 2.13). Differences in proteins bound to the PRE at different times in development or different tissues could be studied directly with this system by sequential ChIP (Loh et al. 2006). This technique would allow performing a first ChIP using the TetR bait and streptavidin beads to purify the transgenic locus from tissues where the TetR is expressed giving tissue specificity, eluting the chromatin from the beads using biotin, and performing a second ChIP on the purified chromatin using a specific antibody, using chromatin from different species as a carrier (O'Neill et al. 2006). This methodology would allow the investigation of the presence of specific factors bound to the chromatin in different situations. However, it would not be possible to find new, previously unidentified factors, since the second pull-down is based on the choice of antibody. Nevertheless, it could provide valuable information about differences at the same locus between tissues where the corresponding regulated gene is active or inactive or it could be used to study kinetics of PcG/TrxG binding by targeted TetR expression at different developmental times. Eventually, a real powerful methodology would be to incorporate the TetR binding sites on the endogenous locus via homologous recombination allowing analysis of the PRE in the context of all endogenous enhancers and regulatory regions.

3.6.2 What is to be modified: re-design of the system

During this work steps of the purification that performed with low efficiency were detected and some could be modified to increase efficiency. However, there were some, identified as critical parts of the system, that could not be modified because they were part of the system design it self. For this reason, in order to improve the performance of the system several aspects of the system design should be adapted.

-Lower expression of TetR. The bait should be expressed in lower amounts in order to increase the proportion of biotinylated protein. It was observed that only 23% of expressed TetR is biotinylated (fig. 2.18), lowering the levels of TetR in the cell might increase the fraction of total TetR that gets biotinylated and hence, the amount of biotinylated TetR bound to the TetO site.

-Nuclear localization sequence on the BirA; adding a NLS on the BirA would allow the enzyme to translocate to the nucleus and further biotinylate TetR in this compartment. In this case, proper controls should be carried out to discard the possibility that inclusion of

BirA in the nucleus would also increase background levels. In this work, it was shown that incubation of pc-NE with recombinant BirA in presence of increasing amounts of biotin had also an effect on the degree of biotinylation of endogenous proteins (fig. 2.21). However, it was not clear whether this effect was caused by endogenous ligases or by BirA. Although this enzyme has been reported to be specific for the Bio-tag (de Boer et al. 2003), this test was performed in mammalian cells. It remains then to be tested in *Drosophila* whether some nuclear endogenous proteins are targets of this enzyme.

-Use of tandem tags on TetR; as an alternative to BirA system tandem tags could be included for the bait purification. For instance a combination of a Strep-tag with a His tag could be implemented (Skerra and Schmidt 1999; Skerra and Schmidt 2000). The use of tandem tag approach would allow elimination of endogenous biotinylated background proteins by performing a first pull-down with streptavidin and re-purifying the pulled material via the His tag. Addition of a Tev cleavage site between tags would allow eluting the specific pulled material from streptavidin beads by protease cleavage leaving behind the biotinylated proteins. The Strep-tag is a short peptide that binds directly to streptavidin without need of biotinylation (Schmidt et al. 1996). Thus, by excluding the BirA from the system the biotinylation efficiency problem would also be solved. A draw back of this approach would be the loss of one level of control over the system, since the uncoupled expression of BirA allows time specific purifications in addition to tissue specific ones. In addition, this would not eliminate the problem of non-chromatin bound TetR (see 2.6) for which a chromatin purification step, like CsCl gradient, would still be needed.

-More TetO sites; arrays of many TetR binding sites could be included to increase pull-down efficiency (Robinett et al. 1996; Vazquez et al. 2006). It was shown that although biotinylation efficiency is 23%, pull-down efficiency is only 2.18 %. Adding more TetO sites to the transgene could have a positive effect on the pull-down efficiency. However, it should be tested whether the presence of so many TetR molecules on the chromatin does not affect PcG/TrxG function since in this work it was shown that binding of TetR to the transgenic locus has a slightly activating effect on the expression of the *miniwhite* reporter gene (fig. 2.10 B).

-Higher number of PRE transgenes in the genome; purification of locus specific complexes relays in the purification of two copies of the locus per cell. One option to increase the amount of proteins purified would be to increase the number of copies of the

transgene by including tandem repeats inserted in the same locus. A disadvantage of this approach could be that PcG/TrxG function might be enhanced by close proximity of more copies of the same PRE.

-Use of recombinases to generate chromatin fragments of proper size; in this work Cre and FLP recombinases were used to generate the proper controls by *in vivo* excision of TetO and PRE (fig. 2.9 and 2.10). Recombination sites for a third recombinase could be included flanking the whole PRE transgene. An enzyme such as the Kw recombinase (Ringrose et al. 1997) could be added *in vitro* to pc-NE for specific excision of the desired fragment. In this work, it was shown that restriction enzyme digest is not efficient to produce homogeneous fragment size containing the transgene and although sonication proved a better solution, it produces a smear of fragments with sizes around 2 kb (fig. 2.20). Using recombinases might help to solve the problem of fragment size. In addition, this method would also constitute an advantage for further purification of the transgene from the chromatin since the excised chromatin fragment is circularized and much smaller than the residual chromatin and it could be separated, for instance, by size exclusion methodology. Due to the presence of endogenous nucleases in the chromatin preparation (fig. 2.20) it would be necessary to test for activity of these enzymes on the circular substrate, which would be digested only by endonucleases but not by exonucleases.

-Optimization in cell culture; the general tests to examine system performance should be carried out first in cell culture, since cells are easier to handle and large amounts of material are easier produced by cell culture. For this, one single cassette containing all transgenes would be transfected into Drosophila cells and stable cell lines would be obtained. Expression of TetR protein would be placed under control of a Cu^{+2} inducible promoter.

-In flies, all different transgenes should be included in the same insertion site; inclusion of all transgenes, TetR, PRE and BirA in the same cassette, in same locus, could contribute to generate more stable fly lines, since inclusion of several transgenes inserted in different sites in the genome in the same fly line, can constitute a great disadvantage producing sick flies that do not grow well as it was detected in this work (see 3.4).

Altogether these modifications in the system design should help improve the efficiency of the system, which constitutes to date probably the only option to analyze the protein composition of a specific locus.

3.7 Conclusion and outlook

In summary, a system was designed to purify proteins bound to a specific locus using external bait. Each transgenic element of the system was brought into flies and all the elements recombined to generate one fly line. Each step in the purification scheme was tested and their efficiencies determined. Unfortunately, the efficiencies of the most critical steps were considerably lower than expected.

During this work, a detailed analysis of each experimental step involved in the system was performed. This analysis enabled the improvement of the steps and the identification of key features of system design that should be modified in the future for successful chromatin bound protein purification. All the information collected in this work was used to propose possible applications of the current system and most importantly to suggest alternatives to different components of the system that might function more efficiently.

In addition to re-designing some aspects of the system, improvement in the sensitivity of current available techniques or development of new techniques for protein analysis will also have great influence on the feasibility and performance of this type of systems. For instance, it is possible that in the near future, mass spectrometry techniques will develop higher sensitivity allowing analysis of single cell proteomes (Woods et al. 2004; Rubakhin and Sweedler 2007; Tsuyama et al. 2008). In fact, micro-devices for single cell manipulation and for extraction of material of one cell are already being developed and implemented in basic biological research (Andersson and van den Berg 2004; Hellmich et al. 2005; Chen et al. 2008). In this scenario, analysis of very small differences between samples, the kind this system could deliver, may prove to be experimentally possible. Until then, the question of PcG/Trx group locus specific regulation and of other factors assisting in the specificity of the cellular memory remains open.

Materials

4. Materials

4.1 Antibodies

Antibody	Source	From	Dilution		
			Western	*Polytenes*	*ChIP*
α-DIG	Mouse	Roche (Nr.11333062910)		1:200	
α-Polycomb	Rabbit	Britta Kock	1:10000	1:100	10 µl
α-Trithorax	Rabbit	Inhua Chen-Muyers			35 µl
α-PHO	Rat	Christian Beisel	1:1000		
α-TetR	Mouse	MoBiTec (Tet02) monoclonal mix	1:1000		10 µl
α-rabbit-Alexa488	Goat	Molecular Probes		1:200	
α-rabbit-Cy3	Goat	Jackson ImmunoResearch		1:200	

4.2 Molecular Weight markers

1 kb DNA ladder	New England Biolabs (NEB)
100 bp DNA ladder.	NEB
Low Range PFGE Marker	NEB
Protein Standard Broad Range	NEB
SeeBlue Plus 2 Prestained	Invitrogen

4.3 Enzymes

Benzonase	Merk
Calf intestinal alkaline phosphatase (CIP)	Merck
Klenow enzyme	Roche
Polynucleotide Kinase	NEB
Proteinase K	Roche
High Fidelity Polymerase	Roche
Restriction enzymes	NEB
RNAse A	Roche
RNAse H	Gibco
Taq Polymerase	Qiagen
T4 DNA ligase	NEB / Roche

4.4 Oligonucleotides

All oligonucleotides were purchased from Sigma, MWG or Biomers.

4.4.1 Oligos and Primers for PRE constructs

Oligo PAZKpn	sense	5’ AGCTTGGTACCCAGCTCGAGG 3’
Oligo PAZKpn	antisense	5’ GATCCCTCGAGCTGGGTACCA 3’
OligoBsiWI	sense	5’ GTACACGTACGACGCGTGTCATGACCGGTAT GCA 3’
OligoBsiWI	antisense	5’ TACCGGTCATGACACGCGTCGTACGT 3’
LOXP	sense	5’AGCTTACTAGTGCGGCCGCATAACTTCGTATAAT GTATGCTATACGAAGTTATGAATTC 3’
LOXP	antisense	5’ TCGAGAATTCATAACTTCGTATAGCATACATTA TACGAAGTTATGCGGCCGCACTAGTA 3’
PRE*Fab7*	Fw	5’ GACTAGTGATGCTATCGCGTTCGATTGTTG 3’
PRE*Fab7*	Rv	5’ ATAATTTAGCGGCCGCTGTGGACTTTTCTTTTA ATGAGCTG 3’
OligoBsu	sense	5’ TCGAGACCGGTCTGACCCTGAGGGCCGGCG 3’
OligoBsu	antisense	5’ GATCCGCCGGCCCTCAGGGTCAGACCGGTC 3’

4.4.2 Oligos and Primers for TetR constructs

3’BioTag	sense	5’ TCGAGT TAGGAGCCGCCGGCGTTCGAGCGCC ACTCCATCTTCTGGGAATCCAGGATCTGGCGCAG CGAGGAGGCG 3’
3’BioTag	antisense	5’ GATCCGCCTCCTCGCTGCGCCAGATCCTGGATT CCCAGAAGATGGAGTGGCGCTCGAACGCCGGCGG CTCCTAAC 3’
5’ BioTag	sense	5’ GGCCGCATGGCCTCCTCGCTGCGCAGATCCTGG ATTCCCAGAAGATGGAGTGGCGCTCGAACGCCGG CGGCTCCATGCCAAAGAG CCCAGACCCT 3’
5’ BioTag	antisense	5’ CTAGAGGGTCTGGGTCTCTTTGGCATGGAGCCG CCGGCGTTCGAGCGCCACTCCATCTTCTGGGAATC CAGGATCTGGCGCAGCGAGGAGGCCATGC 3’
TetR	Fw	5’ATAAGAATGCGGCCGCCGGCGGCTCCATGCCAAA GAGACCCAGACCCTCT 3’
TetR	Rv	5’ CCGCTCGAGTTAGGATCCACTTTCACATTTAAG TTGTTTTTC 3’

4.4.3 Oligos for sequencing of plasmids and cloned fragments

Operator.seq		5’ CGTGACGTCTAAGAAACC 3’
pC4TetOFRT		5’ CAACACTATTATGCCCAC 3’
pUASTetR3		5’ GAACTCTGAATAGGGAAT 3’
pUASTetR5		5’ CACACCACAGAAGTAAG 3’

4.4.4 Primers used to test Cre/loxP and Flp/FRT recombination

PRECreTest	Fw	5' GCTGGCGTAATAGCGAAGAG 3'
PRECreTest	Rv	5' CAGAGAAGGAGGCAAACAGC 3'
∗Fab-7-X-Up	Rv	5' GGCAGACCGAAACACTTTAGCAA 3'
TetOflptest	Fw	5' ACCTCGAGACCGGTACAGTT 3'
TetOflptest	Rv	5' ATCGAACGCGATAGCATCACTA 3'
FLP2	Fw	5' GTAAACTCGAGGGATCCGAAG 3'
ChIP5	Rv	5' GATCGCCGGATCAGCATCT 3'

4.4.5 Primers used to make probes for Southern Blot

PRECreTest	Fw	5' GCTGGCGTAATAGCGAAGAG 3'
*Fab7*pstI *(Fab7 probe)*	Rv	5' CAATTGGGAAAGAAACCCATT 3'
∗WhitepromUp *(miniwhite probe)*	Fw	5' ACCCATCTGCCGAGCATCTGAA 3'
Miniwhite-primer3 *(miniwhite probe)*	Rv	5' GCTGCTGCTCTAAACGACGCA 3'

4.4.6 Additional primers used to check fly stocks

Gal4-1	Fw	5' GAAGGAACACCCTTGGCTATC 3'
Gal4-1	Rv	5' GTGCGGTCTCGTTATTCTCAG 3'
Gal4-2	Fw	5' ATGCCGTCACAGATAGATTGG 3'
Gal4-2	Rv	5' CGTCGCCAAAGAACCCATTATT 3'
BirA3	Fw	5' ACGTGACTGTGCGTTAGGTC3'
BirA1	Rv	5' AGCCTGCTGGTATTCTGCAAT 3'
TetR5'-1	Fw	5' GATTCCCAGAAGATGGAGTGG
TetR5'-1	Rv	5' CCTTCGATTCCGACCTCATTA 3'

4.4.7 Primers used in ChIP

ChIP-1	Rv	5' CCCTCAGGAAGATCGCACT 3'
ChIP-2	Fw	5' GTTACCCAACTTAATCGCCTTG 3'
ChIP-3	Fw	5' CGCAGCCTGAATGGCGAAT 3'

ChIP-3	Rv	5’ TCCCTAGTACACCCGTCAACAA 3’
A-G6PDH	Fw	5’ CGCCCTGGATCTCATAATCA 3’
A-G6PDH	Rv	5’ CCCAAAGATGACGAACGTGT 3’
∗Bxd-Up	Fw	5’ GACGTGCGTAAGAGCGAGATACAG 3’
∗Bxd-Low	Rv	5’ GCACTTAAAACGGCCATTACGAA 3’
24mer		5’ AGAAGCTTGAATTCGAGCAGTCAG 3’
20mer		5’ CTGCTCGAATTCAAGCTTCT 3’

∗Kindly provided by Dr. Sabine Schmitt.

