The immunophilin FKBP39 regulates polycomb group mediated epigenetic control in Drosophila melanogaster

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The immunophilin FKBP39 regulates Polycomb group mediated epigenetic control in *Drosophila melanogaster*

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ETH Zürich

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**Doctor of Sciences (Dr. sc. ETH Zurich)**

Presented by

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Abbreviations

abdA  abdominal-A
abdB  Abdominal-B
ACF   ATP-utilizing chromatin assembly and remodeling factor
ANT-C Antennapedia Complex
Antp  Antennapedia
A-P   anterior-posterior
APS   Ammonium Persulfate
ASH   Absent, small, and homeotic
BRM   Brahma
BSA   Bovine serum albumin
BX-C  Bithorax Complex
bx    bithorax
CaN   calcineurin
CDS   coding sequence
ChIP-seq chromatin immunoprecipitation followed by next generation sequencing
CHRASCH Chromatin associated silencing complex for homeotics
CLD   cyclophilin-like domain
CYP   cyclophilin
dCBP  Drosophila CREB-binding protein
Dfd   Deformed
Dnmt1 DNA methyltransferase1
DSP1  dorsal switch protein1
ESC   Extra sex combs
E(Z)  Enhancer of zeste
EZH2  Enhancer of zeste2
Fab-7 Frontoabdominal-7
FKBP  FK506 binding protein
GAF   GAGA factor
GFP   Green fluorescent protein
GO    gene ontology
GRH   Grainyhead
HAT   histone acetyltransferase
HDAC  histone deacetylase
HMTrase histone methyltransferase
HOX   Homeobox
HP1   heterochromatin protein1
Hsc4  heat-shock cognate 4
Hsp90  heat-shock protein 90
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td><em>iab</em></td>
<td>infra-abdominal</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td><em>ifa</em></td>
<td>irregular facet</td>
</tr>
<tr>
<td><em>lab</em></td>
<td>labial</td>
</tr>
<tr>
<td>MBT</td>
<td>Malignant Brain Tumor</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>N55</td>
<td>Nurf 55</td>
</tr>
<tr>
<td>NoR</td>
<td>nucleolus organizer region</td>
</tr>
<tr>
<td>NoRC</td>
<td>nucleolar remodeling complex</td>
</tr>
<tr>
<td><em>nos</em></td>
<td>nanos</td>
</tr>
<tr>
<td><em>pb</em></td>
<td>proboscipedia</td>
</tr>
<tr>
<td>PC</td>
<td>Polycomb</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb group</td>
</tr>
<tr>
<td>PH</td>
<td>Polyhomeotic</td>
</tr>
<tr>
<td>PHO</td>
<td>Pleiohomeotic</td>
</tr>
<tr>
<td>PHOL</td>
<td>Pleiohomeotic-like</td>
</tr>
<tr>
<td>PhoRC</td>
<td>Pho Repressive Complex</td>
</tr>
<tr>
<td>PPIase</td>
<td>Peptidyl-prolyl isomarase</td>
</tr>
<tr>
<td>PRC</td>
<td>Polycomb group repressive complex</td>
</tr>
<tr>
<td>PRE</td>
<td>Polycomb group response element</td>
</tr>
<tr>
<td>PSC</td>
<td>Posterior sex combs</td>
</tr>
<tr>
<td>PSQ</td>
<td>Pipsqueak</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPKM</td>
<td>reads per kilobase of exon model per million mapped reads</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
<tr>
<td>SCM</td>
<td>Sex comb on midleg</td>
</tr>
<tr>
<td><em>scr</em></td>
<td>sex combs reduced</td>
</tr>
<tr>
<td>SFMBT</td>
<td>Scm-related gene containing four MBT domains</td>
</tr>
<tr>
<td><em>Sra-1</em></td>
<td>specifically Rac-1 associated protein1</td>
</tr>
<tr>
<td>Su(Z)12</td>
<td>Suppressor of zeste 12</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose NonFermentable</td>
</tr>
<tr>
<td>TAF</td>
<td>TBP-associated factor</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
</tr>
<tr>
<td><em>Trl</em></td>
<td>Trithorax-like</td>
</tr>
<tr>
<td>TrxG</td>
<td>Trithorax group</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td><em>Ubx</em></td>
<td>Ultrabithorax</td>
</tr>
<tr>
<td>UTX</td>
<td>ubiquitously transcribed tetratricopeptide repeat X</td>
</tr>
<tr>
<td>TPR</td>
<td>tetratricopeptide repeats</td>
</tr>
<tr>
<td>Z</td>
<td>Zeste</td>
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</table>
Summary

In a multicellular organism, maintenance of cell lineage-specific transcription patterns is critical for normal development. In *Drosophila*, two groups of proteins, the Polycomb group (PcG) and Trithorax group (TrxG), were found to maintain this transcriptional memory at the level of chromatin. Different protein complexes have been characterized for both PcG and TrxG, which antagonize each other by maintaining genes in stably silent or active states, respectively. Recently, gene expression states maintained by PcG have been found to be dynamic and be switched from a silent to an active mode and back. How an active or a silent gene, targeted by PcG proteins, switches its state of expression is not yet fully understood, however, auxiliary factors may be crucial in regulating this switching by modulating PcG proteins or chromatin conformations. Immunophilins represent a class of proteins, which belong to peptidyl-prolyl isomerases (PPIase) catalyzing *cis-trans* isomerization of the peptide bond. They accelerate folding and stimulate conformational changes in folded and unfolded proteins. As several immunophilins have already been linked to PcG/TrxG mediated gene regulation in *Drosophila* and human cells, immunophilins became the co-factor candidates of PcG proteins. The yeast homolog of nuclear immunophilin FKBP39, FPR4 was found to mediate epigenetic gene regulation via controlling the level of H3K36 methylation by isomerizing Proline residues 38 of histone H3. This study set out to investigate a potential link between PcG and FKBP39 in *Drosophila*. In this work I aim to identify the function of FKBP39 and the genome-wide correlation of FKBP39 and PcG proteins at chromatin. This should provide mechanistic insights in our understanding of how PcG proteins may be supported by co-factors to maintain the transcriptional states of target genes.

During this study, the phenotype of *fkbp39* null mutant flies was characterized in detail. FKBP39 can be shown to be important for spermatogenesis, oogenesis and embryonic development of *Drosophila*. Moreover, FKBP39 was also found to link with PcG mediated gene regulation, it was extensively co-localized with PC on chromatin and correlated with Polycomb group repressive complex 1 (PRC1) on the promoter region. The
relative higher expression level of FKBP39 binding genes in PRC1 targets, as well as the enrichment of down-regulated genes within both "FKBP39" and "PRC1 AND FKBP39" targets in FKBP39 knock-down S2 cells, strongly suggests that FKBP39 works as a gene activator and is involved in PcG mediated epigenetic control. Furthermore, FKBP39 was shown to be genetically interacting with Polycomb (PC) acting like a PcG protein. Indeed, FKBP39 was found to be required for activating the expression of the PRC1 genes, *ph* and *Psc*.

The results of this work suggest that FKBP39 is an important protein involved in several developmental processes in *Drosophila*, it is also a gene activator in PcG mediated gene regulation, while still interacting and co-localizing with the components of the PRC1 complex. This demonstrates the possibility of a direct regulation of specific co-factors on the PRC1 core complexes.
Zusammenfassung


Im Rahmen dieser Studie wurde der Phänotyp der Nullmutante fkbp39\(^i\) detailliert beschrieben. FKBP39 ist funktionell an Spermatogenese, Oogenese...

Introduction
1. Introduction

Epigenetic regulation of gene expression is necessary for the correct deployment of developmental programs and for the maintenance of cell fates. *Drosophila* Polycomb group (PcG) and Trithorax group (TrxG) proteins are responsible for the maintenance of stable transcription patterns of many developmental regulators, such as the HOX genes, a set of transcription factors that specify cell identity along the anterior-posterior axis of segmented animals (Duncan, 1982; Lewis, 1978). The expression of HOX genes is established early during embryonic development by the segmentation gene products (Akam, 1987). However, a few hours after these homeotic gene patterns have been established, the segmentation gene products decay, regardless of the fact that the expression patterns of HOX genes need to be maintained throughout development (Schuettengruber and Cavalli, 2009). The transcriptional status of each gene is subsequently maintained throughout the rest of development by the PcG and TrxG proteins (Ringrose and Paro, 2007). PcG and TrxG proteins have long been considered as a cellular memory system that stably controls the expression states of HOX genes for an organism’s whole life span. PcG proteins maintain the silent state, whereas TrxG proteins maintain the active transcription of Hox genes (Ringrose and Paro, 2004). However, in recent years, genome-wide mapping studies of PcG components in several species have led to a comprehensive list of PRC1 and PRC2 target sites, which revealed that besides HOX genes, PcG proteins bind many more genes, mainly comprising transcription factors involved in various cellular functions and developmental pathways (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Negre et al., 2006a; Schwartz et al., 2006; Squazzo et al., 2006; Tolhuis et al., 2006). In addition, PcG proteins exhibit a surprisingly dynamic association with their chromatin targets in photobleaching experiment, the residence time for PcG proteins PC and PH is only 2-6min (Ficz et al., 2005). Also, PcG proteins have been demonstrated to dynamically bind their targets in embryonic stem cells and during subsequent cell lineage commitment events (Boyer et al., 2006; Lee et al., 2006; Mohn et al., 2008). These findings indicates that PcG proteins do not only serve to maintain long term memory of transcriptional states, they are also involved in
the dynamic regulation of genes belonging to a diversity of biological processes and additional components must be involved to achieve gene specificity. Furthermore, it has been demonstrated that the association of PcG proteins with their target genes does not necessarily result in gene silencing (Beisel et al., 2007; Enderle et al., 2011; Papp and Muller, 2006; Schwartz et al., 2006; Schwartz et al., 2010). PcG proteins and TRX were constitutively bound at Drosophila Polycomb response elements in the bithorax complex (BX-C) independently of their activity state (Papp and Muller, 2006), which further suggest that the PcG/TrxG system may be regulated by additional, still unidentified co-factors. Recent discoveries regarding involvement of molecular chaperone Hsp90, immunophilins and cohesin complex in PcG/TrxG mediated regulation have added such additional factors (Anderson et al., 2002; Fair et al., 2001; Hom et al., 2010; Park et al., 2010; Strubbe et al., 2011; Tariq et al., 2009; Wang et al., 2010; Yang et al., 1995; Yang et al., 2001), which highlight the fact that certain auxiliary factors may be crucial in modulation of PcG/TrxG activity. However, the knowledge on the involvement and function of the co-factors in the PcG/TrxG system is still far from complete.

1.1 The specification of cell fates

In the early Drosophila embryo, a hierarchy of genes divides the body along its length into ever-smaller domains. This hierarchy begins with the maternal coordinate genes, whose graded patterns are interpreted into repetitive patterns by three levels of the segmentation genes: the gap, pair rule, and segment polarity genes. The hierarchy ends with the homeotic genes, which give to each parasegment its individual identity (Lawrence and Morata, 1992). The specification of anterior-posterior (A-P) and dorsal-ventral (D-V) polarity, for instance, occurs in the oocyte, even before fertilization (Ingham, 1988; St Johnston and Nusslein-Volhard, 1992). A-P axis formation depends on the tightly localized deposition of maternal mRNAs such as bicoid (bcd) and nanos (nos) at the anterior and posterior poles, respectively, and the localized activation of particular signaling pathways (Driever and Nusslein-Volhard, 1988; Struhl, 1989a). By the time the egg is fertilized, the major A-P axis of
the embryo is already specified. Because fertilization triggers the translation of
the maternal mRNAs, leading to the formation of morphogen gradients along
the A-P axis, this is instrumental in establishing anterior and posterior
signaling centers that direct future development (Billecke et al., 2004).