4.4.8 TetO oligos for beads coupling

~1TetO_ColFw		5’ ThiolC6-TCCCTATCAGTGATAGAGA 3’
1TetO_ColRv		5’ TCTCTATCACTGATAGGGA 3’

4.5 Plasmids

pBKSII	Cloning vector. Invitrogen
pCaSpeR4	Vector for P-mediated transformation in Drosophila. Carries *miniwhite* as transformation marker (Pirrotta 1988).
pSVPAZ11	Contains direct repeats of the FRT sequence separated by 1.1 kb insert and is based on the plasmid pBS (Buchholz et al. 1996) kindly provided by Dr. Leonie Ringrose.
pUHD142-1	Contains the coding sequence of Tet repressor protein with a Nuclear localization sequence on the amino termini (Gossen and Bujard 1992) kindly provided by Prof. Hermman Bujard.
pUC13-3	Contains seven repeats of the TetO sequence. (Gossen and Bujard 1992) kindly provided by Prof. Hermman Bujard.
pUZFab-7	pUZ vector carrying *Fab7* PRE. Kindly provided by Dr. Sabine Schmitt.
pUZ*vg*	pUZ vector carrying *vestigial* PRE between NotI and

	SpeI sites (Lee et al. 2006).
pSVPAZ-TetO-FRT	Vector containing the TetO cassette flanked by FRT sites. This work.
pC4TetOSsp	pCasper4 vector containing TetO-FRT cassette 1.7 kb downstream of the end of the *miniwhite* gene. This work.
pC4TetOLsp	pCasper4 vector containing TetO-FRT cassette 3.1 kb downstream of the end of the *miniwhite* gene. This work.
pBKS-LOXP	Contains 2 direct repeats of the LOXP sites with HindIII, SpeI and NotI cloning sites in between. This work.
pC4TetOSsp-Lox	pC4TetOSsp with LoxP sites. This work.
pC4TetOLsp-Lox	pC4TetOLsp with LoxP sites. This work.
pC4LoxP	pCasper4 vector containing 2 direct repeats of the LOXP sites upstream the *miniwhite* gene. This work.
pC4*Fab7*-2	pCasper4 vector containing the *Fab7* PRE flanked by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 6.8 kb downstream the *miniwhite* gene. This work.
pC4*Fab7*-7	pCasper4 vector containing the *Fab7* PRE flanked by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 5.3 kb downstream the *miniwhite* gene. This work.
pC4*Fab7*-4	pCasper4 vector containing the *Fab7* PRE flanked by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 156 bp upstream the *Fab7* PRE. This work.
pC4*Fab7*-6	pCasper4 vector containing the *Fab7* PRE flanked by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 410 bp upstream the *Fab7* PRE. This work.
pC4*vg*-1	pCasper4 vector containing the *vestigia*l PRE flanked

	by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 6.8 kb downstream the *miniwhite* gene. This work
pC4*vg*-10	pCasper4 vector containing the *vestigial* PRE flanked by LOXP sites upstream the *miniwhite* gene and the TetO site flanked by FRT sites 410 bp upstream the *vg* PRE. This work.
pUASTy	Vector for Gal4 induced expression of Proteins in Drosophila. Contains the *yellow* gene as marker (Brand and Perrimon 1993).
pBKSTetR	Vector containing the TetR coding sequence. This work.
pBKSTetR3'Bio	Vector containing the TetR coding sequence and the biotinylation Tag coding sequence on the 3' end of the TetR (protein amino termini). This work.
pBKSTetR5'Bio	Vector containing the TetR coding sequence and the biotinylation Tag coding sequence on the 5' end of the TetR (protein carboxyl termini). This work.
pUYTetR3'Bio	pUSTy vector carrying TetR and amino termini Bio tag sequences under UAS control region. This work.
pUYTetR5'Bio	pUSTy vector carrying TetR and amino termini Bio tag sequences under UAS control region. This work.
pUChsΔ2-3	Help vector for P-element mediated transformation. Codifies for expression of P-Transposase. Kindly provided by Dr. Stefan Schoenfelder.
pUST(GFP)-MycBirA	pUST vector carrying the coding sequence of BirA biotin ligase enzyme. The protein is myc tagged. (Popp 2004).
pMTGal4	Gal4 expression vector inducible by Cu^{++}. Originally provided by Dr. Peter Soba.

4.6 Bacterial cell lines

SURE 2 e14–(McrA–) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96*

*relA1 lac recB recJ sbcC umuC::*Tn*5* (Kan^r) *uvrC* [F« *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Amy Cam^r], Stratagene

XL1-Blue *E. coli recA* - (*recA1 lac* - *endA1 gyrA96 thi hsdR17 supE44 relA1* { *F' proAB lacIq lacZDM15Tn10*}), Stratagene

4.7 Drosophila culture cells

SL2 SL2 Schneider cell line derived from primary culture of *Drosophila melanogaster* embryos.

4.8 Fly Lines

4.8.1 General Fly stocks

	Donor
Genotype donor y[1]w[1118]	Paro lab Stock collection
YTD: *y[1]w[1118]; + ; TMSb/Dr*	Dr. Leonie Ringrose
YCS: *y[1]w[1118]; CyO/Sp*	Dr. Leonie Ringrose
y[1]w[1118]; BcGla/CyO	Dr. Stefan Schoenfelder
y[1]w[1118]; TM2,Ubx/MKRS	Dr. Stefan Schoenfelder

4.8.2 Gal4 driver lines

Daughterless: *y[1]w[1118]; P{da-Gal4.w-}*	Paro lab Stock collection
Glass (GMR): *w[*]; P{w[+mC]=GAL4-ninaE.GMR}12*	Bloomington Stock Center

4.8.3 Transgenic lines expressing site-specific recombinases

*y[1]w[67c23]; noc[Sco]/CyO, P{w[+mC]=Crew}DH*1	Bloomington Stock Center, No. 1092
*P{ry[+t7.2]=hsFLP}12, y[1] w[*]; noc[Sco]/Cy*O	Bloomington Stock Center, No. 1929

4.8.4 Biotin Ligase (BirA) expressing flies

Y[1]w[1118];P{BirA-myc, GFP}	Christian Popp
Y[1]w[1118];+;P{BirA-myc, GFP}	Christian Popp

4.8.5 *Generated fly lines*

***Fab7* PRE lines**	
6.1 *Fab7*	Contains the p-element pC4*Fab7*-6 inserted in the II chromosome, *y[1] w[1118]; P{ FLP-TetO, FRT-Fab7, w+}.*
6.1.14 ΔFab-7	Is 6.1 *Fab7* after excision of PRE by Cre recombination: *y[1] w[1118]; P{ FLP-TetO, FRT, w+}.*
6.1.55 ΔTetO	Is 6.1 after excision of TetO by FLP recombination: *y[1] w[1118]; P{ FLP, FRT-Fab7, w+}.*
6.1.4 *Fab7*-TB-DaGal (TetO-*Fab7*)	Is 6.1 *Fab7* recombined with 5.5 TetR-BirA. Carries on II chromosome three P-elements. And on III chromosome Daughterless Gal4 driver. *y[1] w[1118]; P{ FLP-TetO, FRT-Fab7, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.*
6.1.14.3 ΔFab-7-TB-DaGal (TetO-Δ*Fab7*)	Is 6.1 Δ*Fab7* recombined with 5.5 TetR-BirA. Carries on II chromosome three P-elements. And on III chromosome Daughterless Gal4 driver. *y[1] w[1118]; P{ FLP-TetO, FRT, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.*
6.1.55.2 ΔTetO-TB-DaGal (ΔTetO-*Fab7*)	Is 6.1.55 ΔTetO recombined with 5.5 TetR-BirA. Carries on II chromosome three P-elements. On the III chromosome Daughterless Gal4 driver. *y[1] w[1118]; P{ FLP, FRT-Fab7, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.*
FBT	Is 6.1 *Fab7* crossed to 5.4(3)TetR-BirA *Y[1]w[1118];P{ FLP-TetO, FRT-Fab7, w+}; P{TetR5'Bio, y^+}, P{BirA-myc, GFP}*
FBT-DaGal4	Is FBT crossed to DaGal4. Is not balanced so the cross was made when needed for control experiments, it was not kept.
6.2 *Fab7*	Contains the p-element pC4*Fab7*-6 inserted in the X chromosome, *y[1] w[1118], P{ FLP-TetO, FRT-Fab7, w+}.*
6.2 ΔFab-7	Is 6.1 *Fab7* after excision of PRE by Cre recombination: *y[1] w[1118], P{ FLP-TetO, FRT, w+}.*
6.2 ΔTetO	Is 6.1 after excision of TetO by FLP recombination:

	y[1] w[1118], P{ FLP, FRT-Fab7, w+}.
6.25 *Fab7*	Contains the p-element pC4*Fab7*-6 inserted in the III chromosome, *y[1] w[1118];+; P{ FLP-TetO, FRT-Fab7, w+}.*
6.25 *Fab7*-TB-DaGal	Is 9.25 *Fab7* recombined on chromosome III with Daughterless Gal4 driver.
6.25Δ*Fab7*-TB-DaGal	Is 9.25 *Fab7*-DaGal after excision of PRE by Cr recombination and Crossing in the TetR-BirA Chromosome on II. *y[1] w[1118]; P{TetR5'Bio, y$^+$}, P{BirA-myc, GFP/Cyo ; P{ FLP-TetO, FRT, w+}, DaGal.*
6.25 ΔTetO-TB-DaGal	Is 9.25 *Fab7*-DaGal after excision of TetO by FLP recombination and crossing in the TetR-BirA Chromosome on II *y[1] w[1118]; P{TetR5'Bio, y$^+$}, P{BirA-myc, GFP/CyO; P{ FLP, FRT-Fab7, w+}, DaGal* .
2.1 *Fab7*	Contains the p-element pC4*Fab7*-2 inserted in the II chromosome, *y[1] w[1118]; P{ FLP-TetO, FRT-Fab7, w+}.*
2.5 *Fab7*	Contains the p-element pC4*Fab7*-2 inserted in the III chromosome, *y[1] w[1118];+; P{ FLP-TetO, FRT-Fab7, w+}.*
4.1 *Fab7*	Contains the p-element pC4*Fab7*-4 inserted in the III chromosome, *y[1] w[1118];+; P{ FLP-TetO, FRT-Fab7, w+}.*
7.2 *Fab7*	Contains the p-element pC4*Fab7*-7 inserted in the II chromosome, *y[1] w[1118]; P{ FLP-TetO, FRT-Fab7, w+}.*
7.6	Contains the p-element pC4*Fab7*-2 inserted in the III chromosome, *y[1] w[1118];+; P{ FLP-TetO, FRT-Fab7, w+}.*
***vg* PRE lines**	
10.7 *vg*	Contains the p-element pC4*vg*-10 inserted in the II chromosome, *y[1] w[1118]; P{ FLP-TetO, FRT-vg, w+}.*

10.7 *vg*-TB	Is 10.7 *vg* recombined on II chromosome with 5.5(8)TetR-BirA. *y[1] w[1118]; P{ FLP-TetO, FRT-vg, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}*
10.7*vg*-TB-DaGal	Is 10.7 *vg*-TB crossed to DaGal4 driver. *y[1] w[1118]; P{ FLP-TetO, FRT-vg, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb*
10.7Δ*vg*-TB-DaGal	Is 10.7-TB after FRT mediated recombination and crossing a DaGal4 driver. *y[1] w[1118]; P{ FLP-TetO, FRT, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb*
10.7ΔTetO-TB-DaGal	Is 10.7-TB after FLP mediated recombination and crossing a DaGal4 driver. *y[1] w[1118]; P{ FLP, FRT-vg, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb*
1.5 *vg*	Contains the p-element pC4*vg*-1 inserted in the II chromosome*, y[1] w[1118]; P{ FLP-TetO, FRT-vg, w+}.*
1.8 *vg*	Contains the p-element pC4*vg*-1 inserted in the III chromosome*, y[1] w[1118];+; P{ FLP-TetO, FRT- vg, w+}.*
TetR lines	
5.5 TetR	Contains the p-element pUYTetR5'Bio inserted in the II chromosome. *y[1] w[1118]; P{TetR5'Bio, y+}.*
5.4 TetR	Contains the p-element pUYTetR5'Bio inserted in the III chromosome. *y[1] w[1118];+; P{TetR5'Bio, y+}.*
5.5(8)TetR-BirA	Is 5.5 TetR recombined with BirA transgenic fly. Both P-elements are on chromosome II. *Y[1]w[1118]; P{TetR5'Bio, y^+}, P{BirA-myc, GFP}*
5.4(3)TetR-BirA	Is 5.5 TetR recombined with BirA transgenic fly. Both P-elements are on chromosome III. *Y[1]w[1118];+; P{TetR5'Bio, y^+}, P{BirA-myc, GFP}*
5.11 TetR	Like 5.5, contains the p-element pUYTetR5'Bio inserted in the chromosome X
3.1 TetR	Contains the p-element pUYTetR3'Bio inserted in the II chromosome. *y[1] w[1118]; P{TetR3'Bio, y+}.*

3.3 TetR	Contains the p-element pUYTetR3'Bio inserted in the III chromosome. *y[1] w[1118]; P{TetR3'Bio, y+}.*
3.1(2) TetR-BirA	Is 3.1 TetR recombined with BirA transgenic fly. *Y[1]w[1118]; P{TetR3'Bio, y^+}, P{BirA-myc, GFP}*
3.3 (3) TetR-BirA	Is 3.1 TetR recombined with BirA transgenic fly. *Y[1]w[1118];+; P{TetR3'Bio, y^+}, P{BirA-myc, GFP}*

4.9 Technical Devices

4.9.1 Microscopes

Microscopes	Olympus BX60
	Leica DMRXA
Stereomicroscopes	Leica MS5
	Leica MS7,5
	Leica MZFLIII
Software	OpenLab 1.7.8

4.9.2 Microinjection

Femtotips	Eppendorf
Microloader tips	Eppendorf
Microinjector	FemtoJeț Eppendorf
Micromanipulator	Leitz
Microscope	Leitz Labovert

4.9.3 Agarose Gel electrophoresis

MUPID agarose gel chamber and powers supply (Helix)

4.9.4 SDS-PAGE gel electrophoresis and Western Blotting

Protean II	BioRad
X-Cell Sure Loc	Invitrogen
Mini Trans Blot	BioRad
Transfer chamber	ZMBH workshop
Voltage source	Pharmacia EPS 500/400

4.9.5 Data processing

Computer	Apple G4, Apple MacBook Pro
Software	Adobe Illustrator CS2
	Adobe Photoshop CS2
	Endnote X.0.2
	Microsoft Excel
	Microsoft PowerPoint
	Microsoft Word

	AIDA DNA Strider 1.4 Gene Construction Kit Biology Workbench 3.2 (SDSC: San Diego super computer center)

4.10 Further Materials

Centrifugation devices	Vivaspin, Sartorius
Centrifuges	Beckmann J2-MC, Thermo Sorvall Wx Ultra, Thermo Sorvall RC6 plus
Concentrator	Eppendorf Concentrator 5301
Coverslits	Menzel
DAPI	Sigma
Dialysis tubing	Snake-skin pleated, Pierce
Dialysis tubing	Cellulose ester, SpectraPor
Double-sided sticky tape	Scotch 3M
Embryo Homogenizer	Yamato LSC
Filters (0.2 µm, 0.45 µm)	Schleicher & Schuell
Filter paper	Whatman, 3 mm
Fly cages	ZMBH fine mechanics workshop
Forceps	A. Dumont & Fils
GeneAmp PCR System 9700	Applied Biosystems
Highspeed Plasmid Midi Kit	Qiagen
Micro Bio-Spin P-30 columns	Biorad
Horizontal shaker	GFL – Gesellschaft für Labortechnik
Magnetic stirrer	Ikamag
Micropestle	Eppendorf
PCR machine	Applied Biosystems GenAmp 9700
Pipetman	IBS Integra Biosciences
Pipettes (1 ml, 200 µl, 10 µl, 2 µl)	Gilson
Petri dishes	Greiner
pH meter	inoLab
E-Box gel documentation system	PeqLab
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Rotator	Heidolph
Slides	Menzel
Spectrophotometers	Witec Nanodrop, BioRad SmartSpec 3000
Sonifier	Branson 250, Bioruptor Diagenode
Syringes	BD Biosciences
Table top centrifuge	Eppendorf
Thermomixer	Eppendorf
U.V crosslinker	Stratagene
Vortex	NeoLab
Waterbath	Julabo EcoTemp EW, Julabo

4.11 Chemicals

Acetic acid	AppliChem
Amonium persulfate	AppliChem
ATP	AppliChem
Agarose ultra pure	GibcoBRL
Aprotinin	AppliChem
Bromophenol blue	Serva
BSA	Serva
Cesium Chloride	Sigma
Citric Acid	Merk
D-Biotin	AppliChem
DMSO	AppliChem
dNTPs	Peqlab
DTT	Merck
DSP	Pierce
DTBP	Pierce
ECL Western Blot Detection reagent	GE Healthcare
EDTA	Merck
EGTA	E. A. Thomas
Ethanol	AppliChem
Ethidium bromide	AppliChem
Formamide	AppliChem
37% Formaldehyde solution	Sigma
Glycerol	AppliChem
Glycine	AppliChem
Hepes	AppliChem
Isopropanol	AppliChem
KCl	Merck
KH_2PO_4	Merck
L-Cystein	Serva
Leupeptin Hemisulfate	AppliChem
LiCl	Sigma
Maleic acid	Fluka
Methanol	AppliChem
Milk powder	AppliChem
$MgCl_2$	Merck
NaCl	Merck
Na-Deoxycholate	Sigma
Na_2HPO_4	Merck
Na-hypochlorite	E. A. Thomas
n-heptane	Riedel-de Haën
Nipagin	Merck
NP-40	Sigma
Orange G	Sigma
Pepstatin A	AppliChem
p-Formaldehyde	Sigma
Phenol-chlorophorm-Isoamylalcohol	AppliChem
PMSF	AppliChem
Poly(L)lysine	Sigma

Propionic acid	Merck
Protein A Sepharose	GE Healthcare
SDS	Sigma
Sodium citrate	AppliChem
Streptavidin Sepharose	GE Healthcare
Sucrose	AppliChem
Tris	AppliChem
Triton X-100	Merck
Tween-20	Sigma
Western Blot Stripping Buffer	Pierce

Methods

5. Methods

5.1 Molecular methods

5.1.1 Phenol-chloroform extraction of DNA

An equivalent volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to a DNA solution and vortexed thoroughly for 30 sec. The emulsion was centrifuged for 8 min at 13.000 rpm at RT (Eppendorf table top centrifuge). The upper aqueous phase containing the DNA was carefully transferred to a new tube. One volume of chloroform was added, vortexed for 30 sec and centrifuged for 8 min at 13.000 rpm at RT. The upper aqueous phase was transferred to a new tube, and the DNA precipitated with ethanol.

5.1.2 Ethanol precipitation of DNA

1/10 volume of 3.5 M sodium acetate pH 5.2 was added to the DNA solution and mixed, if small amounts of DNA were to be precipitated then 1 µl of glycogen (20 mg/ml, Roche) was added as a carrier. Next, 2.5 volumes of 100% ethanol cooled at -20°C were added to the sample, mixed, and incubated for at least 1 h at -20°C or for 20 min at -80°C. The precipitated material was centrifuged for 25 min at 13.000 rpm at 4°C. The supernatant was removed and the precipitated DNA was vacuum-dried and resuspended in Tris-HCl buffer pH 8.0 or ddH_2O.

5.1.3 Analysis of DNA fragments by agarose gel electrophoresis

Agarose gels 0.5-2% (w/v) were used depending on the size of the DNA fragments to be separated. Ethidium bromide was added in the gel to a final concentration of 0.5 µl/ml. Samples were supplemented with DNA sample buffer, loaded on gel and electrophoresis was conducted at 100-150V for 30 min. After separation, the gels were analyzed using a transilluminator with UV light and photographed. For preparative gels, the fragment of interest was cut out under UV light and the DNA was purified.

Buffers:	0.5x TAE buffer:	20 mM Tris-acetate
		0.5 mM EDTA pH 8.0
	Ethidium bromide stock:	10 mg/ml
	6x DNA sample buffer:	0.25% (w/v) OrangeG
		30% (w/v) glycerol
		0.1 mM EDTA

5.1.4 Gel Extraction/ PCR purification

For the purification of DNA from agarose gels, the QIAquick gel extraction kit (Qiagen) was applied as described in the manufacturer's manual. The DNA was eluted in 30-50 µl ddH2O. To purify DNA following PCR reaction or prior to restriction endonuclease digestion, the QIAquick PCR purification kit (Qiagen) was used according to the manufacturer's protocol. The DNA was eluted in 30-50 µl ddH_2O.

5.1.5 Restriction Endonuclease digestion of DNA

For analytical digests, 100-300 ng DNA were digested with 1-10 U of restriction enzyme (New England Biolabs) with its corresponding buffer according to the manufacturer's protocol in a volume of 20 µl. In general, the digests were incubated for 1 h at 37°C. For preparative digests, 5-10 µg DNA were incubated with 10-40 U of restriction enzyme. The digestion were incubated for 1-4 h up to overnight at 37°C. For some reactions, digestion was followed by a phosphatase treatment to prevent re-ligation. Afterwards, the digested DNA was purified using the QIAquick PCR purification kit or isolated by preparative agarose gel electrophoresis.
Digestions with two different restriction enzymes were performed sequentially with one purification step by agarose gel electrophoresis after each digest.

5.1.6 Phosphatase treatment of DNA fragments

To prevent re-circularization of a digested vector DNA in the ligation reaction, the DNA was treated with Calf Intestine Phosphatase (CIP, Roche) to remove the 5' end phosphate group of the DNA. Phosphatase treatment was typically performed directly following a restriction digest in the same buffer, 1 unit of enzyme per 100 pmol of protruding 5' end termini was added, incubated for 15 min at 37°C, and the reaction was stopped by adding EDTA to a final concentration of 5mM. The enzyme was heat inactivated at 75°C for 10 min. Finally the DNA was purified by agarose gel electrophoresis, spin column purification using the PCR purification kit or phenol-chlorophorm extraction.

5.1.7 Ligation of DNA fragments

Ligation reactions were prepared with 300 ng vector DNA and a 3 fold molar excess of insert DNA in 10 μl with 1 U T4 DNA ligase (Roche) according to the manufacturer's instructions. The reaction was incubated overnight at 16°C. For quick ligation steps the

Quick Ligation Kit (New England Biolabs) was used and the ligation was incubated 5 min at RT.

5.1.8 End-filling of DNA protruding ends

1 µl of Klenow fragment (2 U/µl; Roche) and 5 µl 2.5 mM dNTP mix (dATP, dTTP, dCTP, dGTP) were added to 50µl restriction endonuclease digestion reactions, followed by a 15 min incubation at 30°C. Subsequently, the DNA was purified using the QIAquick PCR purification kit (Qiagen).

5.1.9 Annealing of DNA oligos

DNA oligos were resuspended in ddH_2O at 100 pmol/µl as stock solutions. For cloning oligos were diluted to 10 pmol/µl. 5 µl of 3' oligo and 5 µl of 5' oligo were mixed with 1 µl of T_4 PolyNucleotide Kinase (New England Biolabs) and 5 µl of 10X Ligase buffer as ATP source, in a total volume of 50 µl. The reaction mix was incubated at 37°C for 1 h. To stop the reaction EDTA was added to a final concentration of 10 mM and incubated at 70°C for 15 min. The reaction was slowly cooled down to room temperature by switching off the heat block and placing it in the cold room. For cloning purposes 1 to 5 µl of this mix were used in molar excess over vector.

5.1.10 Preparation of agar plates

For agar plates, 1,5% Bacto-Agar was added to the LB medium. After autoclaving, the appropriate antibiotic was supplemented to a final concentration of 100 µg/ml for Ampicillin, and 34 µg/ml for Chloramphenicol.

LB (Luria-Bertani) medium:	1% (w/v) Bacto-tryptone
	0.5% (w/v) yeast extract
	1% (w/v) NaCl pH 7
Ampicillin stock:	100 mg/ml
Chloramphenicol stock:	34 mg/ml

5.1.11 Freezing of bacteria stocks

760 µl of an overnight bacterial culture were added to an eppendorf tube containing 240 µl of sterile glycerol and immediately vortexed, shocked-frozen in liquid nitrogen and stored at -80°C.

5.1.12 Production of E. coli chemo-competent cells

XL1-blue or SURE *E.coli* cells were inoculated into 20 ml LB medium and incubated overnight at 37°C under vigorous shaking. The next day, the culture was diluted in 450 ml of LB medium, supplemented with 0.1% glucose and grown at 37°C in a 3 l Erlenmeyer flasks until a density OD_{600} 0.4-0.5 was reached. All the following steps were conducted at 4°C using pre-cooled material. The culture was centrifuged for 7 min at 5000g. The sedimented cells were resuspended in 50 ml of Solution I, left 20 min on ice and re-centrifuged. The cells were then resuspended in 50 ml of Solution II, incubated on ice for 30 min and centrifuged . Finally, cells were resuspended in 5 ml of Solution III, aliquotted into sterile 1.5 ml Eppendorf tubes, and shock-frozen in liquid nitrogen. Competent cells were stored at -80°C.

Solution I:	100 mM $MgCl_2$
Solution II:	100 mM $CaCl_2$
Solution III:	86 mM $CaCl_2$
	12.3% Glycerol

5.1.13 Transformation of chemo-competent E. coli cells

Competent cells were thawed on ice. The DNA (50-100 ng for a re-transformation, or 2-10 µl of a ligation reaction) was added to a 100 µl cell aliquot and incubated on Ice for 30 min. Cells were heat-pulsed for 80 sec at 42°C and cooled on ice for 2 min. 900 µl LB medium was added and cells were incubated for 30 min at 37°C for recovery on a roller shaker. Different aliquots of transformed cells (50 µl – 1 ml) were plated on LB agar plates supplemented with the appropriate antibiotic and incubated overnight at 37°C.

5.1.14 Isolation of Plasmid DNA from bacteria by alkaline lysis method

A. Small scale DNA preparation (Mini Prep)

A single colony was inoculated into 3 ml LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C under vigorous shaking. 1.5 ml of the overnight culture was transferred into a 1.5 ml Eppendorf tube and centrifuged for 1 min at 13.000 rpm (Eppendorf table top centrifuge). The supernatant was discarded and another 1.5 ml of overnight culture were added, and the centrifugation step was repeated. The cell pellet was resuspended in 150 µl buffer P1, and then 150 µl of buffer P2 were added. After gentle mixing, cell lysis was allowed to proceed for 5 min at RT. Then 150

µl buffer P3 were added and after gentle mixing, the tubes were incubated on ice for 10 min. The bacterial lysate was centrifuged for 10 min at 13.000 rpm at 4°C and the supernatant was transferred into a new tube. The DNA was precipitated by adding 0.7 volume of 100% isopropanol and pelleted by centrifugation at 13.000 rpm for 25 min at 4°C. To wash the DNA pellet, 1 ml 70% ethanol was added and the samples were centrifuged for 10 min at 13.000 rpm at 4°C. The DNA pellet was vacuum-dried and resuspended in 30-50 µl ddH_2O. All buffers used were from Qiagen.

Buffers:
P1: 50 mM Tris-HCl pH 8.0
10mM EDTA
100 µg/ml RNase A
P2: 200 mM NaOH
1% SDS
P3: 3 M potassium acetate pH 5.5

B. Large scale DNA preparation (Midi Prep)

The Highspeed Midi kit from Qiagen was used to isolate larger quantities of pure DNA according to the manufacturer's protocol. The DNA was eluted in ddH_2O.

5.1.15 Polymerase Chain Reaction (PCR)

The DNA was amplified by PCR using Taq polymerase (Qiagen) for semi quantitative purposes or High Fidelity Taq/Pwo-polymerase (Roche) for cloning purposes. Specific sense and antisense oligonucleotides primers were designed, in general, using *Primer 3* tool from the Biology Work Bench (http://seqtool.sdsc.edu).

A typical PCR reaction was prepared as follows:

1-100 ng of Template DNA
5 µl of 10x reaction buffer
5 µl of 2.5 nM dNTP mix (dATP, dTTP, dGTP, dCTP; Peqlab)
5 µl of 3 µM sense primer
5 µl of 3 µM antisense primer
1 U of Polymerase
add to 50 µl ddH_20

The following PCR parameters were adjusted to the appropriate conditions,

depending on the primer, the size of the fragment to be amplified and the desired amount of DNA.

5 min	94°C	
30 sec	94°C	
30 sec	54-62°C	x 15-35 cycles
30 sec-1min	72°C	
10 min	72°C	

5.1.16 Colony PCR

Colony PCR was used for fast screening of large number of transformed *E. coli* colonies for positive clones. PCR reactions using primers within the cloned fragment and the appropriate flanking site of the vector were prepared. Single colonies were picked with a sterile toothpick and dipped in the PCR reaction. The toothpick was used to inoculate in parallel 1.5 ml of liquid cultures. These cultures were grown overnight in LB-medium containing the appropriate selection antibiotic for isolation of plasmid DNA. The PCR reaction was boiled at 95°C for 5 min prior to the PCR in order to lyse the cells and release plasmid DNA. PCR products were analyzed on an agarose gel, only positive clones showed PCR products.

5.1.17 Isolation of genomic DNA (Quick Fly Genomic DNA Prep)

In a standard procedure, 30 flies were anesthetized, collected in eppendorf tubes, and frozen at -80°C. The flies were homogenized with a micropestle (Eppendorf) in 100 µl Buffer A. An additional 100 µl Buffer A was added and grinding continued. After addition of another 200 µl Buffer A, grinding was continued until only cuticles remained. The homogenate was incubated at 65°C for 30 min. 800 µl of freshly prepared LiCl/KAc solution was added, mixed, and left on ice for at least 10 min. After centrifugation at RT/13.000 rpm for 15 min, 1 ml of the supernatant was transferred into a new tube, avoiding floating crud. Following another centrifugation and supernatant transfer step, DNA was precipitated by addition of 600 µl isopropanol, centrifuged at RT/13.000 rpm for 15 min, washed twice with 70% ethanol, and speed vacuum dried for 2-3 min. To prevent any shearing of the DNA, 150 µl ddH_2O was added and the solution was left overnight at 4°C to dissolve. The following day, the DNA was resuspended by carefully pipetting up and down and incubation at

65°C for 15 min. The DNA was then stored at –20°C. If a different number of flies was used, all volumes were adjusted accordingly.

Buffer A:	100 mM Tris-HCl pH 7.5 100 mM EDTA 100 mM NaCl 0.5% SDS
LiCl/KAc:	mix 1 part 5 M KAc with 2.5 parts 6 M LiCl just before use

5.1.18 Single fly PCR

Single flies were squashed in 50 µl Gloor and Engel's extraction buffer (Gloor et al. 1993) in a 1.5 ml Eppendorf tube using a 200 µl pipette tip. The homogenate was incubated for 20 min at 37°C, and then for 2 min at 80°C to inactivate the proteinase K. The DNA was stored at 4°C. Typically, 4 µl of DNA isolated from whole flies, or 5 µl of DNA isolated from single heads was used in a standard PCR reaction.

Gloor and Engel's extraction buffer:	10 mM Tris pH 8.2 1 mM EDTA 25 mM NaCl 200 µg/ml proteinase K (20 mg/ml stock, added freshly)

5.1.19 Southern Blot

Probe synthesis and test hybridization

For probe production, the DIG High Prime DIG labelling kit (Roche) was used according to the manufacturer's instructions. Briefly, 1 µg of template DNA in an 18 µl volume were denatured by boiling for 10 min, then quickly chilled on ice. 4 µl of DIG High Prime mix were added. The reaction was mixed, centrifuged briefly, and then incubated at 37°C overnight. The next day, the labelled DNA probe was purified using the QIAquick PCR purification kit, and eluted in 50 µl ddH_2O. The efficiency of probe synthesis and labelling was tested by spotting 1 µl of serial dilutions of the probe, starting from 1:50 to up to 1:100.000, onto a positively charged nylon membrane (Amersham Bioscience). The DNA was cross-linked to the membrane twice at 1200µJ using an UV Stratalinker (Stratagene). Detection was performed as described below.

Preparation of DNA, restriction endonuclease digestion, and gel electrophoresis

Genomic DNA from flies was isolated as described above. 20 µl of DNA were used which corresponds to 12 flies. The DNA was digested with Restriction enzymes for at least 4 h at 37°C in 75 µl volume. The DNA was ethanol-precipitated for at least 1 h at -20°C as described, with 1 µl glycogen (Roche, 20 mg/ml) added as a carrier, and resuspended in 20 µl ddH_2O.

Embryos genomic DNA was obtained by homogenizing 2 gr of embryos in 5 ml of Restriction Enzyme buffer. The embryo extract was filtrated through 2 layers of miracloth, alicuotted in Eppendorf tubes, transferred into a pre-cooled ultrasound sonicator (Bioruptor UCD-200, Diagenode) and sonicated for 3 cycles 30 sec ON, 30 sec OFF at maximum power (H). After mild shearing to make the chromatin soluble the samples were centrifuged and an aliquot of 90 µl of the supernatant was used for Restriction Enzyme digest.

The DNA samples supplemented with loading buffer were loaded on a 0.8% agarose gel and run overnight at 30-40 V and 4°C.

Denaturation, neutralization, and transfer

To prepare the agarose gel for the transfer to a nylon membrane, the gel was submerged in 0.25 M HCl for 10 min on a shaker at RT and rinsed with ddH_2O prior to the incubation for 2x 15 min in denaturation buffer. After shortly rinsing in ddH_2O, the gel was incubated for 2x 15 min in neutralization buffer, followed by an additional rinse step and 10 min incubation in 20x SSC buffer. For the capillary transfer to the membrane, the gel was placed upside down on Whatman paper (Schleicher & Schuell) on a glass plate, with its left and right ends hanging in 20x SSC buffer. The positively charged nylon membrane (Amersham Bioscience) was moistened with 20x SSC buffer and put on the upper side of the gel. Three layers of Whatman paper and a layer of approximately 10 cm of apura paper were put on top of it and fixed with a weighted glass plate. Transfer was allowed to proceed overnight. The next day, the positions of the gel and the lanes were marked on the membrane using a pencil. Then the membrane was washed for 5 min in ddH_2O prior cross-linking of the DNA with UV light. Cross-linking of the wet membrane was first performed twice on the upper nylon membrane side, then twice on the lower side at 1200 µJ. The blot was either used directly for hybridization or sealed in a nylon bag and stored at 4°C.