The function of the maternal genes is to establish morphogen gradients that
control the expression of zygotic genes in the growing population of nuclei in
the syncytial embryo. The first zygotic genes to be expressed along the
anterior-posterior axis are called gap genes, which define broad domains of
contiguous prospective body segments (Driever and Nusslein-Volhard, 1989;
Gaul and Jackle, 1990; Rivera-Pomar et al., 1995; Struhl, 1989b). The
products of gap genes, in turn, transfer the positional information onto the
pair-rule genes, which are expressed in seven stripes in the syncytial embryo
and define the borders of parasegments (Rivera-Pomar and Jackle, 1996; St
Johnston and Nusslein-Volhard, 1992). The products of the pair rule genes
activate the expression of segment polarity genes in 14 transverse stripes,
and these genes establish and maintain the parasegmental boundaries
between anterior and posterior compartments so that each parasegment
becomes a restricted developmental compartment (Martinez-Arias and
Lawrence, 1985; Nasiadka, 2002).

Each segment of the Drosophila larva is unique, both in terms of its ventral
denticle pattern and its internal organization. Furthermore, segments give rise
to different appendages in the adult fly (Morata and Lawrence, 1975). The
identities of individual parasegments are specified by the expression of a
characteristic pattern of homeotic selector genes, or Hox genes, which give
cells their positional values along the A-P body axis (Kaufman et al., 1990;
Kennison and Tamkun, 1992; Lewis, 1978). Most of the HOX genes are
clustered in the Bithorax (BX-C) or Antennapedia complex (ANT-C) (Figure
1.1). The arrangement of the homeotic genes within these different clusters on
chromosome 3 parallels the body segments they affect along the A-P axis and
this is referred to as “co-linearity” (Rosenfield, 2006). Interestingly, mutations
in these genes do not usually disrupt the segmental arrangement of the body,
but they cause homeosis, the phenomenon in which one body part develops
with the likeness of another (Castelli-Gair and Akam, 1995; McGinnis and Kuziora, 1994).

Figure 1.1 Homeotic genes in the Antennapedia and Bithorax complex

Hox gene expression in the Drosophila melanogaster embryo defines the positions of structures and appendages along the anterior–posterior (A-P) axis of the adult body. Differential expression of the homeotic genes (lab, Pb, Dfd, Scr, Antp, Ubx, abdA, abdB) along the A-P axis specifies segment identities in the embryo. The specific patterns are indicated by different colors in the embryo and in the prospective body parts of the adult fly (Adapted from (Rosenfield and Ziff, 2006)).

The establishment of homeotic gene expression is controlled by transcription factors encoded by the gap and pair-rule genes (Casares and Sanchez-Herrero, 1995; Muller and Bienz, 1992; Qian et al., 1991; Shimell et al., 1994; Zhang et al., 1991). These transcription factors disappear after the first 6
Introduction

hours of development (Frasch et al., 1987; Gaul et al., 1987; Tautz, 1988). Therefore, in order to allow differentiation to proceed correctly, a mechanism has to exist which ensures that the initial homeotic gene expression patterns are transmitted through many rounds of cell division, until differentiation starts.

1.2 Polycomb and Trithorax Group Proteins: Components and regulators of the Cellular Memory mechanism

Once the spatially restricted patterns of homeotic gene expression have been established by transiently expressed transcription factors, these patterns and the determined cell fates they implement are faithfully maintained throughout the rest of development by the action of another set of factors encoded by the Polycomb group (PcG) and trithorax group (trxG) genes. The PcG proteins are required to maintain the transcriptionally inactive state, whereas the TrxG proteins are necessary to counteract silencing and maintain the transcriptionally active state (Orlando et al., 1998). Both PcG and TrxG proteins are thought to function by establishing closed or open chromatin configurations at their target genes (Orlando et al., 1998; Pirrotta, 1997). In addition to these trans-acting factors, extensive genetic studies have identified chromosomal elements which are required in cis to mediate the PcG/TrxG dependent inheritance of transcriptional states (Busturia and Bienz, 1993; Chan et al., 1994; Christen and Bienz, 1994; Poux et al., 1996; Simon et al., 1993; Simon et al., 1990; Zhang and Bienz, 1992). The target genes of the PcG and TrxG proteins carry cis-regulatory elements that enable both groups of proteins to bind and to maintain the status of transcriptional activity of the gene over many cell generations. These elements have a dual function as Trithorax and Polycomb response elements, and have been termed PRE/TREs (Polycomb/Trithorax Response Elements) (Ringrose et al., 2003; Tillib et al., 1999).

The maintenance of transcriptional memory at PRE/TREs is ‘epigenetic’. It is “a change in the state of expression of a gene that does not involve a mutation, but that is nevertheless inherited after cell division in the absence of the signal or event that initiated that change” (Ringrose and Paro, 2007). The
information required to turn gene activity off or on after each new cell division is carried on the PRE/TRE, and copied to both new daughter cells at replication and mitosis (Ringrose and Paro, 2007). The epigenetic nature of PRE/TRE states has been confirmed by several studies that have demonstrated that transgenic PRE/TREs, with their own or foreign promoters, can maintain gene expression states through many cell divisions in the absence of the initial activating or repressing factors (Cavalli and Paro, 1998; Maurange and Paro, 2002; Poux et al., 1996; Rank et al., 2002).

1.2.1 Keeping the silence - the Polycomb group

The PcG proteins will silence by default, unless the TrxG proteins prevent them from doing so (Ringrose and Paro, 2004). There is accumulating evidence that the exact composition of PcG complexes is rather dynamic and that their activities may be subject to tissue-specific and/or developmental regulation (Otte and Kwaks, 2003; Strutt and Paro, 1997). Normally, PcG-mutant Drosophila exhibit anterior to posterior homeotic transformations in the abdomen and homeotic transformation of second and third thoracic segments toward more anterior thoracic segments (Kennison, 2004).

Generally, PcG silencing involves at least three kinds of multiprotein complexes. These are now referred to as the PRC1, PRC2 and PhoRC complexes (Figure 1.2.1). The PRC1 complex, biochemically purified from fly embryos, contains a core quartet of PcG proteins: Polycomb (PC), Polyhomeotic (PH), Posterior sex combs (PSC), and Drosophila Ring1 (dRING1). The chromodomain of the PC subunit of PRC1 binds specifically to trimethylated lysine 27 in the histone H3 tail (H3K27me3) in vitro (Fischle et al., 2003; Min et al., 2003). dRING is ubiquitin E3 ligase that catalyze the monoubiquitylation of histone H2A at lysine 119 (H2AK119ub1), a histone mark that is associated with transcriptional silencing (Buchwald et al., 2006; Cao et al., 2005; Wang et al., 2004a). This histone modification seems to be under tight control, since another PcG complex, PR-DUB containing Calypso and ASX, is involved in actively removing this mark (Scheuermann et al., 2010). The core of PRC1 has also been reconstituted in vitro and was shown to inhibit chromatin remodeling mediated by the human SWI/SNF ATPase.
complex and transcription by RNA polymerase II (King et al., 2002; Saurin et al., 2001; Shao et al., 1999). Moreover, the PRC1 core compacts nucleosomal arrays \textit{in vitro} leading to a chromatin conformation, which could reduce DNA accessibility for key transcription factors at promoters and enhancers in order to repress target genes (Francis et al., 2004; King et al., 2002; Lo et al., 2009). The full PRC1 complex has a reported size in the range of about 1-2 MDa when purified from 0-12h embryos and comprises more than 30 associated proteins. Among them are Sex comb on midleg (SCM), TBP (TATA-box binding protein)-associated factors TAFII250, TAFII110, TAFII85, and TAFII62 and DNA binding protein ZESTE (Levine et al., 2002; Saurin et al., 2001; Shao et al., 1999). The presence of the TAF promoter factors indicates the possibility of a direct interaction between PcG complexes and promoter factors. ZESTE sites are found in PREs (Mulholland et al., 2003), the target sites of GAF (GAGA factor) and PHO (Pleiohomeotic) are also found in PREs, and both proteins can be co-immunoprecipitated with PcG proteins (Horard et al., 2000; Mohd-Sarip et al., 2002; Poux et al., 2001).

Interestingly, in cultured \textit{Drosophila} cells, even when the homeotic genes are silenced by the PcG, transcription factors are still bound at their promoters (Breiling et al., 2001) and TBP (TATA binding protein)-associated factors (dTAFIIIs) can be co-purified with PRC1. This association between PcG proteins and transcription factors must be relatively robust, as it survives purification (Saurin et al., 2001). Thus, it is clear that the PcG do not exclude transcription factors from silenced promoters and this suggests that one aspect of PcG silencing is the direct inhibition of the transcriptional apparatus. Dellino et al. (2004) found that PcG may affect the polymerase itself. When a PRE is placed next to a reporter gene with an inducible heat shock promoter, it silences the gene. At the promoter, transcription factors and RNA polymerase are bound, but the RNA polymerase does not melt the DNA at the start site, and thus transcription is not initiated (Dellino et al., 2004). Furthermore, PRC1 has been proposed to mediate gene repression by stalling Pol II elongation, deletion of Ring1A and Ring1B leads to the loss of ubiquitination of H2A, release of poised RNA Pol II, and subsequent gene de-repression (Stock et al., 2007). Consistent with this finding, PRC1 proteins
were also found to preferentially bind stalled promoters (Enderle et al., 2011). In addition to the “classical” PRC1 complexes, CHRASCH complex (Chromatin associated silencing complex for homeotics) has been purified from *Drosophila* Schneider cells (Huang and Chang, 2004). This complex is related to PRC1 but has an important difference: It contains the PcG protein Pipsqueak (PSQ), which allows the complex to bind specifically to DNA targets from PREs that contain the \((GA)_n\) motif. The CHRASCH complex contains a histone deacetylase (HDAC1), RPD3 in addition to the PC, PH, PSC and PSQ proteins (Huang and Chang, 2004; Huang et al., 2002).

**Figure 1.2.1 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. (Dark blue) Core proteins of PRC1 and PRC2.
The PRC2 complex, originally purified from *Drosophila* embryos, consists of the core components Enhancer of Zeste (E(Z)), Extra Sex Combs (ESC), Suppressor (12) of Zeste (SU(Z)12) and NURF55 (N55), a histone-binding protein that is also associated with the chromatin assembly factor, and other remodeling complexes (Polo and Almouzni, 2006; Taylor-Harding et al., 2004) (Figure 1.2.1). The E(Z) subunit of this complex catalyzes the methylation of histone H3 at lysine 27 (H3K27), a modification which is associated with PREs and other repressed heterochromatic regions (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002), and to a lesser extent catalyzes the methylation of histone H3 at lysine 9. This enzymatic activity depends essentially on association with the Extra sex combs (Esc) and SU(Z)12 proteins (Cao and Zhang, 2004; Muller et al., 2002; Pasini et al., 2004). This complex may be more evolutionarily ancient than PRC1, as its core members are conserved in plants and in *C. elegans* (Ringrose and Paro, 2004; Sawarkar and Paro, 2010).