Solutions:	Denaturation buffer:	0.5 M NaOH
		1.5 M NaCl
	Neutralization buffer:	0.5 M Tris-HCl pH 7.5
		3 M NaCl
	20x SSC:	3 M NaCl
		300 mM Sodium citrate pH 7.0

Hybridization

The DIG Easy Hybridization solution (Roche) was prepared according to the manufacturer's instructions and pre-warmed to 40°C. The nylon membrane was blocked by incubation in Hybridization solution for 30 min at 40°C. The DIG-labelled DNA probe was denatured by boiling at 95°C for 5 min and immediately put on ice to prevent renaturalization. After addition of denatured probe (in general 50 µl) to 8 ml DIG Easy Hybridization solution, the blot was hybridized at 40°C overnight with gentle agitation. The membrane was first washed twice for 5 min in a large volume of wash buffer 1 at RT, then twice for 15 min in wash buffer 2 at 68°C, both steps under constant agitation.

Buffers:	Maleic acid buffer:	0.1 M Maleic acid
		0.15 M NaCl
		adjust to pH 7.5 with NaOH pellets
	Wash buffer 1:	1x SSC
		0.1% (w/v) SDS
	Wash buffer 2:	0.1x SSC
		0.1% (w/v) SDS

Detection

After transferring the membrane into a flat container, it was rinsed 2-5 min in washing buffer and incubated for 30 min in 100 ml freshly prepared blocking solution, 1% (w/v) blocking reagent (Roche) in washing buffer, according to the manufacturer's instructions. The α-DIG-AP conjugate was diluted to 75 mU/ml (1:10.000) in 20 ml blocking solution. Antibody incubation was done for 30 min at RT, then the membrane was washed twice for 15 min in 100 ml washing buffer and equilibrated for 2-5 min with 20 ml detection buffer. For detection, the membrane was transferred into a hybridization bag, 1 ml CSPD (Roche) was added and left for 5 min at RT. To activate

the CSPD, the hybridization bag was sealed and incubated for 10 min at 37°C. The membrane was then exposed to a film (Roche) for 15-30 min.

Buffers:	Washing buffer:	0.1 M Maleic acid
		0.15 M NaCl
		0.3% (v/v) Tween-20
		adjust to pH 7.5
	Detection buffer:	0.1 M Tris-HCl pH 9.5
		0.1 M NaCl
		50 mM $MgCl_2$

5.2 Chromatin methods

5.2.1 In vivo formaldehyde cross-link of Drosophila embryos

Embryos were collected for 12 h on acetic acid agar plates and dechorionated (see 5.3.3). After extensive washes with tap water, excessive liquid was removed and 2 g of embryos were transferred into a 50 ml Falcon tube. After washing once in 0.01% Triton X-100 / PBS, the embryos were cross-linked for 15 min in 10 ml Cross-linking solution in the presence of 30 ml n-heptane. During the incubation time, the samples were shaken vigorously on a roller. Cross-linked embryos were spun down for 1 min at 500 g, and the reaction was stopped by exchanging the supernatant with Stop solution. After spinning down, the supernatant was exchanged by 10 ml Wash A buffer and the embryos were incubated for 10 min on a roller. To remove the Wash A buffer, the embryos were centrifuged again for 1 min at 500 g, resuspended in 10 ml Wash B buffer and incubated another 10 min on a roller. After centrifugation, the supernatant was discarded and the tubes snap-frozen in liquid nitrogen.

Buffers:	PBS:	137 mM NaCl
		2.7 mM KCl
		10 mM Na_2HPO_4
		2 mM KH_2PO_4
		0.01% (v/v) Triton X-100 / PBS
	Cross-linking solution:	50 mM Hepes pH 8
		1 mM EDTA
		0.5 mM EGTA
		100 mM NaCl

	3.6% formaldehyde, add just before cross-linking
Stop Solution:	125 mM glycine / 0.01% (v/v) Triton X-100 / PBS
Wash A:	10 mM Hepes pH 7.6
	10 mM EDTA
	0.5 mM EGTA
	0.25% (v/v) Triton X-100
Wash B:	10 mM Hepes pH 7.6
	200 mM NaCl
	1 mM EDTA
	0.5 mM EGTA
	0.01% (v/v) Triton X-100

5.2.2 Protein-Chromatin nuclear extract (pc-NE)

20-30 gr of cross-linked embryos were thawed in a water bath at 10 °C in the cold room. The embryos were then resuspended in 5 ml/gr of RB buffer and homogenized by three passages through a Yamato homogenizer at 1000 rpm. The homogenate was filtered through 2 layers of Miracloth (Calbiochem) and centrifuged for 15 min at 10.000 g. The supernatant was discharged and the walls of the tubes carefully cleaned with a precision wipe. The nuclei pellet was resuspended in a total of 30 ml of sonication buffer per 10 gr of embryos and aliquot in 15 or 30 ml per falcon tube.

To shear the chromatin to an average size of 3 kb, the Falcon tubes were transferred into a bicker containing ice and ethanol to keep them cool and sonified with a microtip sonifier for 4-5 cycles (30 sec ON- 90 sec OFF) at max output level avoiding foaming. The sheared protein-chromatin samples were then centrifuged for 10 min at approximately 3000 g and the supernatant snap-frozen.

Buffers:	RB buffer:	15 mM Hepes
		10 mM KCl
		5 mM $MgCl_2$
		0.1 mM EDTA
		0.1 mM EGTA
		350 mM Sucrose
		pH 7.6

Sonication buffer:	10 mM Hepes
	1 mM EDTA
	0.5 mM EGTA
	150 mM KCl
	pH 7.6

5.2.3 Chromatin Immunoprecipitacion (ChIP) from Drosophila embryos

Chromatin was prepared as described above. Before centrifugation of sheared chromatin 0.5% N-lauroylsarcosine was added.

To check the quality of the chromatin, a 100 µl aliquot of each sample was extracted and analyzed by agarose gel electrophoresis. First, to digest the RNA, DNase-free RNase was added to each aliquot at a final concentration of 50 µg/ml, followed by a 30 min incubation at 37°C. Then, proteinase K was added to a final concentration of 500 µg/ml, and SDS to a final concentration of 1 % (w/v). To partially reverse cross-link, the samples were incubated for 1 h at 56°C and the DNA was extracted with phenol / chloroform, and precipitated with ethanol, using glycogen as a carrier (see 5.1.1 and 5.1.2). Finally, the DNA was resuspended in ddH_2O, and 3-5 µg were loaded on an agarose gel and concentration was checked with a Nanodrop spectrophotometer.

A. ChIP using antibodies

100 µl Protein A Sepharose (Amersham) slurry per sample were pre-blocked for 2-3 h at 4°C on a rotating wheel using 1 mg/ml BSA and 1 mg/ml herring sperm DNA (Invitrogen) diluted in RIPA buffer. To remove the blocking solution, the samples were centrifuged for 2 min at 3000 g at 4°C. After 3x 5 min washes in RIPA buffer at 4°C, 100-150 µg of chromatin per sample were thawed on ice and adjusted to RIPA conditions:

x µl (=100 µg) chromatin	10 µl 1M Tris pH 8
	28 µl 5 M NaCl
	10 µl 10% Triton X-100
	1 µl 10% SDS
	1 µl 10% Na-Deoxycholate
	10 µl 100 mM PMSF
	ad. 1 ml ddH_2O

1 ml of RIPA-adjusted chromatin was added to 50 µl of pre-blocked Protein A

Sepharose and incubated for 2 h at 4°C on a roller for pre-clearing. After centrifugation (2 min 5500 rpm at 4°C), the pre-cleared chromatin was transferred into a new tube. One aliquot per sample was saved as input control and stored at -20°C. 2-10 µg of antibody were added per IP. The samples were incubated on a rotating wheel at 4°C overnight. To isolate the antibody-chromatin complexes, 50 µl pre-blocked Protein A Sepharose per sample were added and incubated for 3 h at 4°C on a roller. To eliminate non-specific interactions, the samples were then washed 5 x 10 min at 4°C in RIPA buffer, 3 x 10 min at 4°C in LiCl buffer, and 2 x 10min at 4°C in TE buffer. Between the washing steps, the samples were centrifuged for 2 min at 5500 rpm at 4°C. To be able to remove the supernatants without loss of sample, 1 ml syringes with 25 gauge needles were used.

To extract the DNA, the Protein A Sepharose complexes were first resuspended in 100 µl TE buffer, then DNase-free RNase was added to each sample (including the input controls) to a final concentration of 50 µg/ml. RNA digestion was allowed to proceed for 30 min at 37°C, before the samples were adjusted to 0.5% SDS and proteinase K was added to a final concentration of 500 µg/ml. After overnight incubation at 37°C, the samples were heated to 65°C for 6 h to reverse the cross-link. Subsequently, the DNA was extracted using phenol / chloroform (see 5.1.1). The lower phenol phase was back-extracted using an equal volume of 50 mM Tris-HCl pH 8. The aqueous phases from the phenol extraction and the back-extraction were combined and extracted with chloroform. The DNA in the upper aqueous phase was ethanol-precipitated, using glycogen as a carrier (see 5.1.2). The DNA pellets were resuspended in 50 µl ddH_2O and stored at -20°C.

Buffers:	RIPA buffer:	10 mM Tris-HCl pH 8
		140 mM NaCl
		1 mM EDTA
		1% (v/v) Triton X-100
		0.1% (w/v) SDS
		0.1% (w/v) Na-Deoxycholate
		1 mM PMSF (add just before use)
		1 mM PMSF (add just before use)
	LiCl:	10 mM Tris pH 8
		250 mM LiCl

		0.5% (v/v) NP-40
		0.5% (w/v) Na-Deoxycholate
		1 mM EDTA
	TE buffer:	10 mM Tris-HCl pH 7.4
		1 mM EDTA

B. ChIP using Streptavidin Agarose beads

Streptavidin Sepharose beads were blocked as described for Protein A Sepharose. Chromatin was pre-cleared using Protein A Sepharose. An aliquot of pre-cleared chromatin (100-150 μg) was incubated with 80 μl of Streptavidin Sepharose 50% slurry overnight at 4 °C on a rotating wheel. Next day, the samples were washed twice for 10 min at 4°C in FA buffer, twice for 10 min at 4°C LiCl wash buffer, three times for 8 min at room temperature in SDS buffer, and twice for 10 min at 4°C in TE buffer. Between the washing steps, the samples were centrifuged for 2 min at 5500 rpm at 4°C. Finally, the beads were resuspended in 100 μl of TE buffer and the cross-link was reversed as described above.

Buffers:	FA buffer:	50 mM HEPES-KOH, ph 7.5
		150 mM NaCl
		1 mM EDTA
		1% Triton X 100
		0.1% SDS
		0.1% Na deoxycholate
		1 mM PMSF (add fresh)
	LiCl wash buffer:	10 mM Tris-HCl pH 8.0
		1 mM EDTA
		1% Na-deoxycholate
		1% NP40
		0.5 M LiCl
		1 mM PMSF (add fresh)
	SDS wash buffer	10 mM Tris pH 8.0
		1 mM EDTA
		3 % SDS

Linker mediated PCR to amplify sample

In case of necessary, the immunoprecipitated and input DNA were amplified by PCR in a linear manner. For this 7 µl of ChIP material and 7 µl of 1/10 dilution of input material were ligated to pre-annealed linker oligos (24mer-20mer) overnight at 16 °C .

Ligation reaction:	1 µl of annealed oligos
	7 µl IP
	1 µl 10X ligase buffer
	1 µl ligase (Roche)

The next day the ligation mix was used as template in a 20 cycles PCR reaction using the oligo 20mer as primer. The PCR products were separated using QIAquick PCR Purification Kit and resuspended in 50 µl of ddH_2O.

Analysis of IP material by semi-quantitative PCR

Directly after ChIP or after linker mediated amplification, IP and input materials were used as templates for the specific PCR reactions. The PCR was adjusted to conditions under which the reaction was still in the linear range of amplification (usually 28-30 cycles). To quantify the relative amounts of precipitated material, 20 µl of each 50 µl PCR reaction were loaded on a 1.5% agarose gel and scanned using a E-box gel documentation system (Peqlab). The intensities of PCR bands were quantified using the AIDA imaging software.

5.2.4 Chromatin purification by Cesium Chloride gradient

Protein-chromatin nuclear extract was prepared as described before (5.2.2). After sonication of the chromatin, the sample was adjusted to 0.5 % N-lauroylsarcosine, rotated for 10 min at 4°C, and centrifuged 10 min at 2500 g to spin down the debris.

For CsCl gradient, a volume of chromatin corresponding to 1.5 gr of embryos was adjusted to 11.5 ml by adding sonication buffer containing 0.5% of N-lauroylsarcosine. Slowly 7,8 to 7,952 gr of CsCl were added to the sample and the volume was adjusted to 14 ml by adding sonication buffer. Next, the sample was loaded with a 20 ml syringe in polyallomer quick-seal centrifuge tube for the Beckman 70.1 Ti rotor. Up to 12 samples were centrifuged at 55000 rpm for about 40 hs at 20°C.

After centrifugation, the sample was eluted in 700 µl fractions with a peristaltic pump from the bottom of the tube. An aliquot of 70 µl of each fraction was desalted with

Micro Bio-Spin chromatography columns (Bio-Rad), reversed cross-link as described above (5.2.3) and analyzed by agarose gel electrophoresis.
The chromatin containing fractions were pooled and dialyzed overnight at 4°C against Dialysis buffer, changing the buffer repeatedly.

Dialysis buffer (DB):	10 mM Tris-HCl pH 8.0
	4 % Glycerol
	1 mM EDTA
	0.5 mM EGTA

5.2.5 Chromatin digestion with restriction enzymes

Nuclei from *Drosophila* cross-linked embryos were prepared as described in 5.2.2. In order to break the nuclei open without shearing of the chromatin, the nuclei were sonicated with a microtip for 3 cycles of 10 sec at minimum input level and centrifuged to discard debris. The supernatant, un-sheared pc-NE containing the chromatin fraction was used for the digest. For this an aliquot of 90 µl of pc-NE, containing around 1 mg protein, was incubated at 37°C for at least 4 h in the presence of 10 U of Not1 and 10 U of SalI and 45 µl of restriction digest buffer. Control samples were generated by incubation at 37°C without enzymes in the presence or absence of 1.5 mM EDTA. In addition one aliquot was kept frozen at -20°C.

Restriction Digest buffer:	100 mM NaCl
(Buffer 3 NEB)	50 mM TrisCl
	10 mM MgC1
	1 mM DTT
	pH 7.9

After incubation the samples were reversed cross-link as in 5.2.3 and analyzed by agarose gel electrophoresis or southern blot.

5.3 Biochemical methods

*5.3.1 SDS-Polyacrylamide Gel Electrophoresis (***SDS-PAGE***)*

Tris-Tricine gels were prepared according to (Laemmli 1970). Generally, 12% gels were used and poured into SDS-PAGE gel chambers from Hoefer or Biorad. Electrophoresis was started at 150 V until the samples reached the separating gel and continued for 1-2 h at 180 V. Protein samples were supplemented with 5x SDS sample buffer

and boiled for 5 min at 95°C, followed by a 3 min spin at full speed and loading on the gel.

Buffers:	5x Tris glycin SDS buffer:	15.1 g Tris-base
		72 g glycine
		5 g SDS
		add ddH_2O to 1 l
	5x SDS sample buffer:	250 mM Tris-HCl pH 6.8
		10% SDS (w/v)
		50% (w/v) glycerin
		500 mM b-mercaptoethanol or DTT
		0.05% Bromophenol-blue

5.3.2 Bis-Tris-HCl polyacrylamide gel electrophoresis

Separation of proteins by electrophoresis was also carried out using precast 4-12% Bis-Tris gels (Invitrogen) following the manufactures protocol.

20x SDS running buffer	1 M Mes (2-(N-morpholino) ethansufonic acid)
	1 M Tris-base 0.1% SDS
	0.0205 mM EDTA

5.3.3 Coomassie Blue staining of proteins

Following SDS-PAGE, the gel was incubated with Coomassie staining solution on a horizontal shaker for 30-60 min. Afterwards the gel was washed several times with destaining solution, until the nonspecific Coomassie background was removed. The gel was rehydrated in 4% glycerol for 1-2 h and then dried on Whatman paper in a vacuum gel dryer for 1.5 h at 70 °C.

Coomassie staining solution:	0.1% (w/v) Coomassie Brilliant Blue R-250
	45% (v/v) methanol
	10% (v/v) acetic acid
Destaining solution:	45% (v/v) methanol
	10% (v/v) acetic acid

5.3.4 Western Blot (wet blot)

Proteins separated by SDS-PAGE were transferred from the gel to a Hybond

nitrocellulose membrane (Amersham) using electrophoresis. After SDS-PAGE, the gel was equilibrated in transfer buffer for 2 min and all other components were soaked in transfer buffer. The gel and the membrane were sandwiched between perforated plastic plates as follows:

Anode (+), sponge pad
3 Whatman sheets nitrocellulose membrane SDS gel 3 Whatman sheets sponge pad
Cathode (-)

The transfer was performed in a blotting tank for 2 h at 200 mA at 4°C.

Transfer buffer:	39 mM glycine
	48 mM Tris-base
	0.0357% SDS
	20% methanol

Ponceau S staining

Proteins immobilized on a membrane can be reversibly stained with the Ponceau S dye to evaluate the efficiency of protein transfer after Western Blotting. The nitrocellulose membrane was stained in Ponceau S solution (Sigma) for less than 1 min. The membrane staining could be completely removed by incubation in PBS.

Detection

After Western Blot transfer of the proteins to the nitrocellulose membrane, the membrane was blocked for 1 h in PBST / 5% (w/v) skimmed milk or BSA. The primary antibody was diluted in PBST to the appropriate concentration and the blot was incubated overnight at 4 °C. The membrane was then washed three times with PBST for 10 min. Incubation of the blot with the secondary antibody conjugated to horseradish peroxidase (HRP) was carried out for 1-2 h at RT. The membrane was washed again for 3x 12 min with PBST prior to detection. For this purpose, the membrane was incubated with the ECL (Amersham) reagent for 1 min and the chemiluminiscent signal was visualized by exposure of Hyperfilm ECL (Amersham) films. In the case of very weak signals, the blot was washed in PBST and incubated for 3 min with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) before exposure was repeated.

For detection of biotinylated proteins, the membrane was incubated after blocking with

Streptavidin or Neutravidin HRP in PBST 0.2% for 1h at RT. The membrane was washed 3 x 12 min in PBST 0.2% and 1 x 12 min with PBST 0.2% NaCl 500 mM and the signal detected as described above.

10x PBS (Phosphate buffered saline):	10 mM Na_2HPO_4
	2 mM KH_2PO_4
	137 mM NaCl
	2.7 mM KCl adjusted to pH 7.4 (hydrochloric acid)
PBST (PBS- Tween20):	PBS
	0.05%-0.2% Tween20

Re-probing of western blot membranes

Prior to re-probing the membrane with different antibodies, the previous antibodies had to be removed. Therefore, the membrane was incubated in stripping buffer (Restore™ Western Blot Stripping Buffer, Pierce) on a horizontal shaker for 15 min at 37°C and extensively washed with PBS. The blot was blocked again for 1 h and a new primary antibody could be applied as described.

5.3.5 Streptavidin-Shift

Protein samples from pull-down material or pc-NE were incubated on SDS-sample buffer at 95°C to reverse cross-link. After incubation the samples were cooled to room temperature and 8 µg of Streptavidin were added to 40-50 µg of protein and incubated for 5 min at RT. Next, the samples were analyzed by SDS-PAGE and Western Blotting.

5.3.6 Cross-link of Drosophila embryos with DSP

Drosophila embryos were collected as described in 5.5.3. The embryos were cross-linked for 30 min as described in 5.2.1 with addition of DSP. For this, a 5 mM stock solution of DSP in DMSO was prepared and diluted 1:4 in cross-linking solution containing formaldehyde. After cross-link the embryos were washed and stored as described above (5.2.1). The chromatin was prepared by direct shearing of the embryos with a sonifier without pre-purification of nuclei. Reversal of cross-link was carried by heating the samples to reverse formaldehyde cross-link and by addition of sample buffer with ß-mercaptoethanol to reverse DSP cross-link. The un-reversed controls were

prepared by avoiding heating and using 300 µM of Iodacetamide instead of ß-mercaptoethanol in the sample buffer.

5.3.7 Cross-link of Drosophila nuclei with DTBP and streptavidin pull-down

Drosophila nuclei from formaldehyde cross-linked embryos were prepared as described in 5.2.2 and resuspended in 3gr of embryos / 3ml of buffer B. DTBP solution was added to 2.5 ml of nuclei suspension and incubated for 2 hs at 4°C on rocking. To stop the reaction, 180 mM final concentration of Tris pH 7.6 was added. After that, the nuclei were recovered by spinning and washed with Buffer B. Finally, the nuclei were resuspended in Dialysis buffer and sonified to shear the chromatin.

The streptavidin pull-down from DTBP cross-linked chromatin was performed as described in 5.3.11

Buffer B:	10 mM Hepes
	10 mM KCl
	5 mM $MgCl_2$
	0.1 mM EDTA
	0.5 mM EGTA

5.3.8 In-Vitro biotinylation of nuclear extract

Protein-chromatin nuclear extract was prepared as described before (5.2.2). For all biotinylation experiments, the extracts contained no EDTA.

An aliquot of pc-NE (120 µg protein) was incubated in the presence of 10 µg of recombinant BirA enzyme (kindly provided by Dr. Christian Beisel), BirA buffer and D-Biotin (up to 5 mM). The reaction was incubated at 30 °C for at least 30 min and the samples analyzed after reversal of cross-link by SDS-PAGE using Streptavidin-HRP.

Buffers:	10 X BirA buffer:	10 mM Hepes pH 7.6
		100 mM ATP
		100 mM MgOAc
	Biotin stock Solution:	50 mM D-Biotin

For ChIP, 15 ml of pc-NE were incubated in the presence of BirA (250 µg) and 1mM D-Biotin for 1.30 h at 30°C. After biotinylation reaction, the chromatin was purified by CsCl gradient and used in Streptavidin ChIP experiments as described in 5.2.3 and 5.2.4.

5.3.9 Protein pull-down using Streptavidin beads.

Streptavidin Beads were washed in Dialysis buffer and blocked as described in 5.2.3 B. Pull-down experiments were performed by incubation of pc-NE or CsCl purified chromatin, with Streptavidin beads in Dialysis buffer O/N at 4°C in the presence of 0.05-0.25% of NP40, complete EDTA free protease inhibitors and 1mM PMSF. The next day, the unbound fraction was separated by centrifugation and the beads were washed thoroughly with Dialysis buffer with 250mM of NaCl. After washing, the bound proteins were recovered by direct boiling of the beads with sample buffer o by elution with Elution buffer (see 5.3.14)

5.3.10 Salt separation of chromatin from soluble nuclear proteins

Cross-linked embryos were homogenized and nuclei were prepared as described in 5.2.2. An amount of nuclei corresponding to 5 gr of embryos, were resuspended in 15 ml of NB buffer and sonicated in mild conditions (3 cycles of 20 sec at middle output level) in a micro tip sonicator to break open the nuclei without shearing the chromatin. Next, the extracts were centrifuged 20 min at 13.000 rpm at 4 °C and the supernatant (nuclear proteins) separated from the pellet (chromatin). The pellet was then resuspended with a pipette tip in 15 ml of NW buffer and incubated 10 min in a rotator at 4°C. After that, the chromatin was pelleted by centrifugation. The washing step was repeated a second time with 10 mM Tris-HCl buffer pH 8.0. After washing, the chromatin pellet was resuspended in 3 ml 1X NEB buffer 3, sonicated 4 cycles of 15 sec (middle output level) and centrifuged producing a final pellet. Aliquots of all steps (pellets and supernatant) were analyzed, after reversal of cross-link, by agarose gel electrophoresis.

Buffers:	NB (Nuclear Break) buffer:	15 mM Hepes pH 7.6
		400 mM NaCl
		5 mM MgCl
		0.1 mM EDTA
		0.5 mM EGTA
		10 mM KCl
		25 % Glycerol
		Protease inhibitors
	NW (Nuclear Wash) buffer:	Equal to NB buffer without NaCl.

5.3.11 Ultrafiltration of pc-NE

For Ultrafiltration two different molecular weight cut off (MWCO) devices were tested, Vivaspin 300kDa and Vivaspin 1000kDa (Sartorius). Before use, the centrifugation devices were rinsed with ddH_2O and then loaded with pc-NE. The 300kDa has a capacity of 5 ml, thus it was loaded sequentially with 5 ml and 4 ml of pc-NE. The 1000kDa has a capacity of 20 ml, thus it was loaded sequentially with 10 ml and 5 ml of pc-NE. Centrifugation was carried out at 9°C for 2.45 hs and DB buffer (1 and 2 ml) was added to the devices to re-dilute the samples, which were then centrifuged for additional 30 min. The final volumes were 3 ml and 5 ml that constituted 1/3 of input volume. Protein concentrations were determined in input material, flow-through and final sample. Aliquots of each sample were analyzed after reversal of cross-link by SDS-PAGE and Western Blotting with α-TetR antibody.

5.3.12 Chromatin dialysis

Protein-chromatin nuclear extracts were dialyzed in DB buffer with 6% sucrose at 4°C using a Spectra/Por Cellulose ester membrane with a MWCO of 300 KDa. Before dialysis the tubing was rinsed in DB buffer. 2 ml of pc-NE were dialysed in 5 cm of tubing. In some cases the starting NaCl concentration of the extracts and dialysis buffer was adjusted to 1 M and the salt concentration of the dialysis buffer was decreased gradually with each buffer change. The buffer was replaced at least 4 times and the final NaCl concentration was 125 mM. For some experiments the tubing was replaced 2 times during dialysis.

After dialysis the extracts were recovered and 2 aliquots were separated for chromatin and protein analysis. All the samples were centrifuged 10 min at 13.000 rpm at 4°C and pellet and supernatant were analyzed separately.

5.3.13 Depletion of TetR protein by binding to TetO beads

Preparation of TetO beads

DNA oligos carrying a single TetO binding site (1-TetO_Col Fw and Rv) were coupled via a covalent link to SulfoLink® Coupling Gel (Pierce). For this, the Fw DNA oligo was synthesized with a Thiol group at the 5' end. This free sulfhydryl group can react with iodoacetyl groups on the SulfoLink® gel forming a Thioether bond.

First, 2 ml of SulfoLink® Coupling Gel (50% slurry) were equilibrated with 4 gel-bed volumes of Coupling buffer. Next 30 µMoles of annealed double stranded oligo in 2 ml of Coupling buffer were incubated with 1 ml of gel-bed at RT for 15 min with shaking followed by additional 30 min without shaking. After that, the supernatant was removed and saved for DNA concentration determination and the beads were washed with 3 bed volumes of Coupling buffer. The coupling efficiency, was determined by comparing the DNA concentration of the non-coupled fraction (supernatant) to the starting DNA sample.

After coupling reaction, the nonspecific binding sites on the beads were blocked by incubation with one bed volume of Quenching Reagent for 15 min at RT with mixing and additional 30 min with no shaking. The beads were then washed with at least 6 bed volumes of Wash Solution followed by 2 gel volumes of Storage buffer and stored at 4°C.

Coupling buffer:	50 mM Tris 5 mM EDTA-Na pH 8.5 Prepare a volume equal to 20 times the volume of SulfoLink® Gel to be used.
Quenching Reagent:	50 mM L-cysteine-HCl in Coupling buffer (prepare fresh)
Wash Solution:	1 M NaCl
Storage buffer:	PBS 0.05% Sodium azide

TetR depletion experiment

For TetR depletion from pc-NE, 140 µl of extract (12.5 ng protein / µl) were incubated with 100 µl of TetO beads, containing 2400 pMol TetO in the presence of 10 mM Mg^{+2} and complete EDTA free protease inhibitors during 30 min at 30°C. Next, the beads were centrifuged and the supernatant (un-bound) was recovered. The beads were washed 4 times 150 µl of buffer with no Mg^{+2} and resuspended in 140 µl of sample buffer. Sample buffer was added to all fractions and the samples were incubated 40 min at 95°C for reversal of cross-link after which they were analyzed by SDS-PAGE.

As control, 12 ng of pure TetR protein were incubated with 50 µl of beads (1200 pMol TetO) in the same conditions as described above.

5.3.14 Elution of proteins from streptavidin beads and TCA precipitation

For elution of pulled material, the beads were resuspended in 250 µl of buffer with benzonase and incubated 10 min at RT for DNA digestion. After that, the supernatant was removed and the beads incubated at 95°C for 30min with 250 µl of elution buffer containing 95% formamide. Next, the supernatant was recovered, and the beads washed several times with buffer. All the fractions were poled and the final volume divided in 3 eppendorf tubes for TCA precipitation. At this point, an aliquot of input and unbound fraction were included.

To precipitate the proteins the samples were incubated with 1:4 volume of TCA-DOC for 30 min on ice followed by centrifugation for 15 min at 4°C at 13000 rpm. The pellet was the washed with Acetone by sonication and incubation during 10 min at RT. Then, the samples were centrifuged and the washing procedure was repeated. Finally, the protein pellets were resuspended in sample buffer, pooled and reversed cross-link for 30' at 95°C. After centrifugation, the supernatant was loaded on a pre-casted polyacrilamide gel. Proteins were analyzed by incubation with different antibodies.

Elution buffer:	95% formamide
	10 mM EDTA pH 8.2
TCA-DOC	100% TCA
	4 mg/ml Sodium Deoxycholate

5.4 Cloning strategies

All clones were checked by digestion with restriction enzymes for proper orientation and length of the cloned fragments or by PCR with appropriate primers, and the final constructs were sequenced. Cloning was performed using XL1-Blue or SURE *E. coli* host cells.

5.4.1 PRE constructs

Many different constructs were generated bearing the *Fab-7* PRE and the *vg* PRE. In the different constructs the TetO site was placed in different distances from the PRE, downstream or upstream of it.

A. Generation of TetO-FRT cassette

The TetO cassette consisting of seven repeats of the palindromic TetR binding site was obtained by restriction digest with XhoI and KpnI from the plasmid pUHC13.3 kindly

provided by Dr. Bujard. In order to generate a TetO cassette flanked by FRT sites, the pSVPAZ-FRT plasmid was digested with HindIII and BamHI and the annealed oligo OligoKpn was cloned in this site. The TetO cassette was then cloned in between XhoI-KpnI sites, resulting in pSVPAZ-TetO-FRT.

B. Cloning of TetO-FRT in pCaSpeR4

The TetO-FRT cassette was cloned downstream of the *miniwhite* gene *(mw*) of pCaSpeR4. Two different constructs were generated which differ in the length of the spacing between the end of mw gene and the beginning of the TetO.

To facilitate the cloning the OligoBsiWI was inserted into BsiWI-AgeI sites of the pCaSpeR4. Next, the TetO-FRT cassette was extracted from pSVPAZ-TetO-FRT by digestion with AgeI-MluI or with AgeI-BsiWI resulting in a 1.7 kb and 3.1 kb fragments respectively, and inserted in pCaSpeR4 generating the plasmids pC4TetOSsp and pC4TetOLsp.

C. Insertion of LoxP sites

The LoxP sequence was obtained as an oligo and cloned twice in tandem into pBKSII with BamHI-HindIII the first insert and HindIII-XhoI the second, resulting in pBKSLoxP (see 7.1 Vector Maps). Next, the LoxP cassette consisting of two repeats was liberated from pBKSLoxP with SpeI-XbaI and cloned into pC4TetO (Lsp and Ssp) SpeI-EcoRI site upstream of *miniwhite* reporter gene.

D. Cloning of pC4Fab7-2, pC4Fab7-7 and pC4vg-1

A 3.6 kb *Fab7* PRE fragment was obtained by PCR amplification using pre*Fab7* Fw and Rv plasmids and pUZ-*Fab7* DNA as a template (kindly provided by S. Schmitt). The PCR product was digested with restriction enzymes and cloned into SpeI-NotI sites in the pC4TetO plasmids.