In flies, different approaches and purification schemes have resulted in purification of PRC2 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 1.2.1). The most prevalent complex is the 600 kDa PRC2 purified from *Drosophila* embryos (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Ng et al., 2000). 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 (Papp and Muller, 2006; Tie et al., 2003). Other larger complex appears at later developmental stages and contains SIR2, the homologue of yeast SIR2 Nad+ dependent Histone deacetylase (Furuyama et al., 2004; Furuyama et al., 2003). 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. Also RPD3, a histone deacetylase, has been reported to co-purify with some of the larger complexes (Figure 1.2.1) (Czermin et al., 2002; Furuyama et al., 2004; Tie et al., 2003).
On the other hand, the Pleiohomeotic (PHO) protein, which specifically binds to DNA motifs, is commonly found in PREs. It can interact with the PRC1 subunits PC and PH (Mohd-Sarip et al., 2002), and with PRC2 through binding to E(Z) (Wang et al., 2004b). An attractive model emerging from multiple studies is that the sequence-specific binding of PHO is the initial event during the establishment of PcG repression. Subsequently, PRC2 complexes become recruited via the interaction with E(Z), which in turn methylates H3K27. It has been shown that this methylation mark is specifically bound by the chromodomain of PC, albeit weakly (Fischle et al., 2003). Thus, the recognition of methylated H3K27 by PC, together with the direct interaction of PC and PH with DNA-bound PHO is thought to be responsible for the tethering of PRC1 to the chromatin specifically at PREs (Cao et al., 2002; Fischle et al., 2003; Mohd-Sarip et al., 2002; Wang et al., 2004b) (Lee et al., 2007). However, recent genome-wide profiling of histone modifications and mapping of chromatin binding proteins challenged the view of PC recruitment by H3K27me3. First, the known and presumptive PREs seem to be depleted of H3 methylation, probably because they are depleted of nucleosomes (Kahn et al., 2006; Mohd-Sarip et al., 2006; Papp and Muller, 2006; Schwartz et al., 2006), which also shows in their hypersensitivity to DNase I nuclease digestion (Mishra et al., 2001; Schwartz and Pirrotta, 2007). Second, the distribution of trimethylated H3K27 at a silenced gene extends over the entire transcription unit and the upstream regulatory region, frequently involving many tens of kilobases (Kahn et al., 2006; Papp and Muller, 2006; Schwartz et al., 2006). Although the distribution of PC itself is generally broader than that of the other PcG proteins, and tails gradually from the PRE peak, it certainly does not parallel the distribution of methylation. Third, PRC1 can stay on the chromatin for some time even if H3K27me3 is completely lost (Ohno et al., 2008). So, the most reasonable conclusion would be that, although K27 methylation is important for PC binding, it is not sufficient. If the role of H3K27me3 is not the recruitment of PRC1 then the function of the chromodomain of PC might be to facilitate transient, long range interactions between the PRE and their target promoters (“looping”) (Comet et al., 2006; Lanzuolo et al., 2007; Muller and Kassis, 2006; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007; Wang et al., 2004a). This kind of
interaction could be responsible for the wide spreading of H3K27me3 mark. Consistent with this, PC itself appears to be spread over wider regions than other PRC1 and PRC2 components (Beisel et al., 2007; Papp and Muller, 2006). This could be due to transient interactions during PRE looping, fixed during ChIP experiments measuring interaction of mixed populations.

The PHO-containing complex, PhoRC (Figure 1.2.1), is involved in homeotic gene silencing. In Drosophila, PHO and Pleiohomeotic-like (PHOL) function redundantly to maintain Hox genes silencing (Brown et al., 2003). PhoRC includes an MBT-domain protein, Drosophila Scm-related gene containing four mbt domains (SFMBT). Its Malignant Brain Tumor (MBT) repeats bind specifically to mono- and dimethylated H3K9 and H4K20 (Klymenko et al., 2006), which could also be involved in PRE looping. 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). PhoRC occupies short PREs of a large set of developmental regulator genes in both embryos and larvae. The majority of these PREs are co-occupied by the PcG complexes PRC1 and PRC2 (Oktaba et al., 2008).

In addition to PHO, many other proteins with DNA-binding affinities including Zeste, GAGA factor (GAF), dorsal switch protein 1 (DSP1), Sp1/Klf family members, Pipsqueak (PSQ) and Grainyhead (GRH) were connected to PcG silencing on the basis of genetic interactions, biochemical co-purification, functional assays and/or co-localization on PREs (Beisel and Paro, 2011; Muller and Kassis, 2006; Ringrose and Paro, 2007).

1.2.2 Propagating the active state - the Trithorax group

The PcG target genes that have been studied are also known to be positively regulated by Trithorax and other proteins including ASH1 and ASH2, which work antagonistically to the PcG proteins (Klymenko and Muller, 2004; Poux et al., 2002). Mutants of trxG genes often exhibit anterior transformations in the abdomen, partial transformation of halteres to wings and transformations of first legs to second (Kennison, 1993; Kennison, 1995).
There are two TrxG proteins, namely GAGA factor (GAF) and Zeste (Z), are found to be sequence-specific DNA-binding proteins (Chen and Pirrotta, 1993; Katsani et al., 1999). Both GAF and Zeste are not exclusively devoted to the regulation of PcG/TrxG target genes, as their DNA recognition sequences can be found in the promoters of many genes known not to be controlled by the cellular memory system (Ringrose et al., 2003). Bejarano and Busturia proposed that GAF has a role in establishing a chromatin ground state needed for gene regulation by either PcG or TrxG (Bejarano and Busturia, 2004). This suggestion is supported by the observation that GAF facilitates binding of PHO to a chromatinized template (Mahmoudi et al., 2003) and Trithorax-like (the gene encoding the GAF) mutations enhance PcG mutations or are needed for silencing (Busturia et al., 2001; Gildea et al., 2000; Hagstrom et al., 1997; Horard et al., 2000; Mishra et al., 2003; Strutt et al., 1997). Similar dual functions have been described for Zeste. For example, Zeste can recruit the TrxG protein Brahma (BRM) to a chromatin template in vitro (Kal et al., 2000), and Zeste is also needed to recruit the BRM complex (SWI/SNF complex) to target gene regulatory maintenance elements in vivo (Dejardin and Cavalli, 2004). In contrast, Zeste is also a member of PRC1 (Saurin et al., 2001) and enhances PRC1 function (Mulholland et al., 2003).

TrxG proteins form a somewhat heterogeneous group (Figure 1.2.2). One class of TrxG factors, involved in general transcriptional processes, include components of ATP-dependent chromatin remodeling complexes like the BRM, NURF and ACF complexes. So, their function is not limited to epigenetic maintenance (Collins and Treisman, 2000; Smith et al., 2004). The BRM complex is highly related to the yeast SWI/SNF nucleosome-remodeling complex, and the BRM protein (SWI2/SNF2 in yeast) functions as the ATPase subunit of this complex, using the energy of ATP hydrolysis to move histones (Papoulas et al., 1998). In addition to BRM, two other general chromatin remodeling complexes might contribute to TrxG function. Both complexes contain ISWI protein as ATP dependent chromatin remodeling engine. In one complex ISWI is associated with several NURF proteins (Badenhorst et al.,
2002) and in the other with ACF protein (Figure 1.2.2) (Fyodorov et al., 2004; Fyodorov and Kadonaga, 2002).

A second class of TrxG members is composed of SET domain factors like *Drosophila* TRX and ASH1, as well as their associated proteins (Schuettengruber et al., 2007). TRX and ASH1 proteins are involved more specifically in regulation at PREs (Chinwalla et al., 1995; Rozovskaia et al., 1999). ASH1 and ASH2 (absent, small, and homeotic) are related proteins, which are present in different high molecular weight complexes (Papoulas et al., 1998). ASH1 exists in a 2MDa complex in *Drosophila* embryos. Via its SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain, it catalyzes the methylation of lysines 4 and 9 in histone H3 (H3K4, H3K9) and of lysine 20 in histone H4 (H4K20), which is required for the maintenance of HOX gene expression *in vivo*. Additionally, ASH1 is also able to methylate H3K36 (Beisel et al., 2002; Byrd and Shearn, 2003; Klymenko and Muller, 2004; Tanaka et al., 2007). The TRX protein is a SET-domain histone methyltransferase (HMTase) that methylates H3K4 which is typically associated with active chromatin (Lachner et al., 2003). Similar to ASH1, the HMTase activity of TRX is required to prevent the PcG-mediated repression of the homeotic gene *Ultrabithorax (Ubx)* within its normal expression domains *in vivo* (Klymenko and Muller, 2004). Besides TRX, the TAC1 complex of 1MDa also contains the histone acetyltransferase CBP (CREB binding protein) and the phosphatase-related protein SBF1 (Petruk et al., 2001; Smith et al., 2004). dCBP, encoded by the *nejire* gene, is a histone acetyltransferase that has also been associated to ASH1 (Bantignies et al., 2000). The presence of dCBP together with ASH1 and TRX suggests that both complexes may act by combining the histone acetylation function of dCBP with H3K4 trimethylation activity of TRX and ASH1.
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Recent study showed that TRX was proteolytically cleaved into an N-terminal and a C-terminal domain by Taspase (Capotosti et al., 2007). Genome-wide mapping studies using two different antibodies, one against TRX-C (which recognizes all isoforms) and one against TRX-N (which recognizes only two isoforms), have provided further insight into how this switch in PcG target gene activity might happen (Schuettengruber and Cavalli, 2009). TRX-C showed high affinity to PcG binding sites, whereas TRX-N bound mainly to active promoter regions trimethylated on H3K4 (Schuettengruber et al., 2009).

Figure 1.2.2 Trithorax Group complexes involved in transcriptional activation.

The TrxG complexes, ASH1, ASH2 and TAC1 directly counteract the default state of silencing of target genes by PcG proteins. Other TrxG complexes, like BRM, NURF and ACF, are involved in chromatin remodeling and might assist TrxG in their function (question marks represent unidentified components of the complexes).
Thus, the isoforms of TRX or the N-terminal / C-terminal part of the protein might have different functions. At PREs, TRX-C might regulate the expression level of PcG targets together with PcG proteins and the binding of TRX-C might allow the target genes to switch their state in response to transcription-inducing signals. At promoter regions that are not occupied by PcG factors, TRX-N might work as a general transcription co-factor and constitutively activate the transcription (Petruk et al., 2006; Petruk et al., 2008).

In summary, the TrxG proteins involved in chromatin remodeling such as the BRM complex and NURF complex presumably function as co-activators, which explains their roles in the regulation of global transcription. The ASH and TRX containing complexes, on the other hand, directly counteract the default state of silencing of target genes by PcG proteins (Klymenko and Muller, 2004).

1.2.3 Polycomb/Trithorax group Response Elements (PRE/TREs):
Cis-acting regulatory elements
In the *Drosophila* Bithorax complex, the studies of the regulation of homeotic genes uncovered two classes of cis-regulatory DNA elements with different behavior: initiator elements (parasegment-specific enhancers) and maintenance elements (PRE/TREs) (Busturia et al., 1989; Chan et al., 1994; Chiang et al., 1995; Simon et al., 1993; Simon et al., 1990). These two types of elements regulate the expression patterns of the homeotic genes at different stages of embryonic development. In the first three hours of development, the expression of each homeotic gene depends on the local concentrations of segmentation gene products and the initiator elements are in control. After these homeotic gene patterns have been established and the segmentation gene products degrade, the maintenance elements come into control and the transcriptional status of each gene is subsequently maintained throughout the rest of development, and into adulthood, by the ubiquitously expressed PcG and TrxG proteins (Maeda and Karch, 2006; Ringrose and Paro, 2007). They remember and maintain the expression patterns of the
homeotic genes in the BX-C by binding to the bx, bxd, iab2, iab3, Mcp, iab6, Fab-7, iab8 and the putative iab9 PRE (Figure 1.2.3) (Barges et al., 2000; Beisel et al., 2007; Busturia and Bienz, 1993; Busturia et al., 1997; Chan et al., 1994; Chiang et al., 1995; Christen and Bienz, 1994; Gyurkovics et al., 1990) and work antagonistically to maintain silenced or active transcriptional states (Chinwalla et al., 1995; Moehrle and Paro, 1994; Orlando et al., 1998). The cis-acting elements have been identified at many loci by functional assays: in the Bithorax (BX-C) and Antennapedia (ANT-C) complexes containing the homeotic genes (Gindhart and Kaufman, 1995; Orlando and Paro, 1995; Zink et al., 1991), the polyhomeotic locus (Bloyer et al., 2003), the engrailed locus (Kassis, 1994), the hedgehog locus (Maurange and Paro, 2002), the cyclin A (Martinez et al., 2006) and invected locus (Cunningham et al., 2010). Hence, the PREs are considered to be epigenetic switchable elements. They maintain the previously determined transcriptional state of their associated genes over many cell generations, thus ensuring a memory of transcriptional history (Ringrose et al., 2003).