The *vestigial* (*vg*) PRE, 3.2 kb, was obtained by restriction digest of the pUZ-*vg* plasmid (kindly provided by N. Lee) and inserted into the pC4TetO plasmids as described above. Of the resulting plasmids, three were injected in embryos:

pC4*Fab7*-2, pC4*Fab7*-7 and pC4*vg*-1 (See 7.1 Vector Maps)

E. Cloning of pC4Fab7-6 and pC4vg-9

To generate PREs constructs where the TetO is positioned upstream of the PRE, the pC4TetOLspLoxP was digested with AgeI-BsiWI and the TetO-FRT cassette was

liberated. The plasmid ends were filled-in and the plasmid was re-ligated generating pC4LoxP. The oligoBsu was then introduced into XhoI-BamHI sites of this plasmid and the TetO-FRT cassette (from pSVPAZ-TetO-FRT) was re cloned in. The resulting plasmid, pC4TetOLoxP, contains 400 bp spacing between the TetO cassette and the PRE. The *Fab7* and *vestigial* PREs were inserted as described in 5.4.1 D.
The resulting plasmids, pC4*Fab7*-6 and pC4*vg*-9, were injected in embryos (See 7.1 Vector Maps).

F. TetR constructs, pUYTetR3'bio and pUYTetR5'bio

The TetR DNA was obtained by PCR amplification using TetR Fw and Rv plasmids and pUHD142-1 plasmid (kindly provided by Dr. Bujard) as a template. The purified PCR fragment was digested with NotI and XhoI and cloned into pBKSII plasmid. The resulting plasmid bearing the TetR sequence was digested with XhoI-BamHI or NotI-XbaI to introduce the BioTag in the 3' or 5' end of the gene to generate pBKSTetR3'Bio and pBKSTetR5'Bio respectively (See 7.1 Vector Maps).
The Bio-tagged TetR sequences were then introduced into XhoI-NotI sites of the pUASTy2 vector to produce the final constructs pUyTetR3'bio and pUyTetR5'bio that were injected in *drosophila* embryos (See 7.1 Vector Maps).

5.5 Drosophila handling and genetic methods

5.5.1 Drosophila handling

Fly stocks were raised on standard fly food on plastic vials (Greiner) and crossed at 25°C with 60-70% relative humidity. Fly stocks were maintained at 18°C with 60-70% relative humidity.

Standard fly food:

10 l H2O
80 g Agar-agar
180 g dry yeast
100 g soy flour
220 g beet syrup
800 g corn meal
24 g nipagin
(methyl-4-hydroxybenzoate, Merck)
62.5 ml propionic acid (Sigma)

Vials:	Small: 6.4 x 2.6 cm
	Medium: 8.3 x 3.6 cm
	Big: 10 x 5.3 cm

5.5.2 Preparation of acetic acid agar plates

33gr Agar-agar in 1 l ddH_2O were autoclaved and allowed to cool down to approximately 60°C. 14 g sucrose and 3 ml 100% acetic acid were added before the mixture was poured into Petri dishes. After solidification, acetic agar plates were stored at 4°C.

5.5.3 Drosophila embryo collection

For embryo collection for injection, adult flies were transferred into medium-sized (9 cm diameter, 12 cm height) cages sealed at the bottom with an agar plate.

For collection of material for ChIP or biochemical methods, big-sized (50 cm long x 35 cm wide x 35 cm high) cages were used. Typically 180-240 big vials of flies/cage were transferred into the cages containing paper to keep them dry and 8-10 agar plates (145/20) containing freshly prepared yeast to stimulate egg deposition (figure 5.1). Cages were placed at 25°C with 30% relative humidity (figure 5.1). After 12 h of collection, the plates were replaced with new ones. The embryos on the agar plate were flooded with water and transferred using a brush to a stack of sieves (1mm, 400 μM and 100 μM stainless mesh) where washing with water was continued. Excessive liquid was removed and embryos were dechorionated by incubating them for 2.5 m in a 3% Na-hypochlorite / tap water solution. After thorough washing in tap water, excessive liquid was removed. Using a spatula, embryos were transferred into Falcon tubes and weighed.

Figure 5.1 *Drosophila* cages for embryo collection. A-B) Each cage was filled with 180-240 big vials of freshly hatched flies. **C)** 6-8 plates of acetic acid agar plates with yeast were introduced in the cages and exchanged every 12 hs. **D)** The cages were incubated at 25°C with 30% relative humidity.

5.5.4 Generation of transgenic flies by P-element mediated germ line transformation

Transgenic flies carrying the construct of interest were generated by P-element mediated germ line transformation (Rubin and Spradling 1982; Spradling and Rubin 1982). The constructs for injection were prepared as follows: 9 µg of plasmid DNA together with 3 µg of pUChsΔ2-3 in a final volume of 30 µl of injection buffer. Prior to injection, the DNA mixture was centrifuged for 30 min at 13.000 rpm (Eppendorf table top centrifuge) at 4°C, and 2.5 µl of the supernatant were loaded into a Femtotip needle (Eppendorf). The injection needle was submerged in oil to avoid clogging.

Adult $y^1 w^{1118}$ flies were transferred to medium size cages (9 cm diameter, 12 cm height)

and were allowed to lay eggs on acetic acid agar plates for 25min at 25°C. Using a brush and tap water, embryos were transferred into a sieve and washed extensively. After dechorionation by 2.5 min incubation in bleach and extensive washing in tap water, 80-100 embryos were transferred onto a 1 cm x 3 cm block of acetic acid agar and aligned under a binocular. The aligned embryos were then transferred onto a double-sided sticky tape (3M, Scotch) mounted on a coverslip, with the posterior pole pointing toward the edge of the coverslip. Embryos were dehydrated in a closed chamber containing Silica gel for 7 min and covered with Voltalef 10S oil (Lehmann & Voss & Co.). Microinjection of DNA dissolved in injection buffer was performed under a stereomicroscope at 18°C with the Femtotip needle using an Eppendorf FemtoJet Microinjector at 200-600 hPa injection pressure. In general, about 300-400 embryos were injected per construct and were allowed to develop in oil in a humid chamber at 18°C. Only embryos in which the pole cells were not yet visible were injected, all embryos older than stage 2 (Campos-Ortega and Hartenstein 1997) were discarded. Freshly hatched first instar larvae were transferred into a vial containing standard fly food enriched with yeast paste, and were kept at 25°C until the founder G_0 generation hatched.

Injection buffer:	5 mM KCl
	0.1 mM NaH_2PO4, pH 6.8
PBS:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4 pH 7.4
Bleach:	3% Na-hypochlorite in PBS

5.5.5 Establishing of transgenic lines and mapping of integration chromosome

The hatched founder G_0 generation flies were crossed to y^1w^{1118} virgins or males and progenies were then screened for the transformation marker *miniwhite*, i.e. pigmented eyes or *yellow*, i.e. darker pigmented body. Depending on the construct and the insertion site, the eye colour varied from almost white to orange, for *yellow* marker there were no differences on the body colour depending on insertion site. F_1 generation transformants were then back-crossed twice to y^1w^{1118}*; sp / CyO* flies and y^1w^{1118} *; + ; TMSb / Dr* to map the chromosomal insertion site.

After the chromosomal insertion site was mapped, homozygous fly lines were

established. For this, heterozygous balanced flies were either crossed directly with one another and homozygous individuals identified by the absence of the dominant marker of the respective Balancer. Single insertions were verified by Southern Blotting.

5.5.6 Flp/FRT site-specific germ line recombination

To excise the TetO site from the pC4*Fab7*-6 and pC4*vg*-9 transgenes, the transgenic lines were crossed with a line expressing the Flp recombinase under the control of a heat-shock promoter at 18°C (G_0) (Chou and Perrimon 1996) (Figure 5.2).

G_0 **yw hsFLP ; noc / CyO** ♀ **X** **w ; P (w^+)** ♂
F_1 **yw hsFLP ; P (w^+) / CyO** ♂ **X** **w ; sp / CyO** ♀
F_2 **w ; P (w^+) / CyO** ♂ **X** **w ; sp / CyO** ♀
F_3 **w ; P (w^+) / CyO** ♂ **X** **w ; P (w^+) / CyO** ♀
F_4 **w ; P (w^+)**

Figure 5.2 Crossing scheme to excise the *TetO* by Flp/FRT specific recombination

During the first 64 h of development, the Flp recombinase was induced in the F_1 progeny by heat-shocking for 2 h at 37°C. 24 h later, a second heat-shock was applied, and the embryos were transferred to 25°C until adulthood. Males of the hatched progeny, carrying the hsFlp transgene as well as the reporter construct *(P (w+))* were crossed to *Sp/CyO* balancer virgins. The males hatching in the next generation (F_2, *P* (w+)*) were again crossed to *Sp/CyO* balancer virgins in single pair matting. Individuals of the F_3 generation were tested for successful recombination by single fly PCR. Recombinants *(P* (w+))* were crossed with each other to establish homozygous stocks (F_4).

5.5.7 Cre/loxP site-specific germ line recombination

To excise the *Fab-7* PRE and the *vestigial* PRE from the pC4*Fab7*-6 and pC4*vg*-9 transgenes, transgenic males were crossed to virgins expressing the Cre recombinase (G_0) (Figure 5.3).

G_0 **yw ; noc / CyO Cre ♀ X w ; P (w^+) ♂**

F_1 **yw ; P (w^+) / noc ♂ X w ; sp / CyO ♀**

F_2 **w ; P (w^+) / CyO ♂ X w ; P (w^+) / CyO ♀**

F_3 **w ; P (w^+) / CyO ♂ X w ; P (w^+) / CyO ♀**

F_4 **w ; P (w^+)**

Figure 5.3: Crossing scheme to excise the *Fab-7* or the *vestigial PRE* by Cre/loxP specific recombination

The *hsp70-Mos1* promoter driving the expression of the Cre recombinase is active in both somatic and germ line tissues (Siegal and Hartl 1996). Due to the strong maternal effect of Cre recombinase expression, males hatching in the next generation (F_1), bearing the transgene *(P (w+))* but not the *CyO*-marked Cre recombinase, were crossed to *sp/CyO* balancer virgins. Males hatching in the F_2 generation were again back-crossed to *sp/CyO* balancer virgins in single pair mating. In the F_3 generation, successful recombinants *(P* (w+))* were identified by single fly PCR and homozygous stocks (F_4) were established.

5.5.8 Determination of eye pigment in adult flies

Freshly hatched females were collected and left at 25°C to age for 24 h, 10 flies were then transferred into an Eppendorf tube. For decapitation, the flies were flash-frozen in liquid nitrogen and vortexed for about 20 sec. The heads were isolated with a brush and transferred into a fresh Eppendorf tube. For homogenization, 100 µl EPE buffer were added and the heads were grounded using an Eppendorf micropestle. Additional 150 µl EPE buffer were used to wash the micropestle, and extraction was allowed to proceed in the dark at 25°C for 1 h. The samples were then centrifuged for 2 min at 13.000 rpm (Eppendorf table top centrifuge) to remove the head debris. 150µl of clear supernatant were transferred into a new tube; The centrifugation step was repeated and 120 µl of the supernatant transferred into a new tube. The relative levels of eye pigmentation were determined by measuring the absorbance at 480 nm in a photometer. As a negative control, heads of w^{1118} flies were used.

EPE buffer: 30% ethanol (adjust to pH 2 with HCl)

To photograph adult fly eyes, freshly hatched flies were left additional 24 h at 25°C and then either photographed directly or stored at -20°C.

5.6 Drosophila histological methods

5.6.1 DNA FISH on larval polytene chromosomes

Chromosome squashes

This protocol was adapted from (Lavrov et al. 2004). Larvae were grown on standard *Drosophila* medium supplemented with fresh yeast paste in big vials not to crowded and at 18°C. 11-12 days after egg deposition wandering third instar larvae were collected in PBS on ice and the females were selected (generally, the salivary glands of females are bigger than those of males). Two pairs of salivary glands were dissected in solution 1, removing fat body cells as much as possible. Using forceps, the two pairs of salivary glands were transferred into a drop (approximately 40 µl) of solution 2 on a slide. While continuously stirring, the glands were homogeneously fixed for 10 sec. Subsequently, the glands were transferred into a drop of solution 3 on a 18 cm x 18 cm coverslip and left for 2 min 10 sec. Using a poly(L)lysine coated slide, the glands were taken up and then squashed to spread the chromosomes. To do this, a pencil was tapped onto the coverslip with moderate force and constant moving of the coverslip (1-2 mm). Excess liquid was removed by turning the slide upside down and pressing it onto Whatman paper. The quality of the preparation was examined immediately under phase contrast. The position of the coverslip was marked on the slide using a diamond pencil, before it was flash-frozen in liquid nitrogen. The coverslip was removed using a razorblade and the slide was then stored in PBS on ice (for storage up to one week, slides were transferred into a jar containing 100% methanol at 4°C). The slides were washed twice in PBS for 15 min with constant rocking. Subsequently, the chromosomes were dehydrated by passing them through an increasing series of ethanol (2x 5 min 70%; 2x 5 min 96%). Finally, the slides were air-dried and stored flat at 4°C at least overnight, as this markedly increased the adhesion of the chromosomes to the slide, and thus the quality of the DNA FISH signals.

Buffers:	Solution 1:	0.1% (v/v) Triton X-100 / PBS
	Solution 2:	50 µl 10% (v/v) Triton X-100 400 µl PBS 50 µl 37% p-formaldehyde; this solution must be prepared fresh and can be used for 2-3 h when kept on ice.

37% p-formaldehyde stock:	1.85 g p-formaldehyde was weighed into a final volume of 5 ml ddH2O, and 70 µl 1N KOH were added to adjust to pH 7.4. The powder was dissolved by heating in a 60-65°C waterbath. 100 µl aliquots were stored at -80°C. Before use, aliquots were thawed by heating at 60-65°C. If precipitates formed, the solution was discarded.
Solution 3:	50 µl 37% p-formaldehyde 200 µl ddH2O 250 µl 100% acetic acid; this solution also has to be prepared fresh every time and is stable for 2-3 h when kept on ice.

Preparation of the DIG-labelled DNA probe

1 µg DNA (linear or circular plasmid DNA) was used as a template in a standard labelling assay according to the manual provided with the DIG-High Prime labelling kit (Roche). Briefly, the template DNA was dissolved in a volume of 16 µl and denatured by boiling for 10 min. The DIG-High Prime labelling mix was mixed thoroughly and 4 µl added to the denatured DNA. After short centrifugation, the reaction was incubated overnight at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8) or heating for 10 min at 65°C, the DIG-labelled DNA was ethanol-precipitated and resuspended in 20 µl TE buffer (pH 7.4). 200 µl hybridization buffer were added and the probe was stored for several months at -20°C.

Hybridization buffer:	50% formamide 2x SSC 10% dextran sulfate 400 µg/ml herring sperm DNA (Invitrogen) pH 7 store at -20°C

Pre-treatment and Hybridization

Just before hybridization, the slides were put into a Coplin Jar containing 2x SSC at RT. The jar was then transferred into a 70°C waterbath and incubated for 40 min. Next, the

slides were dehydrated by passing through ethanol series (2x 5 min 70%; 2x 5 min 96%) and air-dried. To denature the chromosomes, they were incubated for in 0.1 M NaOH for 10 min and then washed three times (1 min, 1 min, 5 min) in 2x SSC, followed by dehydration and air-drying. One aliquot of DIG labelled DNA probe (in hybridization buffer, around 15 µl per sample) was denatured by heating for 5 min at 80°C, then quickly chilled on ice, pre-warmed to 37°C, and pipetted onto a 22 mm x 22 mm coverslip. The slide covered with polytene chromosomes was turned upside down to take up the coverslip with the probe. To prevent liquid evaporation, the coverslips were sealed with Fixogum and the slides were hybridized overnight in a humid chamber in a 37°C waterbath.