Figure 1.2.3 Regulatory regions of the BX-C.

Interspersed in the complex are parasegment-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-7 and iab8; iab9 is a putative PRE) indicated with red arrows in the bithorax complex (Adapted from (Gilbert, 2003)).

Several short DNA sequence motifs that are required for PRE function have been identified. They are the binding sites for three sequence-specific DNA
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binding proteins: the Pleiohomeotic protein (PHO), a PcG member (Brown et al., 1998; Mihaly et al., 1998), the GAGA factor (GAF) (Strutt et al., 1997) and the Zeste protein (Z) (Hur et al., 2002; Saurin et al., 2001). Each of these motifs occurs at least once in all known PREs. Nevertheless, before (Ringrose et al., 2003) reported the definition of sequence criteria that distinguish PREs from non-PREs, only very few PREs had been identified, due to the lack of a defining consensus sequence and the little similarity between known PRE sequences. Ringrose et al. (2003) identified 167 candidate PREs in Drosophila genome, which map to genes involved in development and cell proliferation. Some of these PREs were shown to be bound and regulated by Polycomb proteins in vivo (Ringrose et al., 2003). However recent genome-wide mapping of PcG distribution in Drosophila indicate that, only 20% of the detected PcG binding peaks are predicted by the algorithm, the majority are not. Conversely, a similar majority of the predicted PREs fail to bind PcG proteins (Negre et al., 2006a; Schwartz et al., 2006; Tolhuis et al., 2006). It is likely that GAF, PHO and ZESTE are not the only DNA-binding proteins that are associated with PREs, their presence is not sufficient to define a functional PRE. Clues to further pieces in the puzzle of PRE design come from some additional recent studies, showing that the high mobility group (HMG)-like dorsal switch protein 1 (DSP1) (Dejardin et al., 2005), Grainy head (Grh) (Blastyak et al., 2006) and Sp1/KLF DNA-binding proteins (Brown et al., 2005) are also vital for recruiting the PcG proteins to specific PREs. The inclusion of Dsp1 or Sp1/KLF sites increases the number of experimentally defined Polycomb-binding sites that are hit by a prediction (Fiedler and Rehmsmeier, 2006).

Interestingly, a novel anti-silencing mechanism via PRE was also found. The transcription through endogenous PREs was shown to be required continuously as an anti-silencing mechanism to prevent the access of repressive PcG complexes to the chromatin (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Sanchez-Elsner et al., 2006; Schmitt et al., 2005). The non-coding transcript of bxd PREs is found to recruit ASH1 to Ubx in Drosophila and is indispensable for maintenance of Ubx expression (Sanchez-Elsner et al., 2006). Thus, non-coding transcription in
memory element seems to play an important role in switching the epigenetic state of the PRE/TRE from repressed to active and in recruiting HMT-containing TrxG complexes.

1.2.4 Genome-wide distribution and dynamic regulation of PcG proteins

PcG proteins were long considered to represent epigenetic gatekeepers of cellular memory processes (Ringrose and Paro, 2004). However, recent genome-wide mapping studies of PcG components widened our view on chromatin profiles of PcG proteins: they revealed that PcG proteins bind preferentially to genes encoding transcription factors. Many of these transcription factors, including many homeodomain-containing proteins, are involved in developmental patterning, morphogenesis and organogenesis (Bracken et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006). This supports the idea that PcG mediated epigenetic mechanisms play a global role to coordinate many pathways necessary for the development of a multicellular adult organism (Schuettengruber et al., 2007). Most recently, PcG proteins were also shown to bind the promoters of primary transcripts of microRNA genes, which expands the scope of PcG control to miRNA genes essential for development, apoptosis and growth (Enderle et al., 2011). Furthermore, PcG proteins are also capable of tissue-specific and dynamic gene regulation during fly development (Kwong et al., 2008; Negre et al., 2006a; Oktaba et al., 2008). Especially, testis-specific transcription factors have been shown to work against PcG protein complex-mediated silencing by selectively removing PcG protein complexes from target promoters to activate testis-specific genes (Chen et al., 2005). Interestingly, the PcG protein-binding patterns showed dynamic behavior during embryonic development, new binding sites appeared during later stages and the sites present in early embryos disappeared in older ones (Negre et al., 2006a). It suggests that PcG repression is a more dynamic process than previously thought.

Dynamic gene regulation by PcG proteins is even more prominent in mammalian ES (embryonic stem) cells. PRC1 and PRC2 targets were
mapped by ChIP-on-chip in murine and ES cells, which demonstrated that PcG complexes are mainly bound at genes important for development, morphogenesis, organogenesis, neurogenesis and transcriptional regulation (Boyer et al., 2006; Lee et al., 2006). Many of these regulator genes are repressed in ES cells. During differentiation occupancy of PcG complexes at the previously identified targets is drastically reduced, showing that PcG-repressed genes in ES cells are able to become activated when cells lose their pluripotent state (Boyer et al., 2006; Lee et al., 2006; Mohn et al., 2008; Schuettengruber and Cavalli, 2009), which indicates a crucial role for Pc proteins in the dynamic regulation of stem cell identity and cell fate determination. Furthermore, PcG proteins not only prevent differentiation by repressing specific genes, but also enable and modulate differentiation in response to appropriate signals (Schuettengruber and Cavalli, 2009). ES cells with impaired SUZ12 (mammalian ortholog of SU(Z)12) function fail to repress pluripotency genes efficiently during differentiation, and differentiation markers are not derepressed completely (Pasini et al., 2007; Schuettengruber and Cavalli, 2009). In addition, the expression of BMI1 and RING1B (mammalian homolog of PSC and dRING) is also required for proper neuronal differentiation (Cui et al., 2006; Roman-Trufero et al., 2009). Therefore, PcG proteins play a vital, but context dependent role in the maintenance of stem cell proliferation and in differentiation processes, they are involved in the dynamic regulation of genes belonging to variety of biological processes and additional components must be involved to achieve the gene specificity.

Recently, the identification of H3K27 demethylases: two JmjC domain-containing proteins, JMJD3 and UTX, further confirmed that PcG protein-dependent histone modifications can be actively removed, which enables the activation and the dynamic regulation of genes repressed by PcG protein complexes (Agger et al., 2007; De Santa et al., 2007; Hong et al., 2007; Swigut and Wysocka, 2007).

Furthermore, it was found that the TRX binding did not necessarily coincide with active transcription, and also, PcG-bound genes can be repressed or actively transcribed (Beisel et al., 2007; Enderle et al., 2011; Papp and Muller,
2006; Schwartz et al., 2006; Schwartz et al., 2010). In addition, PcG proteins and TRX could constitutively bind at Drosophila PREs in BX-C, independently of their ON or OFF state (Papp and Muller, 2006). This finding further suggests that the PcG/TrxG system may be regulated by additional co-factors.

1.2.5 Gene regulation by PcG/TrxG: additional players come into action

To date, the finding of an involvement of proteins as different as the cohesin complex, molecular chaperones like Hsp90, Hsc4, Droj2 and immunophilins in the PcG/TrxG mediated gene regulation already suggested the PcG/TrxG-system to be an intricate and carefully regulated network (Anderson et al., 2002; Mollaaghababa et al., 2001; Strubbe et al., 2011; Tariq et al., 2009; Wang and Brock, 2003; Yang et al., 1995; Yang et al., 2001).

Cohesin is a protein complex that regulates the separation of sister chromatids during cell division and has recently been linked to the PcG/TrxG based cellular memory system by genetic interaction with TrxG mutations (Hallson et al., 2008) and by coregulation of PcG target genes (Rollins et al., 1999; Schaaf et al., 2009; Strubbe et al., 2011). Nipped-B, loading cohesin onto chromosome, participates in activation by remote enhancers in the cut and Ultrabithorax genes (Rollins et al., 1999). Knock-down of cohesin subunits Rad21, SA or Smc1 increases cut expression (Dorsett et al., 2005; Rollins et al., 2004). Genes located in the invected-engrailed gene complexes in BG3 cells increase their transcription after loss of cohesin (Schaaf et al., 2009). Therefore, the influence of cohesin on gene regulation includes both activation and silencing, which might be context dependent. Although cohesin associates preferentially with active genes, and is generally absent from regions in which histone H3 is methylated by the E(z) Polycomb group silencing protein (Misulovin et al., 2008), there are two exceptional cases, where cohesin and the E(z)-mediated histone methylation simultaneously coat the entire Enhancer of split and invected-engrailed gene complexes in cells derived from Drosophila central nervous system (Schaaf et al., 2009). Most recently, Strubbe et al. (2011) further supported the connection between
cohesin and PcG by providing evidence for a biochemical interaction of PC with Cohesin in early embryonic development. However, it is not clear why this interaction does not lead to co-localization on chromatin of BG3 or salivary gland cells (Misulovin et al., 2008; Strubbe et al., 2011).

Interestingly, some molecular chaperones were also shown to interact with PcG and TrxG. Molecular chaperones are a class of proteins that, by preventing improper associations, assist in the correct folding or assembly of other proteins in vivo (Deuerling and Bukau, 2004). They facilitate developmental signaling and can be indispensable for survival in unpredictable environments (Rutherford, 2003). Sollars et al. (2003) found morphological alterations occur in Drosophila melanogaster when function of Hsp90 is compromised during development and mutations in nine different genes of TrxG also induce the same abnormal phenotype. This suggests that Hsp90 may have a role in TrxG mediated gene regulation (Sollars et al., 2003).

Recently it was shown that Hsp90 cooperated with TRX at chromatin for maintaining the active expression state of targets like the Hox genes, the inhibition of Hsp90 results in degradation of TRX and a concomitant down-regulation of homeotic gene expression (Tariq et al., 2009). Furthermore, the heat shock cognate 4 (Hsc4) molecular chaperone has been shown to be a part of the PcG multiprotein complex and hsc mutant flies have a Pc-like phenotype (Mollaaghababa et al., 2001). Similarly, the molecular chaperone Hsc4 and a novel Drosophila J class chaperone (Droj2) were found stably associated with Polyhomeotic in Drosophila Kc167 cells (Wang and Brock, 2003). These findings suggest that PcG/TrxG mediated regulation requires many other co-factors to complete and regulate their activities. However, the knowledge about additional co-factors in the PcG/TrxG system is still far from complete. Moreover, it was found that immunophilins, a group of protein containing peptidyl-prolyl cis-trans isomerase activity were also involved in PcG/TrxG mediated gene regulation.
1.3 Immunophilins: biological diversity and functions

Immunophilins are a varied family of chaperones found throughout all known taxonomic groups. These proteins are also known as peptidyl-prolyl cis-trans isomerases (PPIase) for their ability to convert proline bonds from cis to trans form, a rate-limiting step in protein folding (Figure 1.3) (Davies and Sanchez, 2005; Galat, 1993; Harding et al., 1989; Kay, 1996; Schiene-Fischer and Yu, 2001; Schiene and Fischer, 2000; Standaert et al., 1990). They were originally discovered as targets of the structurally unrelated drugs cyclosporine A (CsA) and FK-506, which are powerful immunosuppressants used prophylactically following transplant surgery to prevent organ rejection. Both drugs bind to receptors that have therefore been named “immunophilins” to describe their immunosuppressant binding activity (Wiederrecht, 1994). Immunophilins can be divided into two subfamilies based on their ability to bind specific immunosuppressive drugs. Those that bind cyclosporine A (CsA) are known as cyclophilins (CYPs), while those that associate with FK506, rapamycin are known as the FK506-binding proteins (FKBPs) (Galat, 2003; Liu, 1993; Sigal and Dumont, 1992; Somarelli et al., 2008). The formation of FKBPG FK506 and CYP/CsA complexes inhibits not only the PPIase activity of FKBPG CYP but also the phosphatase activity of the secondary target calcineurin (CaN), thereby preventing the dephosphorylation of NF-AT that is required for IL-2 gene expression and T-cell activation (Huai et al., 2002; Ke and Huai, 2003). Hence, immunophilins are essential and indispensable for the survival of the organisms.

In human, fifteen FKBPs have been identified, whose segments have significant homology with the 12 kDa FKBP protein (Galat, 2003). The 12 kDa FKBP, is the smallest and most comprehensively studied FKBP, which is 108 amino acids in length and contains just one FKBP domain (Kang et al., 2008), whereas other FKBPs possess from one to four FKBP domains and have masses varying from 13 to 135 kDa (Somarelli et al., 2008). The human genome contains also at least sixteen genes encoding proteins comprising one cyclosporine-A (CsA) binding domain (cyclophilin-like domain, CLD) called cyclophilins, whose masses vary from 17 to 324 kDa. Conversely, the C.
elegans, D. melanogaster and S. cerevisiae genomes encode a less diverse spectrum of immunophilins (Arevalo-Rodriguez et al., 2004; Galat, 2003).

Figure 1.3 cis-trans prolyl isomerization of peptide chains
PPIase interconverts the cis and trans isomers of peptide bonds with the amino acid Proline (Pro). A sharp bend is introduced into the polypeptide backbone of a protein when Proline adopts the cis configuration. The isomerisation reaction can lead to substantial conformational changes in the protein (Derived from (Freeman, 2001)).

Cyclophilins are ubiquitous proteins (Galat, 1999) present in all subcellular compartments and involved in a wide variety of processes (Romano et al., 2004), including receptor complex stabilization (Leverson and Ness, 1998), receptor signaling (Brazin et al., 2002; Yurchenko et al., 2002), protein trafficking and maturation (Ferreira et al., 1996; Shieh et al., 1989), apoptosis (Leung et al., 2008; Lin and Lechleiter, 2002), RNA processing (Krzywicka et al., 2001), and spliceosome assembly (Bourquin et al., 1997; Horowitz et al., 2002; Mortillaro and Berezney, 1998). In cyclophilins, some other domains besides the cyclophilin-like domain are defined; for instance, the RNA recognition motif (RRM) domain previously mentioned suggests an RNA targeting function (Wang et al., 2008), the U-box motif implies involvement in ubiquitin conjugation pathways (Hatakeyama et al., 2001), the WD-40 repeat and tetratricopeptide repeat (TPR) motif might confers a protein-protein interaction surface (Arevalo-Rodriguez et al., 2004; Davis et al., 2010; Davis et al., 2008; Li et al., 2007).
The FKBPs are found in a wide variety of organisms and help performing numerous cellular functions including protein folding (Davies and Sanchez, 2005; Galat, 1993; Harding et al., 1989; Kay, 1996; Schiene-Fischer and Yu, 2001; Schiene and Fischer, 2000; Standaert et al., 1990), regulation of cytokines (Galat, 1993; Liu et al., 1991; Rao et al., 1997), transport of steroid receptor complexes (Kurek et al., 2002; Sinares et al., 2003), maintenance of meiotic recombination checkpoint (Hochwagen et al., 2005), nucleic acid binding (Alnemri et al., 1994; Riviere et al., 1993; Somarelli et al., 2007), histone assembly (Xiao et al., 2006), and modulation of apoptosis (Edlich et al., 2005; Maestre-Martinez et al., 2006; Wang et al., 2005). These functions are mediated by specific domains that adopt distinct tertiary conformations. FKBPs possess up to four FKBP domains along with several additional functional motifs (Somarelli et al., 2008), including nucleic acid binding regions (Riviere et al., 1993), tetratricopeptide repeat (TPR) motifs (Davies and Sanchez, 2005), Ef-hand (EfH) calcium-binding domains (Nakamura et al., 1998), nucleolin-related domain (Arevalo-Rodriguez et al., 2004) as well as transmembrane (Rulten et al., 2006), nuclear localization (Jin et al., 1992; Riviere et al., 1993) and endoplasmic reticulum (ER) signal sequences (Jin et al., 1991).

1.3.1 Immunophilins in chromatin transcriptional regulation

Since the majority of immunophilins are cytoplasmic proteins, this suggests primary functions of immunophilins in the regulation of a wide variety of cytosolic events. However, nuclear immunophilins clearly exist as well, and some of them have been shown to play important roles in chromatin transcriptional regulation.

For example, in yeast, cyclophilin A was shown to interact with the Sin3-Rpd3 histone deacetylase complex and increase the disruption of gene silencing. (Arevalo-Rodriguez et al., 2000; Smith et al., 1999; Sun and Hampsey, 1999). The yeast cyclophilin 40 homologs Cpr6 and Cpr7 were also shown to interact directly with Rpd3 in vitro and in vivo (Duina et al., 1996b). Nuclear cyclophilin A physically associates with the Set3C histone deacetylase and controls
meiosis in *S. cerevisiae* (Arevalo-Rodriguez and Heitman, 2005). The DNA-binding activity of the c-Myb transcription factor is negatively regulated by a stable interaction with the nuclear immunophilin CyP40 (Leverson and Ness, 1998). In addition, a WD40 domain cyclophilin, CYP71, which functions in gene repression and organogenesis in *Arabidopsis thaliana*, was shown to associate with the chromatin of homeotic gene loci and physically interact with histone H3. Interestingly, the *cyp71* mutant showed reduced methylation of H3K27 at several target loci, consistent with the derepression of the corresponding homeotic genes (Li et al., 2007).

Furthermore, Kuzuhara and colleagues (2004) found that a nuclear FK506-binding protein, SpFkbp39p, the *S. pombe* yeast homolog of FKbp39 (Himukai et al., 1999), acts as a histone chaperone. The histone chaperone activity is also conserved in Fpr4 (Xiao et al., 2006), the budding yeast homologue of SpFkbp39p. Fpr4p localizes in the nucleus, and is specifically enriched in the nucleolus and associates with chromatin at the rDNA loci. The histone chaperone domain of Fpr4 is essential for silencing at the rDNA locus and the PPIase domain has a regulatory role in the silencing process (Kuzuhara and Horikoshi, 2004). Moreover, Fpr4 is regulating the level of H3K36 methylation by isomerizing Proline residues near Lysine 36 of histone H3. Importantly, abrogation of Fpr4 catalytic activity *in vivo* results in increased levels of H3K36 methylation and delayed transcriptional induction kinetics of specific genes in yeast (Nelson et al., 2006). Thus, already we can find several examples, where immunophilins function as essential co-factors for regulation of gene expression.

### 1.3.2 Immunophilins and PcG/TrxG proteins

To date, several nuclear cyclophilins and FKBP5s have been found to interact with PcG/TrxG proteins and affect their transcriptional activities. In mammalian cells, Cyclophilin A and FKBP12 were found to interact with the zinc finger transcription factor YY1, the human ortholog of *Drosophila* PHO, a member of the PcG. An overexpression of Cyclophilin A and FKBP12 represses the transcription from SV40 promoter containing YY1 binding site (Yang et al., 1995). This suggests that immunophilins may regulate the activity
of YY1 (Yang et al., 1995). Another immunophilin, FKBP25 has been shown to physically associate with the histone deacetylases HDAC1, HDAC2 and the HDAC-binding transcriptional regulator YY1. The DNA-binding activity of YY1 is dramatically increased by FKBP25 (Yang et al., 2001).

Moreover, Mi and colleagues (1996) discovered a novel nuclear cyclophilin (hCyP33) from human T cells. The hCyP33 has an RNA-recognition motif (RRM), which combined for the first time RNA binding with protein folding in one protein. The RRM domain of Cyp33 includes two conserved sub-motifs: RNP-1 (RGFAGVEF) and RNP-2 (LYVGGL), and is separated from the cyclophilin domain by a 50-amino-acid spacer (Mi et al., 1996). Cyp33 associates with polyribonucleotide poly(A) and poly(U), with a particular preference for a consensus AAUAAA motif (Wang et al., 2008). Recently a Drosophila cyclophilin, dCyp33 was shown to interact in vitro through its RRM domain with the third PHD finger of Trithorax (Anderson et al., 2002). Overexpression of dCyp33 in Drosophila SL1 cells, was shown to correlate with a decrease of Abdominal-B expression, a well-described PcG-target gene in the BX-C (Anderson et al., 2002). Consistently, the interaction between human Cyp33 (hCyp33) and MLL, the human homolog of Trithorax, is mediated through the hCyp33 RRM domain and the 3rd PHD finger of MLL. This interaction was found to be required for repression of HOXC8 and HOXC9 genes in vivo (Fair et al., 2001; Hom et al., 2010; Park et al., 2010; Wang et al., 2010). These findings suggest that immunophilins indeed play a role in PcG and TrxG mediated gene regulation. However, the exact mechanism of immunophilins in this type of transcriptional memory control is not fully understood.

1.4 Technique: Genome-wide analysis with next generation sequencing

Over the past years, next generation sequencing has made it relatively easy and fast to produce comprehensive DNA sequence information. Compare to classical sequencers, the ‘next-generation sequencers’ produce data at a rate several orders of magnitude higher. We are now able to more thoroughly
analyze and characterize gene regulation, structure, and function in a global and high-throughput manner (Fox, 2009). Most next-generation sequencers produce millions of short sequence reads, which limits their value for sequencing and assembly of unknown repeat-rich genomes. However, they are perfect for quantifying DNA or RNA molecules by counting, which uncover the binding profiles of the proteins or transcriptomes with an extreme high resolution, down to 1 base (Johnson et al., 2007). RNA-Seq (quantification of transcripts) and ChIP-Seq (quantification of protein-bound genomic DNA) are two very popular examples of counting applications (Fox, 2009; Werner, 2010; Zhou et al., 2010).