20x SSC:	3M NaCl
	300mM sodium citrate pH 7

Washing and detection

After hybridization, the slides were immersed in pre-warmed 2x SSC to loosen the coverslips and the fixogum was removed with forceps (during the post-hybridization washes, cooling down of the samples should be avoided). The slides were then transferred into a Coplin Jar and washed 3x 5 min in 2x SSC at 42°C, then once for 5 min in 2x SSC at RT with rocking. Next, the slides were washed 5 min in TNT buffer on a shaker, then 100 µl of TNB buffer were pipetted onto each slide which was then covered with a 24 mm x 44 mm coverslip and incubated for 30 min at RT. The coverslips were removed by immersing the slides in TNT buffer. Mouse α-DIG antibody (Roche) was diluted in TNB buffer to a final concentration of 1:200, and 20 µl were then pipetted onto a 22 mm x 22 mm coverslip. The slide was turned upside down to take up the coverslip, then laid down flat and incubated in a humid chamber for 90 min at RT or overnight at 4°C. After 3x 5 min washes in TNT buffer on a shaker, the secondary antibody (goat α-mouse-Cy3, JacksonImmunoResearch) was diluted 1:200 in TNB buffer, and 20 µl were pipetted onto a 22 mm x 22 mm coverslip, which was then taken up by turning the slide upside down. The slides were incubated 60-90 min in a humid chamber at RT in the dark. To prevent bleaching of the fluorophore, all following steps were also carried out in the dark. After 3x 5 min washes with TNT buffer on a shaker, the DNA was counterstained with DAPI 1 µg/ml diluted in TNT buffer, 10 min incubation at RT in the dark. The slides were washed once for 5 min in TNT buffer, and mounted in Mowiol or Fluoromount G (SouthernBiotech).

Buffers:	TNT buffer:	100 mM Tris-HCl pH 7.4
		150 mM NaCl
		0.05% (v/v) Tween-20
	TNB buffer:	100 mM Tris-HCl pH 7.4
		150 mM NaCl
		4% BSA

5.7 Handling Drosophila *Cells*

5.7.1 Cultivation of Schneider SL2 cells

The cells were cultivated in T-75 cell culture flasks (Cellstar) with 20 ml growth medium. At 80-90% confluency, the adherent SL2 cells detach and proliferate in suspension and have to be passaged. Cells were resuspended until a single cell suspension was present, an aliquot (1:10-1:50) thereof was transferred into a new flask containing 20 ml fresh medium and equally distributed. Cells were cultivated at 25 °C under normal atmosphere.

Culture Medium:	Schneider´s Medium (Invitrogen)
	1% Penicillin/Streptomycin
	10% FCS

5.7.2 Freezing of cells for long term storage

Cells at 70-90% confluency were resuspended and transferred into 15 ml Falcon tubes with 10 ml of fresh medium. The cells were sedimented at 300g for 5 min and resuspended in 3-4.5 ml 10% (v/v) DMSO in growth medium with 20% FCS. 1.5 ml aliquots were transferred into cryotubes (Nunc) and incubated on ice for 1-1.5 h. The vials were stored overnight at -80°C and then transferred into a liquid nitrogen tank for long-term storage.

5.7.3 Thawing of frozen cells

Cells frozen in liquid nitrogen were thawed quickly in a 37°C waterbath. Cells were transferred into a 15 ml Falcon tube with 10 ml of fresh medium and centrifuged for 5 min at 300g. The cells were resuspended in 5 ml normal growth medium and transferred to 9 cm culture dishes containing 10 ml growth medium.

5.7.4 Transient transfection of SL2 cells with Effectene

S2 cells were plated in 6 well dishes at 30-50 % confluency the day before transfection. 2-3 h before transfection, the growth medium was replaced with 1.6 ml fresh medium, and cells were transfected with Effectene (Qiagen) according to the manufacturer's protocol as follows:

1 µg DNA (1µg each plasmid: pmtGal4, pUASp(EGFP)BirA, pUYTetRBio3 or pUYTetRBio5)

200 µl Enhancer buffer

16 µl Enhancer (Enhancer:DNA = 8:1)

The mixture was vortexed for 1 sec and incubated for 5 min at RT. 25 µl Effectene was added by pipetting up and down five times and incubated for 10 min at RT. 580 µl S2 growth medium was added, the mixture was pipetted up and down twice, directly added drop wise to the cells and incubated at 25 °C. Expression was induced 72 h after transfection by adding $CuSO_4$ (0.5 M stock in ddH_2O) to a final concentration of 500 µM. Cells were generally analyzed 48 h after induction.

5.7.5 Cell Lysis for western blot analysis

Cells were scraped by pipetting up and down with a 1 ml pipette tip, transferred into a 15 ml falcon tube and centrifuged at 800x g for 10 min at 4°C . Next the cells were washed once with cold PBS, resuspended in 1 ml of PBS supplemented with protease inhibitors (complete EDTA free protease inhibitors, Roche) and stored at -20°C. For SDS-Page analysis 100 µl of cells were centrifuged and resuspended in 20 µl of 2x SDS sample buffer. 0.5µl bezonase was added and the samples were first incubated 5 min at RT and then heated for 5 min at 95°C.

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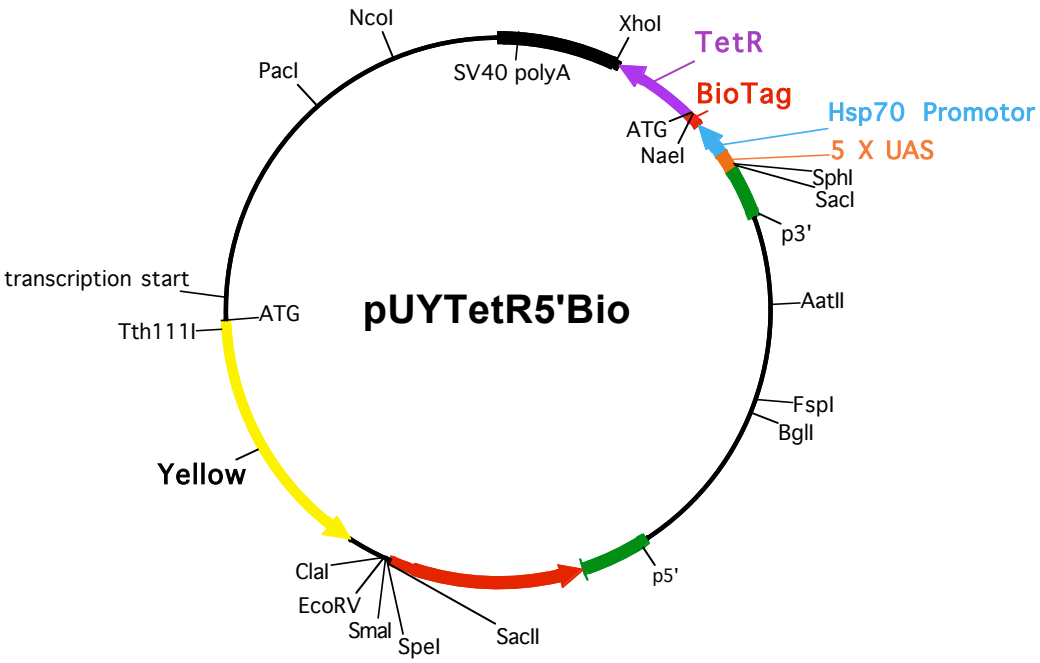
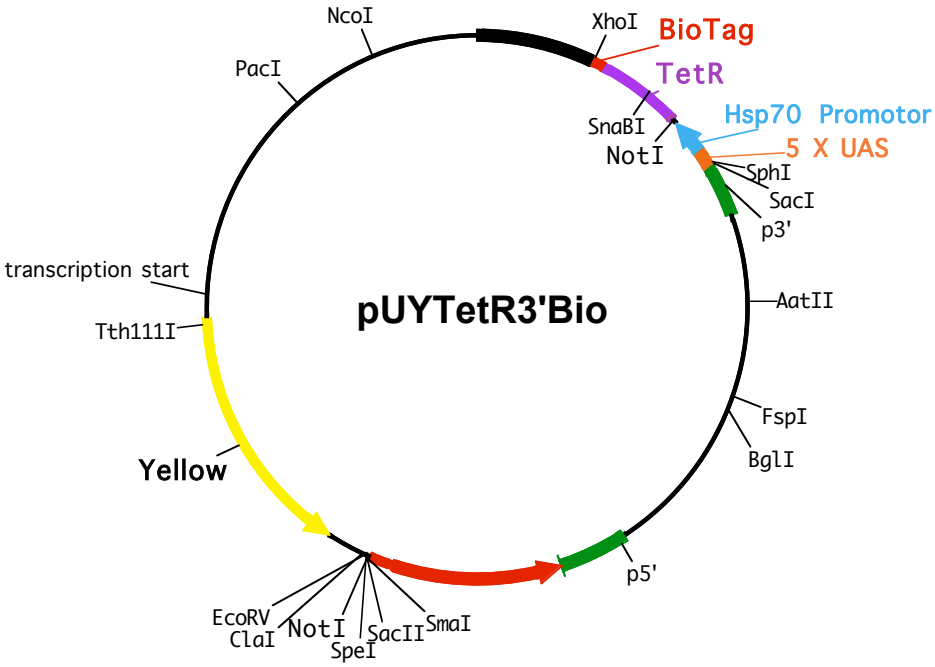
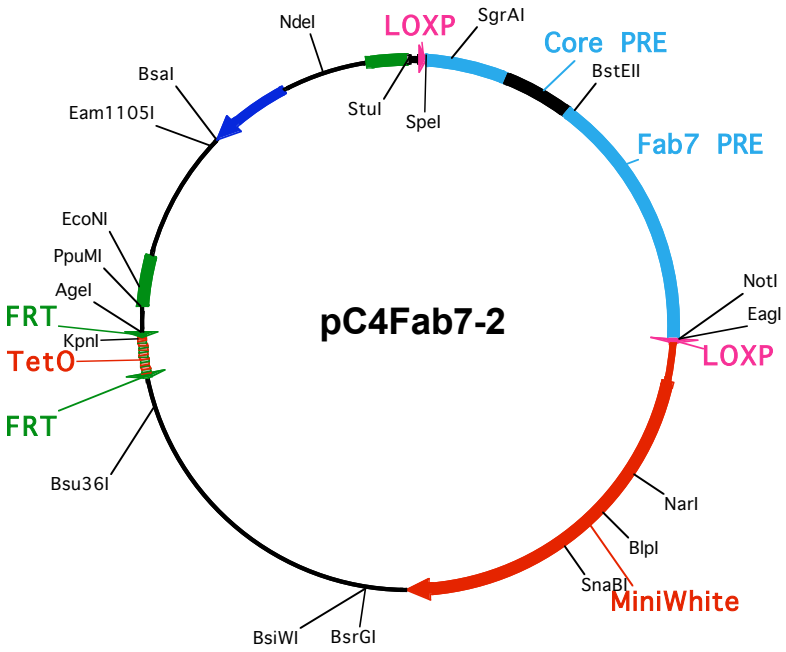
Appendix