**ChIP-seq**
Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) couples the commonly used chromatin immunoprecipitation procedure, in which DNA–protein complexes are cross-linked and precipitated using an antibody (Gilmour and Lis, 1984), to next-generation sequencing of DNA fragments bound to the precipitated protein (Johnson et al., 2007). To date, ChIP-Seq has been applied to the identification of transcription factor binding sites as well as histone modifications on a genome-wide scale (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Robertson et al., 2007). Previously, microarray was the dominant method for analyzing DNA sequences interacting with proteins such as transcription factors *in vivo* (Sandmann et al., 2006). In such microarray studies, typically called ChIP-on-chip, a transcription factor is isolated by immunoprecipitation along with the DNA fragment to which it is bound. The co-immunoprecipitated DNA is labeled, hybridized to a DNA microarray, and the resulting hybridization signal data are analyzed (Mockler et al., 2005; Negre et al., 2006b). ChIP-seq offers several important advantages over microarrays including increased sequence information, sensitivity, and the need for less starting material (Fox, 2009; Mardis, 2007).
RNA-seq
The identification and quantification of mRNA species under different conditions or in different cell types have long been of interest to biologists. Two different approaches to high throughput gene expression profiling have emerged in the past decade to allow the simultaneous examination of gene expression levels on a genome-wide scale (Morozova and Marra, 2008). One method is based on microarray, in which cDNA is hybridized to an array of complementary oligonucleotide probes corresponding to genes of interest, and the abundance of a particular mRNA species is estimated from its hybridization intensity to the relevant probe (Schena et al., 1995). Another method, termed RNA-Seq (RNA sequencing), has clear advantages over microarray approaches and is expected to revolutionize the manner in which eukaryotic transcriptomes are analyzed (Wang et al., 2009). The sequencing of cDNA rather than genomic DNA focuses analysis on the transcribed portion of the genome. Transcriptome sequencing has been used for applications ranging from gene expression profiling, genome annotation, and rearrangement detection to noncoding RNA discovery and quantification (Morozova and Marra, 2008). Compare to microarray analysis, RNA-Seq has several advantages: (i) It is independent of prior knowledge, (ii) it features a bigger dynamic range, both on end of sparsely expressed transcript all the way to a much better quantification even of highly expressed transcripts (Wang et al., 2009), (iii) it identifies transcripts directly and covers all these transcripts over the entirely length rather than interrogating with a few pre-selected probes and (iv) it allows the identification of detailed transcript structures such as alternative promoters and alternative splicing events (Werner, 2010).

1.5 Aims of the thesis
The antagonistic role played by PcG and TrxG proteins in maintaining cellular memory during fly development involves different protein complexes as well as cis acting DNA elements called PREs. In a recent genome wide mapping of PcG proteins, it was found that PcG was highly enriched at repressed
PREs and the corresponding target gene promoters whereas the binding levels were significantly decreased at active loci (Enderle et al., 2011; Schwartz et al., 2006). This indicates that the epigenetic state of a PRE may primarily be regulated through the modulation of PcG binding activity. However, it has also been shown that PcG proteins and TRX were constitutively bound at Drosophila Polycomb response elements in BX-C independently of their activity state (Papp and Muller, 2006). This co-occupation of activating and repressing factors might have the advantage of allowing PcG target genes to switch rapidly from an inactive to an active state in response to an incoming signal. Therefore, this implies that additional components must be critically involved in controlling PcG-dependent repression. The recent findings about the function of immunophilins in chromatin biology make them good candidates for such a regulatory role. In particular, the immunophilin Fpr4 was shown in yeasts to affect specific histone tail modifications and subsequently switch gene expression states. I hypothesized that such fine-tuning of protein conformation may also play a major role in PcG protein mediated epigenetic control. I have chosen Drosophila FKBP39 (The Drosophila homolog of Fpr4) to study a potential interaction with the PcG system.

All together, the aim of this project was to investigate the function of FKBP39 in PcG mediated regulation system. During my thesis, following specific aspects would be investigated to achieve set goals/objectives while characterizing molecular interaction of FKBP39 with PcG proteins.

- **Characterize genetic interactions of FKbp39 with Polycomb**
  Genetic mutants of FKbp39 would be crossed to Pc mutants to elucidate if FKbp39 mutations either enhance or suppress classical homeotic phenotypes caused by Pc mutants. A null mutant of FKbp39 would be generated for characterizing the presence of any homeotic phenotype and analyzing the function of FKBP39.
Introduction

- **Characterize chromatin association of FKBP39**
  The predicted nuclear localization would be assessed using fluorescently tagged FKBP39 in transgenic *Drosophila*. In addition, chromatin binding of FKBP39 would be analyzed by immunostaining of polytene chromosomes. Polytene chromosomes would also be used to co-stain FKBP39-EGFP and PC to evaluate if FKBP39 co-localize with PC protein. The chromatin association of FKBP39 with PC would be further validated by chromatin immunoprecipitation and finally PCR amplifying selected PcG target genes. In order to identify genome-wide binding sites of FKBP39, chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) would be performed.

- **Characterize FKBP39 chromatin association in relation to PcG**
  Immunostaining of polytene chromosomes would provide initial evidence for co-localization of FKBP39 with PC protein. However, ChIP-seq data would provide an opportunity to characterize binding sites of FKBP39 in relation to global PcG binding sites. Specifically, I would compare the binding profile of FKBP39 to PRC1 (Polycomb Repression Complex 1) components, Polycomb (PC), Polyhomeotic (PH) and Posterior sex combs (PSC) (Shao et al., 1999). The biochemical interaction between FKBP39 and PcG proteins would be characterized by using transgenic flies expressing epitope tagged FKBP39. This would also be validated in wild type flies by using FKBP39 specific antibody which would be generated during this study.

- **Characterization of FKBP39 function in transcription**
  The characterization of *fkbp39* null mutants should allow assigning a function to FKBP39 in specific developmental processes. In addition, I would also perform the genome-wide expression profiling by knocking down FKBP39 in *Drosophila* embryonic S2 cell line. The ChIP-seq data of FKBP39 and PRC1 in combination with RNA-seq data would enable me to decipher specific functional link between FKBP39 and PcG mediated gene regulation.
Results
2. Results

2.1 Generation of transgenic flies expressing epitope tagged FKBP39

In the absence of specific antibodies against a given protein, epitope tagged fusion proteins are an easy and quick way to characterize a protein at the molecular and cellular level. In order to investigate the sub-cellular localization as well as isolate interacting partners of FKBP39, full-length coding sequence (CDS) of **FKbp39** was cloned in frame with EGFP and Flag-tag to generate fusion proteins. Both the EGFP as well as Flag-tagged fusion constructs were placed under control of a tubulin promoter in a pCaSpeR4 vector to have constitutive ectopic expression of **Fkbp39** in flies. The EGFP-tag was fused to the C-terminus of **FKbp39** CDS and the Flag-tag fused to N-terminus of the **FKbp39** CDS (Figure 2.1). The constructs were injected into *Drosophila yw* embryos to generate transgenic flies following the standard injection protocol (for details, see Yuje Chen Master thesis 2006, University of Heidelberg). With FKBP39-EGFP transgenic flies, the sub-cellular localization of FKBP39 was detected in salivary gland, ovary and testis. In the Flag-FKBP39 transgenic flies, the interaction between PC and FKBP39 was detected. These results will be described in detail later.
Figure 2.1 Map of plasmid pCaSpeR4-tubulin-FKbp39-EGFP and pCaSpeR4-tubulin-Flag-FKbp39

EGFP was fused to C-terminal of FKbp39 CDS and the Flag tag was fused to N-terminal of the FKbp39 CDS. The white gene was used as a marker. SV40 Term: SV40 termination signal.

2.2 Generation and purification of FKBP39 antibody

In order to detect endogenous FKBP39 protein as well as protein-interaction partners and validate the results from experiments with epitope-tagged proteins, a specific antibody against FKBP39 was generated. The N-terminus of the FKbp39 coding sequence, which does not contain conserved PPlase domains (Figure 2.2.1), was cloned in frame with a thioredoxin tag at its N-terminus and a 6xHIS tag at C-termini in a pET32 expression vector (cloned by M. Tariq). The HIS-tagged fusion protein was expressed in the BL21 codon plus bacterial strain and purified with Nickel-NTA beads (see methods). A polyclonal anti-FKBP39 antiserum was generated by immunizing a rabbit with the antigen and the serum was purified by antigen affinity purification (Figure 2.2.2A). The purified antibody was tested by Western blot with purified antigen
and wild type (WT) embryonic nuclear extract. 2µl embryonic nuclear extract, 25ng purified tagged FKBP39 and tagged PPIase domain were used in this test. A 1:2500 dilution and a short exposure time already give a very strong signal for the endogenous FKBP39 from embryonic nuclear extract suggesting that the FKBP39 antibody is highly specific (Figure 2.2.2B).

**Figure 2.2.1 FKBP39 protein**
The picture denotes the length of FKBP39 in kDa and the FKBP39 protein contains a conserved peptidyl-prolyl isomerase (PPIase) domain at C-termini.

**Figure 2.2.2 Workflow of affinity purification for anti-FKBP39**

(A) Before purification, the anti-His tag, anti-thioredoxin (Trx) tag antibody and the antibodies with cross activity to PPIase domain should be removed. Use the PPIase domain-6x His protein, to clean the anti-His and anti-PPIase antibody. Incubate the serums in the PPIase-6His coupled NHS-column, collect the flow through and load onto the antigen-coupled column to purify anti-FKBP39 antibody. (B) Purified anti-FKBP39 was tested by Western blot with 25ng purified antigens (thioredoxin and His tagged FKBP39/ thioredoxin and His tagged PPIase domain) and 2µl WT embryonic nuclear extract (NE)(16µg/µl) in different dilutions, exposure time: 1 sec.
2.3 Characterization of the $fkbp39^{5\text{-HA-2440}}$ mutant and $fkbp39$ null mutant

2.3.1 $FKbp39$ is down-regulated in the $fkbp39^{5\text{-HA-2440}}$ mutant

A transgenic line carrying a P-element inserted in the 5’UTR of $FKbp39$, $FK506-bp1^{5\text{-HA-2440}}$ (here we call it $fkbp39^{5\text{-HA-2440}}$) was characterized for misregulation of $FKbp39$ gene due to the P-element insertion (Juhasz et al., 2007). This transgenic line is viable when it is homozygous for the P-element insertion. However, there is only a distance of 136 bp between the 5’-end of $Fkbp39$ and the neighboring gene $Sra-1$, which encodes a Rho GTPase binding protein $Sra-1$ (Specifically Rac-1 associated protein1). $FKbp39$ and $Sra1$ are organized in the genome in opposite orientation (Figure 2.3.1). Due to the close proximity of the two neighboring genes, the potential deleterious effect of the P-element insertion on $Sra-1$ could not be ignored. Therefore, the transcriptional status of both the genes, $FKbp39$ and $Sra1$, was analyzed in this mutant and compared with wild type flies. A semi-quantitative polymerase chain reaction (see Methods) using complementary DNA (cDNA) made from total RNA of mutant and wild type flies was used in this test. An equal amplification of the tubulin gene on the same templates indicated equal amounts of the templates used for both wild type and mutant (Figure 2.3.2A). The transcription level of $FKbp39$ showed a drastic reduction in the mutant as compared to wild type flies. Conversely, no effect of the P-element insertion on the transcription of $Sra-1$ can be observed in the $fkbp39^{5\text{-HA-2440}}$ mutant, when compared to wild type flies (Figure 2.3.2A). This suggests that the insertion of the P-element results in a mutation of $FKbp39$, but has no effect on the $Sra-1$ gene. Furthermore, $fkbp39^{5\text{-HA-2440}}$ mutant showed an expected reduction of FKBP39 protein in Western blot analysis, but no effect on Tubulin control protein (Figure 2.3.2B).
Results

Figure 2.3.1 Genomic region of FKbp39 and location of P-element insertion

The *fkbp39* null allele was generated from a P-element mutant *fkbp39* \(^{5\text{-HA-2440}}\). The white arrow indicates the gene region and the transcriptional direction of FKbp39, the orange arrows indicate the mRNA of FKbp39, the P-element insert site is marked by a red line and the excised region in *fkbp39* null mutant is indicated with a yellow rectangle. The pink arrows indicate the neighbor genes *mRpL9* and *Sra-1*.

2.3.2 Phenotype of *fkbp39* \(^{5\text{-HA-2440}}\) mutants

The homozygous *fkbp39* \(^{5\text{-HA-2440}}\) mutant flies were analyzed for any visible phenotype. When closely examined, 3.85% of *fkbp39* \(^{5\text{-HA-2440}}\) mutant flies showed a misregulated abdominal segments phenotype. No such phenotype was found in *w* \(^{1118}\) flies (n=180), which were analyzed as a control group (Fig. 2.3.3 B,C). This suggests that FKBP39 may play a role in the development of *Drosophila*. During amplification of *fkbp39* \(^{5\text{-HA-2440}}\) homozygous mutant flies, it was frequently observed that in spite of laying a number of eggs in a vial, no larvae or adult flies developed. It was assumed that mutations in the FKbp39 gene may affect the fertility of flies. In order to analyze sterility in mutant flies, homozygous *fkbp39* \(^{5\text{-HA-2440}}\) mutants were reciprocally crossed to *w* \(^{1118}\) flies. In these reciprocal crosses either a single mutant male or female was crossed to multiple females or males of *w* \(^{1118}\) respectively.

Interestingly it was found that 16 of 55 *fkbp39* \(^{5\text{-HA-2440}}\) males showed sterility since no progeny developed from those crosses, while all 56
**Results**

fkbp39\(^{5\text{-HA-2440}}\) / fkbp39\(^{5\text{-HA-2440}}\) females crossed to \(w^{1118}\) males developed normal progeny.

![Image of RT-PCR results](image)

**Figure 2.3.2 FKbp39 and Sra-1 expression in fkbp39 mutants**

(A) FKbp39 is down regulated and Sra-1 is almost not affected in fkbp39\(^{5\text{-HA-2440}}\) mutant. 5 µl of 1:5 diluted cDNA from \(w^{1118}\) and fkbp39\(^{5\text{-HA-2440}}\) mutant were used in RT-PCR with FKbp39 and Sra-1 specific primers. Tubulin was used as an internal control. -: template from the reverse transcription reaction without reverse transcriptase, +: cDNA from the reverse transcription reaction with reverse transcriptase. (B) fkbp39\(^{5\text{-HA-2440}}\) mutant (FKbp39 P-element mutant) shows a partial reduction of FKBP39 protein levels, while the fkbp39\(^{1}\) null mutant depicts a complete depletion of the protein in a Western blot analysis. Tubulin is used as a control. The proteins extracted from fly heads are detected with anti-FKBP39 and anti-Tubulin antibody. (C) Sra-1 is only down regulated 10% in fkbp39\(^{1}\) null mutant. 1:100 diluted cDNA from \(w^{1118}\) and fkbp39\(^{1}\) null mutant were used in Real-time RT-PCR with Sra-1 specific primers. ATPase was used as an internal control.

### 2.3.3 Generation of a fkbp39 null mutant

To confirm the specificity of the loss-of-function phenotype in fkbp39\(^{5\text{-HA-2440}}\) mutant and get a more stringent picture of the genetic function of FKBP39, an fkbp39 null allele was generated by imprecise excision. Female fkbp39\(^{5\text{-HA-2440}}\) mutants were crossed to male flies carrying a gene expressing the Δ2-3 transposase. The flies were kept at 25°C, the optimum temperature for Δ2-3 activity. The recombination occurs especially during the generation of the
Results

To remove the transposase, \( fkbp39^{5-HA-2440} / \Delta 2-3 \) TM3Sb flies were crossed to TM3Sb/Dr; the recombinants with imprecise excision among the progeny were screened by PCR. From 200 P-element excised flies, 1 mutant with a deletion of the FKbp39 5’UTR and the 1st exon, but no deletion of the neighboring Sra-1 and mRpL9 genes was obtained (Figure 2.3.1). The protein level of FKBP39 was analyzed by Western blot and a complete depletion of FKBP39 was detected in this null mutant (from now on called \( fkbp39^{1} \) null mutant) (Figure 2.3.2B). Furthermore, the expression of the Sra-1 gene in the \( fkbp39^{1} \) null mutant was analyzed by Real-time RT-PCR and only about 10% downregulation on Sra-1 expression could be detected (Figure 2.3.2C).

2.3.4 Phenotype of the \( fkbp39^{1} \) null mutant

Homozygous \( fkbp39^{1} \) null mutant flies are viable and could therefore be analyzed for any visible developmental phenotypes. Similar to \( fkbp39^{5-HA-2440} \) mutant flies, the adult flies of \( fkbp39^{1} \) null mutants also showed a misregulated abdominal segments phenotype (Figure 2.3.3B). Moreover, we noticed that the penetrance of this phenotype was related to the depletion level of FKBP39. In \( fkbp39^{5-HA-2440} \) mutants, 3.85% flies had misregulated abdominal segments; in the \( fkbp39^{1} \) null mutant, 8.65% flies showed this phenotype. In the \( fkbp39^{1} \) null mutant, with a removed maternal component, the penetrance increased dramatically to 32.09% (Figure 2.3.3C). Furthermore, the developmental phenotype of the \( fkbp39^{1} \) null mutant was also analyzed at embryonic stages: 63.5% of the \( fkbp39^{1} \) lethal embryos (in \( fkbp39^{1} \) around 60% of the embryos are lethal) showed misregulated segments (n=130) (Figure 2.3.3A), which implies a function early in embryonic development.
Figure 2.3.3 Segmental misregulation phenotype in \textit{fkbp39} mutants

(A) Cuticles of \textit{fkbp39}\textsuperscript{1} null mutants were prepared from lethal embryos. The analysis of the pattern of embryonic ventral epidermis revealed that 63.5\% ($n = 130$) of the \textit{fkbp39}\textsuperscript{1} null mutant lethal embryos have misregulated segments. The cuticles of wild type embryos, which showed normal segments, were used as a control. (B) The adult flies of \textit{fkbp39}\textsuperscript{5-HA-2440} and \textit{fkbp39}\textsuperscript{1} null mutant showed misregulated abdominal segments. The abdomens of wild type female and male flies, which show normal segments, were used as a control. (C) The penetrance of the misregulated abdominal segments phenotype was increased according to the depletion of FKBP39 protein. 180 \textit{fkbp39}\textsuperscript{5-HA-2440} flies from two independent crossings were analyzed, 3.85\% showed misregulated abdominal segments; 130 \textit{fkbp39}\textsuperscript{1} null mutant homozygous offspring from \textit{fkbp39}\textsuperscript{1} null/TM3sb female crossing to \textit{fkbp39}\textsuperscript{1} null/TM3sb male were analyzed, the mean values of two independent experiments are presented, 8.65\% showed misregulated abdominal segments; 170 \textit{fkbp39}\textsuperscript{1} null mutant homozygous offspring from \textit{fkbp39}\textsuperscript{1} null/fkbp39\textsuperscript{1} null female crossing to \textit{fkbp39}\textsuperscript{1} null/TM3sb male were also analyzed, the mean values of two independent experiments are presented, 32.09\% showed misregulated abdominal segments.
In spite of laying many eggs, homozygous \textit{fkbp39} \textsuperscript{1} \textit{null} mutants also showed much less fertility than wild type flies. To quantify this sterility in null mutant flies, homozygous \textit{fkbp39} \textsuperscript{1} \textit{null} mutants were reciprocally crossed to \textit{w} \textsuperscript{1118} flies. In these reciprocal crosses either a single mutant male or female was crossed to three females or males of \textit{w} \textsuperscript{1118} respectively. 11 days after crossing, the hatched files and unhatched pupae were counted as the progeny number, it was found that both male and female \textit{fkbp39} \textsuperscript{1} \textit{null} mutants were defective in fertilization (Figure 2.3.4). When the male mutant was crossed to the female mutant, very few progeny (larvae or pupae) could be found.

\textbf{Figure 2.3.4 \textit{fkbp39} \textsuperscript{1} \textit{null} mutant flies are defective in fertilization}

Single \textit{w} \textsuperscript{1118} male crossed to three \textit{w} \textsuperscript{1118} female and single \textit{w} \textsuperscript{1118} female crossed to three \textit{w} \textsuperscript{1118} male were used as controls, the mean values of progeny number from two independent crossings are presented; Single \textit{fkbp39} \textsuperscript{1} null mutant male crossed to three \textit{w} \textsuperscript{1118} female and single \textit{fkbp39} \textsuperscript{1} null mutant female crossed to three \textit{w} \textsuperscript{1118} male were analyzed, the mean values of progeny number from ten independent crossings are presented; Single \textit{fkbp39} \textsuperscript{1} null mutant male crossed to three \textit{fkbp39} \textsuperscript{1} null mutant female and single \textit{fkbp39} \textsuperscript{1} null mutant female crossed to three \textit{fkbp39} \textsuperscript{1} null mutant male were also analyzed, the mean values of progeny number from two independent crossings are presented.
Results

Figure 2.3.5 Phenotype of *fkbp39* null mutant in testis

(A, B) DAPI staining for the chromatin in testes of flies visualizes the spermatogenesis stages, which include: spermatogonia, spermatocytes, spermatids with round nuclei, spermatids with young elongating nuclei, spermatids with canoe-shaped nuclei and the spermatids with fully shaped (needle-shaped) nuclei (left to right). Scale bar: 10µm. (A) The testes of *w1118* were used as a control. (B) In *fkbp39* null mutant, the spermatids with canoe-shaped nuclei and fully shaped nuclei were missing. (C) Bundles of fully shaped spermatids were counted in the testes of *w1118* and *fkbp39* null mutant flies. The testis has been classified into 4 classes, 0-3, 4-10, 11-20 and >20, which means contain 0-3, 4-10, 11-20 and more than 20 bundles of fully shaped spermatids in one testis. The mean value from 3 independent experiments are presented, total 58 testes were counted.

Besides the lethality of embryos, 83% eggs from homozygous *fkbp39* null mutant parents and 49% eggs from homozygous *fkbp39* null female crossed to heterozygous *fkbp39* null male flies were found to be unfertilized by DAPI staining of embryos. This is another reason for the small amount of progeny in *fkbp39* mutants, which indicates the defect in the germline of the mutant parents. Thus, both testis and ovary of *fkbp39* null mutants were analyzed in this study. *fkbp39* mutants were found defective in nuclear condensation.
Results

during spermatogenesis (Figure 2.3.5) and had defective nucleolar structures in nurse cells during oogenesis (Figure 2.3.7). In mutant testes, many spermatids could not reach the canoe-shape stage and fully condensed stage, the development was stopped at the young elongating stage (Figure 2.3.5 A, B). 60 testes from 3 independent experiments were analyzed, 77.4% testis had only 0-3 bundles of fully-shaped (condensed) spermatids and none of the testis had more than 20 condensed spermatids. However, all the testes of w^{1118} contained more than 20 bundles of fully-shaped spermatids (Figure 2.3.5 C).