7. Appendix

7.1 Vector maps



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Fab7 PRE
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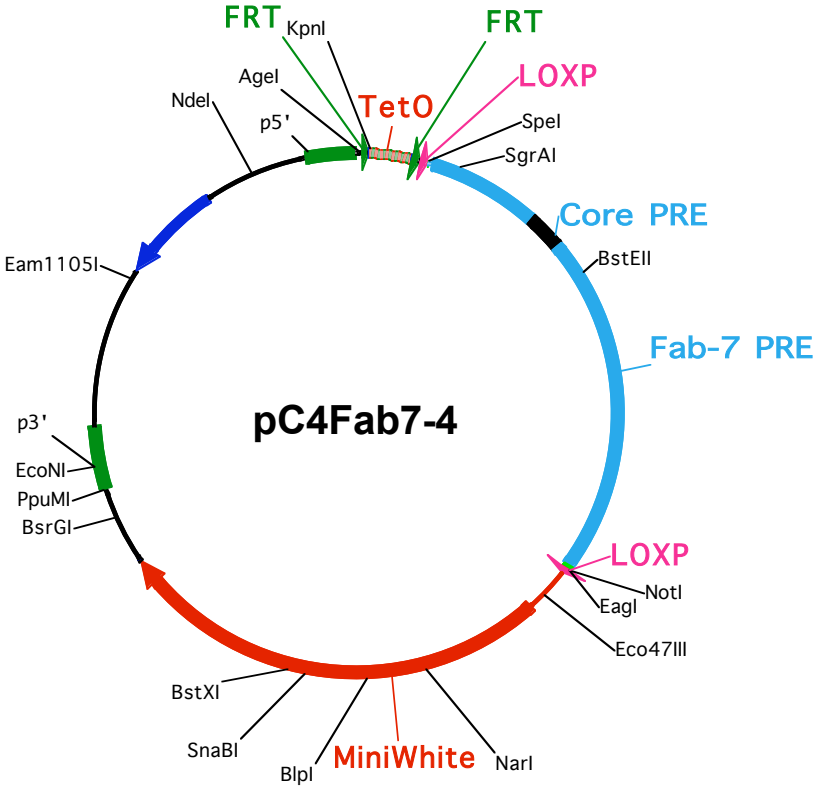
SnaBI
MiniWhite
BsiWI
BsrGI
Bsu36I
FRT
TetO
FRT
KpnI
AgeI
PpuMI
EcoNI
Eam1105I
BsaI
StuI
SpeI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SgrAI
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Eam1105I
BstEII
Fab-7 PRE
p3'
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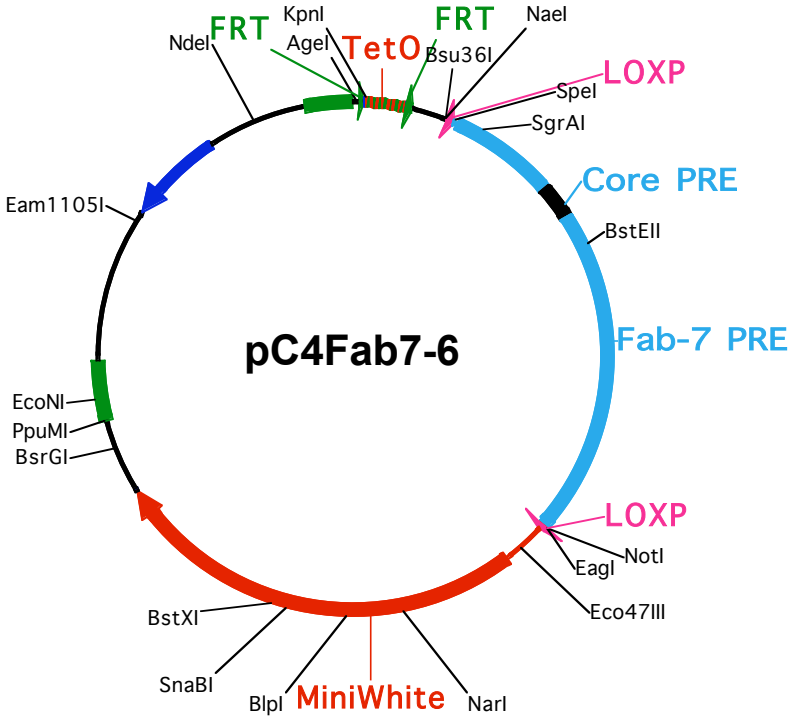
NarI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Eam1105I
Fab-7 PRE
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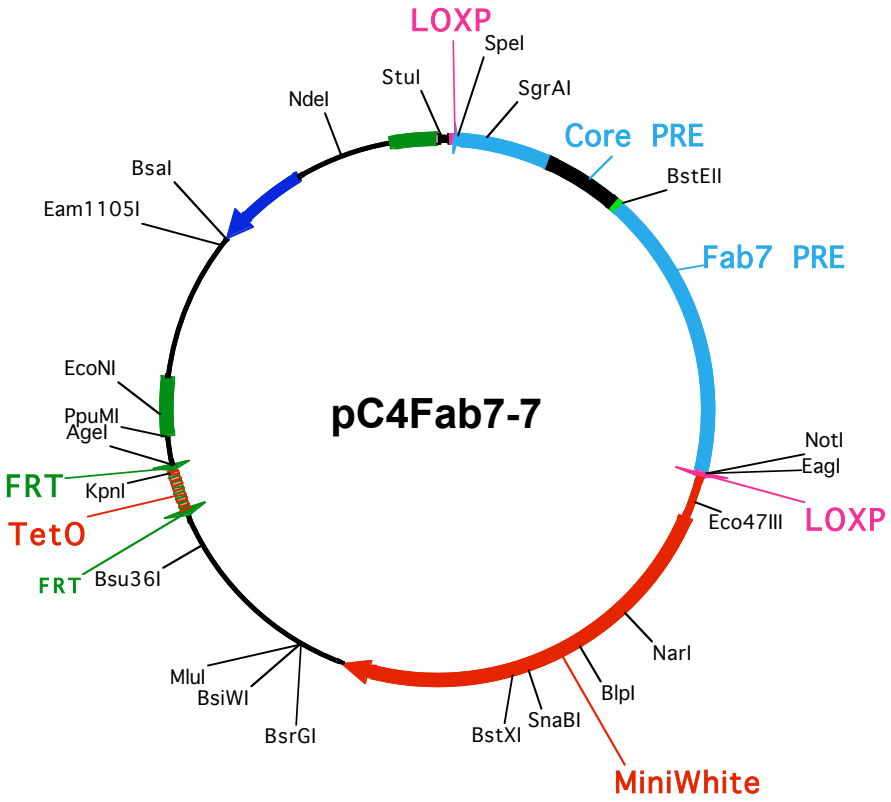
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Fab7 PRE
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AgeI
NotI
EagI
FRT
KpnI
Eco47III
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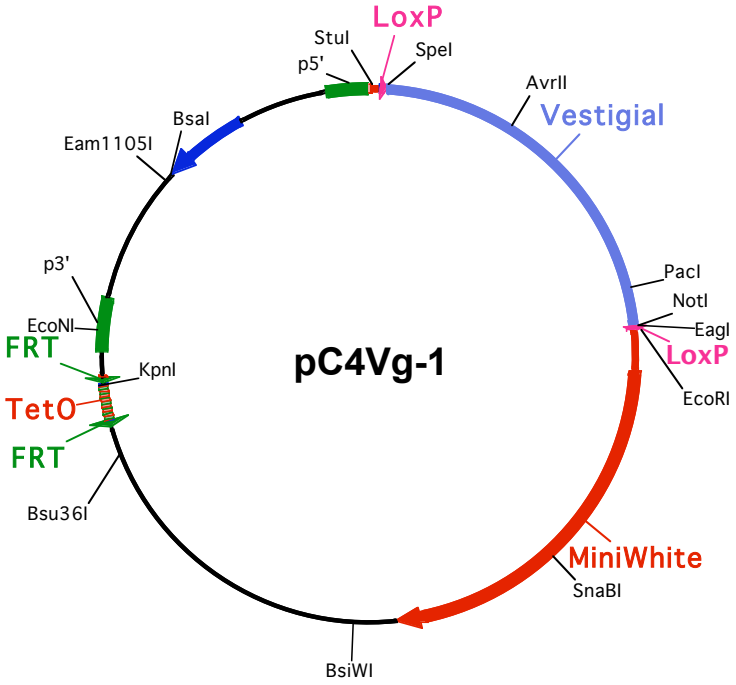
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StuI
SpeI
p5'
AvrII
Vestigial
BsaI
Eam1105I
p3'
PacI
NotI
EagI
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FRT
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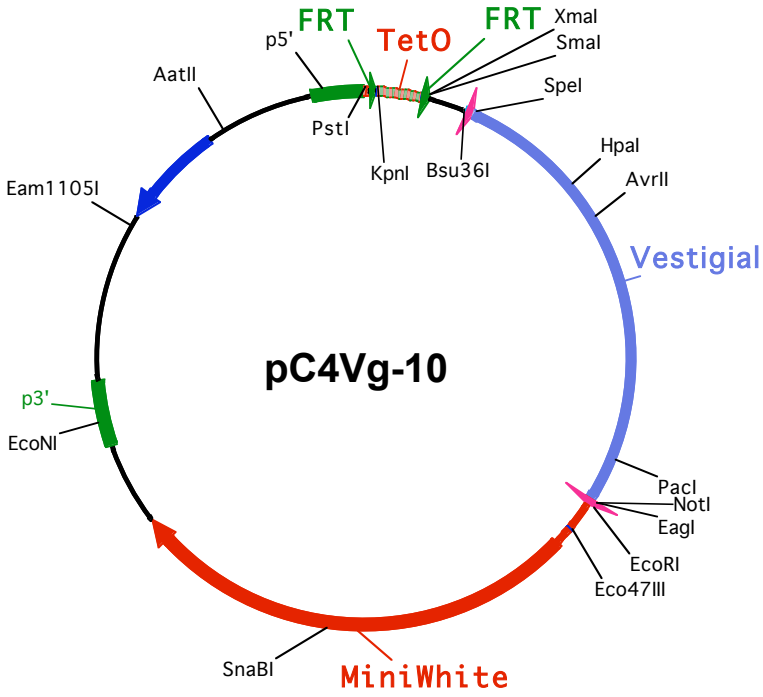
BsiWI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Eam1105I
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Eco47III
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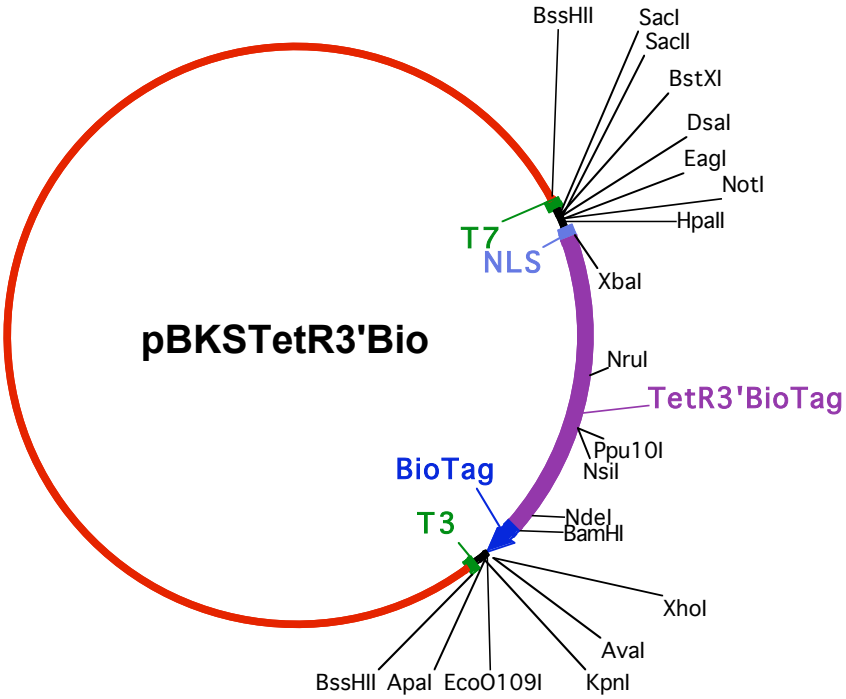
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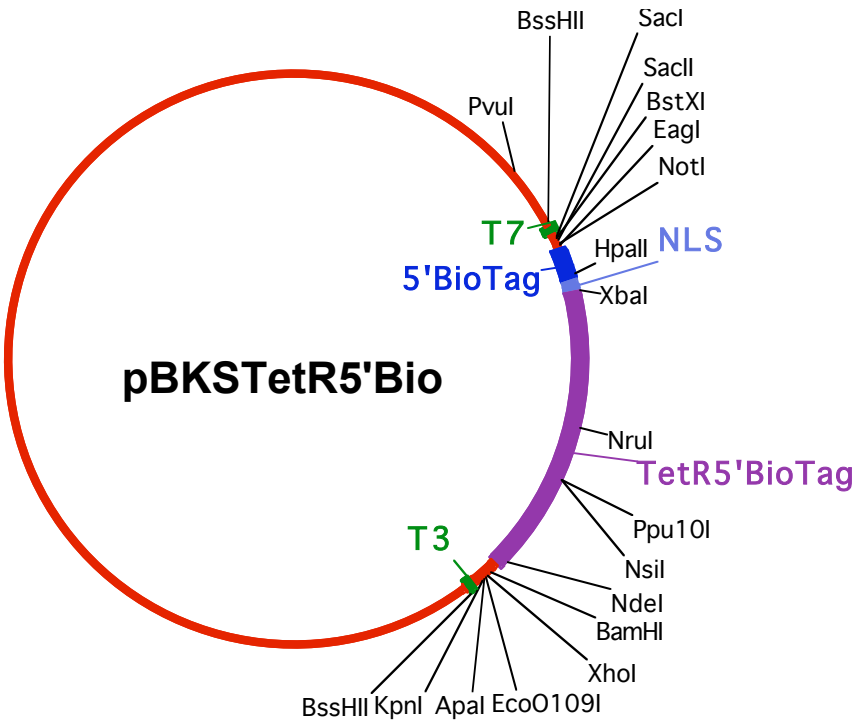
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XbaI
NruI
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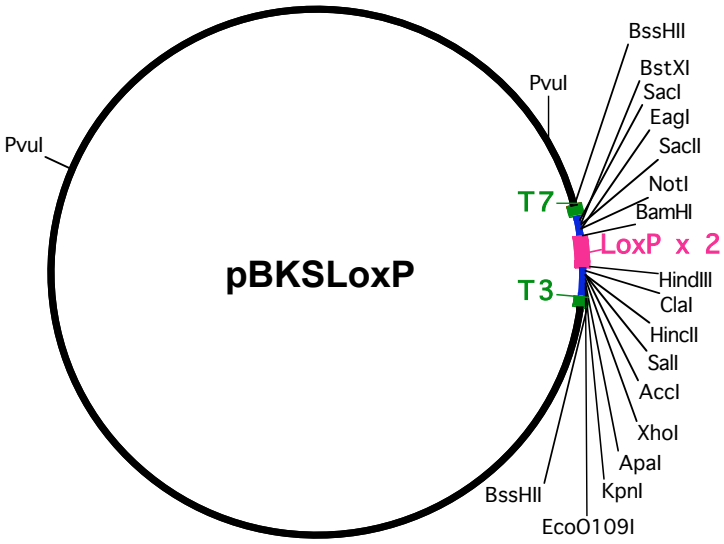

7.2 General crossing scheme for generation of transgenic lines

A- Generation of TetR/BirA flies:

-Y[1]w[1118]; P{TetR5'Bio, y^+}, P{BirA-myc, GFP}

G_0 yw ; TetR (y^+) / TetR (y^+) ♀ X yw ; BirA (GFP^+) / BirA (GFP^+) ♂

F_1 yw ; TetR (y^+) / BirA (GFP^+) ♀ X yw ; sp / CyO ♂

F_2 yw ; TetR (y^+), BirA (GFP^+) / CyO

B-Generation of PRE/TetR/BirA flies:

- y[1] w[1118]; P{ FLP-TetO, FRT-PRE, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}
-y[1] w[1118]; P{ FLP-TetO, FRT, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}
-y[1] w[1118]; P{ FLP, FRT-PRE, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}

G_0 yw ; PRE (w^+) ♀ X yw ; TetR (y^+), BirA (GFP^+) ♂

F_1 yw ; PRE (w^+) / TetR (y^+), BirA (GFP^+) ♀ X yw ; sp / CyO ♂

F_2 yw ; PRE (w^+),TetR (y^+), BirA (GFP^+) / CyO

C-Generation of PRE/TetR/Bir;DaGal4 flies:

- y[1] w[1118]; P{ FLP-TetO, FRT-Fab7, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.

-y[1] w[1118]; P{ FLP-TetO, FRT, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.

- y[1] w[1118]; P{ FLP, FRT-Fab7, w+}, P{TetR5'Bio, y+}, P{BirA-myc, GFP}; DaGal4/TMSb.

G_0 yw; PRE (w^+), TetR (y^+), BirA (GFP^+); Dr/TMSb ♀ X
yw ; CyO/Sp; DaGal4 ♂

F_1 yw; PRE (w^+), TetR (y^+), BirA (GFP^+)/ CyO; DaGal/ TMSb

7.3 Abbreviations

7.3.1 Genes, chromosomal markers, proteins and protein domains

abdA	*Abdominal-A*
AbdB	*Abdominal-B*
ANT-C	Antennapedia Complex
Antp	Antennapedia
ASH	Absent, small and homeotic
Bcd	*bicoid*
BRM	Brahma
BMI1	BMI1 polycomb ring finger oncogene
Bx	Bithorax
CHRASCH	Chromatin associated silencing complex for homeotics
dCBP	*Drosophila* CREB-binding protein
Cre	Causes recombination
DSP1	Dorsal switch protein 1
dTAFII	*Drosophila* TFIID associated factor
en	Engrailed
ESC	Extra sex combs
Ey	*eyeless*
E(Z)	Enhancer of zeste
EZH2	Enhancer of zeste 2
EED	Embryonic ectoderm development
Fab-7	*Frontoabdominal-7*
Flp	flipase
FRT	*Flp recombination target*
GAF	GAGA factor
GFP	Green fluorescent protein
HAT	histone acetyltransferase
HDAC	histone deacetylase

hb	*hunchback*
hh	*hedgehog*
HMTase	histone methyltransferase
Hox	*Homeobox*
iab	*infraabdominal*
LHP1	Like Heterochromatin Protein 1
loxP	*locus of crossing over (x), P1*
Mcp	*Miscadestral pigmentation*
MLL	Mixed lineage leukemia
MOF	Males absent on the first
MOR	Moira
mw	*miniwhite*
nos	*nanos*
PC	Polycomb
PCL	Polycomb like
PcG	Polycomb group
PH	Polyhomeotic
PHD	Polyhomeotic distal
PHO	Pleiohomeotic
PHP	Polyhomeotic proximal
PHOL	Pleihomeotic-Like
PRC	Polycomb group repressive complex
PRE	Polycomb group response element
PSC	Posterior sex combs
PSQ	Pipsqueak
RNAPII	RNA polymerase II
Su(Z)12	Suppressor of zeste 12
SCM	Sex Comb on midleg
TBP	TATA-box binding protein
TAF	Transcription factor
TetR	Tetracycline Repressor
TetO	Tetracycline Operator
TRX	Trithorax
UAS	*upstream activating sequence*
Ubx	*Ultrabithorax*
3'UTR	*3' untranslated region*
vg	*vestigial*
wg	*wingless*
yw	*yellow-white*
Z	Zeste

7.3.2 Others

aa	amino acid
Amp	ampicillin
AP	alkaline phosphatase
A-P	anterior-posterior
ATP	adenosine-5'-triphosphate

bp	basepair
BSA	bovine serum albumin
ChIP	Chromatin Immunoprecipitation
CIP	calf intestinal phosphatase
cm	centimeter
CsCl	Cesium Chloride
DB	Dialysis buffer
ddH_2O	Double distil water
DEAE	Diethylaminoethyl
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTO	Deoxynucleotide triphosphate
DOC	Sodium Deoxycholate
ds	Double-stranded
DSP	Dithiobis[sulfosuccinimidylpropionate]
DTBP	Dimethyl 3,3'-dithiobispropionimidate-2HCl
DTT	Dithio-DL-threit(ol)
D-V	dorsal-ventral
E.coli	*Escherichia coli*
EDTA	Ethylendiaminotetraacetic acid
EGTA	Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Fig	Figure
FISH	fluorescent *in situ* hybridization
g	gravitation
h	hour
Hepes	N-(2-Hydroxyethyl)-piperazin-N'(2-ethansulfonic acid)
hPa	Hectopascal
HRP	Horseradish peroxidase
kb	kilobase
kDa	kiloDalton
LB	Luria Bertani
Lsp	Long spacer
M	Molar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MWCO	Molecular weight cut off
NB	Nuclear break buffer
NE	Nuclear extract
NEB	New England Biolab
NP-40	Nonidet P-40
nt	nucleotide
NW	Nuclear wash buffer
OD	optical density
PAGES	Polyacrilamide gel electrophoresis
PBS	phosphate-buffered saline

PBST	PBS-Tween
pc-NE	Protein chromatin nuclear extract
PCR	polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SB	sample buffer
SDS	Sodium duodecil sulfate
sec	second
SP	Sulphopropyl
ss	single-stranded
TAE	Tris-acetate-EDTA
TCA	Trichloroacetic acid
TE	Tris-EDTA
U	units
UV	ultraviolet
V	voltage
v/v	volume per volume
w/v	weight per volume
µJ	microjoule
µg	microgram
µm	micrometer
µM	micromolar

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfsmittel durchgeführt habe.

Heidelberg, 17.06.2008

…….

Ana Laura Monqaut

Erratum

In the Results section, on page 49 of the present manuscript "Locus specific analysis of PcG/TrxG proteins using Bio-tagging technology" in Figure 2.13 A, TetO-ΔFab7 and ΔTetO-Fab7 charts are interchanged.

The correct figure follows.