![Figure 2.3.6 FKBP39-EGFP in testis](image)

**Figure 2.3.6 FKBP39-EGFP in testis**

Fixed testes from FKbp39-EGFP transgenic flies were stained with DAPI (blue). (A) FKBP39-EGFP in a testis. (B) DAPI staining of a testis. (C) An intact testis expressed FKBP39-EGFP (green) stained with DAPI (blue). (D) FKBP39-EGFP in spermatocytes, the white arrow indicates the nucleolar subcompartment in a spermatocyte. (E) Spermatocytes stained with DAPI. (F) Spermatocytes expressed FKBP39-EGFP (green) stained with DAPI (blue). Scale bar: 10 µm.
Furthermore, the expression pattern of FKBP39-EGFP was investigated in testis. It was found that FKBP39-EGFP was expressed from spermatogonia stage to spermatids with round nuclei stage (Figure 2.3.6 A, B, C), localized on chromosomes and in nucleoli (Figure 2.3.6 D, E, F). It suggests that the chromosome and nucleoli association with FKBP39 in early spermatogenesis stages maybe required for the chromosome structure alteration in later stages.

Figure 2.3.7 Phenotype of *fkbp39* null mutant in ovary

Immunostaining of ovaries with anti-Fibrillarin (red), a nucleolar marker in all cells. DNA stained with DAPI (blue). (A, C) Ovaries immunostained with anti-Fibrillarin (red) and DAPI (blue). Scale bar: 50µm. (B, D) Single nurse cell nucleus immunostained with anti-Fibrillarin (red) and DAPI (blue). Scale bar: 10µm. (A,B) *w*¹¹¹⁸ ovaries. (C, D) *fkbp39* null mutant ovaries.
In ovaries, the nucleoli of nurse cells were marked by anti-Fibrillarin, multiple nucleoli were loosely and evenly distributed in w1118 nurse cell (Figure 2.3.7 A, B). However, aggregated and centrally localized single nucleolus were found in fkbp391 null mutant nurse cells (Figure 2.3.7 C, D). The nucleolar structure of oocytes could not be detected due to the bad penetration of DAPI and the anti-Fibrillarin antibody. In the ovaries of FKbp39-EGFP transgenic flies, FKBP39-EGFP was expressed in nurse cells through all stages of oogenesis (Figure 2.3.8 A, B, C). The protein was localized in the nuclei and enriched in the nucleolar subcompartment of the nurse cells (Figure 2.3.8 D, E). Interestingly, it was also found that the RNA level of 28S was significantly down regulated in the ovaries of fkbp391 mutants (Figure 2.3.9). All together, these findings are suggestive of a function of FKBP39 in nucleolar structure maintenance and rRNA expression.

**Figure 2.3.8 FKBP39-EGFP in ovary**

Fixed ovaries from FKbp39-EGFP transgenic flies were immunostained with anti-GFP (green) and DAPI (blue). (A) Ovaries in stage 1-8, (B) Ovaries in stage 8-10, (C) Ovary of stage 13, (D, E) Single nurse cell nucleus stained with anti-GFP (green) and DAPI. Scale bar: 10µm.
Figure 2.3.9 Expression level of 28Sa and 28Sb in \textit{fkbp39}\textsuperscript{1} mutant ovary

The RNA level of 28Sa and 28Sb was detected in the ovaries of \textit{fkbp39}\textsuperscript{1} mutants by real-time PCR, 28Sa and 28Sb were down regulated in \textit{fkbp39}\textsuperscript{1} mutants. Ovaries from \textit{w}\textsuperscript{1118} were used as a control.

### 2.4 The link between FKBP39 and PcG proteins

#### 2.4.1 FKBP39 associated with PC on chromatin

**Nuclear localization of FKBP39**

The specific sub-cellular localization of FKBP39 in germline cells suggests that FKBP39 may bind to chromatin. To study the chromatin association of FKBP39 in detail, the chromosomal site of action of FKBP39 was first identified in salivary glands containing giant polytene chromosomes. In \textit{FKbp39-EGFP} transgenic flies, the analysis of unfixed salivary glands revealed that FKBP39-EGFP was a nuclear protein, it also abundantly localized in the nucleolus (Kaufmann, 1937; Marinho et al., 2011; Nemeth and Langst, 2011) (Figure 2.4.1.1).
**Figure 2.4.1.1 FKBP39-EGFP in salivary gland**

Unfixed salivary gland from *FKbp39-EGFP* transgenic fly stained with DAPI (blue). (A-C) FKBP39-EGFP (green) localizes in nuclei and enriched in nucleoli. (A) DAPI, (B) FKBP39-EGFP, (C) Overlap of A and B. (D-F) Single nucleus of salivary gland cell stained with DAPI. (D) DAPI, (E) FKBP39-EGFP, (F) Overlap of D and E.

**FKBP39 co-localize with PC on polytene chromosomes**

Next, immunostaining of polytene chromosomes from third instar larvae of *FKbp39-EGFP* transgenic flies with anti-GFP antibody revealed the association of FKBP39-EGFP with chromosomes (Figure 2.4.1.2 A). In order to find out the link between FKBP39 and the PcG control system, the polytene chromosomes were co-stained with anti-GFP and anti-PC, which showed the co-localization of FKBP39 and PC at many sites (Figure 2.4.1.2 B).
Figure 2.4.1.2 FKBP39 co-localize with PC on polytene chromosomes

Polytene chromosomes from third instar larvae of FKbp39-EGFP transgenic flies stained with anti-GFP(A)/anti-GFP and anti-PC (B) showed FKBP39-EGFP (green) bound to chromatin and co-localized with PC (red). The arrows indicate the overlapping bands of FKBP39-EGFP and PC.

Genome-wide co-localization of FKBP39 and PC

To investigate these findings at a higher resolution we performed two replicate FKBP39 ChIP-Seq experiments (see methods) in D. melanogaster S2 tissue culture cells using the Illumina Genome Analyzer platform. All sequence reads passing the quality filter were aligned to the Drosophila melanogaster reference genome using the Bowtie short read aligner (Langmead et al., 2009). Following the alignment, genomic regions enriched in short reads were detected by the MACS algorithm (Zhang et al., 2008) in the process denoted as peak calling. Short reads coming from each replicate were processed in the same way. In batch A, totally 7.42 million base pairs (bp) region was recognized as FKBP39 enriched region; in batch B, 4.98 million bp region was recognized as FKBP39 enriched region. For a more precise characterization of the genomic binding specificity of the FKBP39 protein we computed the intersection of all peaks coming from the two replicates. As the replicated and independent detection of a given region was evidence enough to assume the presence of the FKBP39 protein. The set containing exactly the intersection of all peaks encompassed a total of 3254 genomic regions (3.15 million bp in
Results

total) represented the FKBP39 binding sites. The intersecting peaks from two different batches of ChIP-seq experiments showed a significant correlation (Figure 2.4.1.3 A). The analysis of the fold enrichment of the intersecting peaks revealed that the peaks common to the two biological replicates are these show higher fold enrichment (Figure 2.4.1.3 B). All together, it suggests that the intersecting peaks from the ChIP-seq experiments are reliable enough for further analysis.

![Figure 2.4.1.3 Comparison of intersecting peaks](image)

**Figure 2.4.1.3 Comparison of intersecting peaks**

(A) A scatter plot showing fold enrichment of FKBP39 ChIP signal calculated within peak regions common to the two biological replicates, and plotted against each ther. A Pearson correlation coefficient is shown. (B) The fold enrichment of the peaks common to the two biological replicates is compared to the total peaks in FKBP39 ChIP-seq batch A. The intersecting peaks are indicated in red, the total peaks in FKBP39 ChIP-seq batch A are indicated in black.

Furthermore, the integration of previously published ChIP-Seq data sets (Enderle et al., 2011), measuring binding specificity of PC, revealed that 40.6% FKBP39 peaks overlapped with PC on a genome-wide scale (Figure 2.4.1.4 A ). However, the correlation between the enrichment of FKBP39 and PC is low (Figure 2.4.1.4 B), it suggests that FKBP39 and PC do not affect the chromatin binding of each other.
Results

Figure 2.4.1.4 Genome-wide co-localization and correlation of FKBP39 and PC

(A) Intersection peaks from replicated ChIP-seq data were showed for FKBP39 and PC. The number in the Venn diagram indicates the FKBP39 peaks which overlapped with PC. (B) A scatter plot showing fold enrichment of PC and FKBP39 ChIP signal calculated within peak regions co-occupied by both proteins, and plotted against each other. A Pearson correlation coefficient is shown.

FKBP39 interact with PC

To verify the co-localization result, we further investigated the existence of potential physical interaction between FKBP39 and PC by the performance of a co-immunoprecipitation from wild type embryonic nuclear extracts. While PC appears to be robustly associated with FKBP39, the interaction with TRX is faint, which suggests a weak interaction between FKBP39 and TRX (Figure 2.4.1.5 A). The interaction between FKBP39 and PC was also confirmed in the embryonic nuclear extract of Flag-FKbp39 transgenic flies (Figure 2.4.1.5 B) and the Kc cells which transiently transfected with Flag–FKbp39 by anti-Flag immunoprecipitation followed by Western blot with anti-PC antibody (Figure 2.4.1.5 C). All together, it suggests that FKBP39 is interacting with PC on chromatin.
Figure 2.4.1.5 FKBP39 interacting with PC

(A) Wild type (WT) embryonic nuclear extract (NE) was immunoprecipitated with anti-FKBP39 polyclonal antibody, the Western blot analysis of the immunoprecipitated material with anti-PC/anti-TRX antibody reveals that FKBP39 robustly interacts with PC and only weakly with TRX. The reciprocal immunoprecipitation also indicates an interaction between FKBP39 and PC. anti-β-Gal antibody was used as a negative control. (B) Embryonic nuclear extract from Flag-FKbp39 transgenic flies was immunoprecipitated with anti-Flag antibody, the Western blot was probed with anti-PC antibody. WT embryonic nuclear extract was used as a mock. (C) The lysate of Kc cells, which had been transiently transfected with Flag-FKbp39, was immunoprecipitated with anti-Flag antibodies, the Western blot was analyzed with anti-PC antibody. The lysate of mock transfected cells was used as a negative control.
2.4.2 FKBP39 correlates with PRC1 at transcription start sites

In order to gain deeper insights about the sites of action of FKBP39 in the process of chromatin-controlled gene regulation, we investigated the genomic context of the previously identified FKBP39 peaks. We found that a large fraction of the FKBP39 peaks can be associated with at least one transcription start site (TSS). A peak is associated with a particular TSS if it overlaps with the genomic interval, defined by the TSS and the 100 bases upstream, by at least one base. The *Drosophila melanogaster* Ensemble annotation BDGP 5.25 was used for this analysis. Since a given FKBP39 peak can meet the above stated association requirements for more than one TSS simultaneously, the set of all FKBP39 peaks that were associated with at least one TSS will be denoted by ‘allTSS’. In total 44% of the FKBP39 peaks could be classified into this set (Figure 2.4.2.1). For a more refined analysis the subset of all peaks that associate with exactly one TSS was computed in addition and will be denoted by ‘uniTSS’. This set contains 30% of all FKBP39 peaks (Figure 2.4.2.1). The observation that a substantial fraction of all FKBP39 peaks can be associated with promoter proximal regions of annotated transcripts suggests that it might contribute to transcriptional control at the promoter.

![Figure 2.4.2.1 FKbp39 binds to transcription start site](image)

**Figure 2.4.2.1 FKbp39 binds to transcription start site**

The association of FKBP39 peaks with transcription start sites is summarized in the Venn diagram. The green circle indicates the total peaks of FKBP39, the yellow circle indicates the FKBP39 peaks associate with TSS (allTSS), and the FKBP39 peaks correlated with only one TSS (uniTSS) are shown in the orange circle. allTSS means at least one TSS (could be more than one TSS) are associated. uniTSS means exactly one TSS are associated.
It was noticed that FKBP39 peaks were frequently co-localized with PcG proteins (Figure 2.4.2.2 A). Thus, we integrated the binding information of the three PRC1 (Polycomb repressive complex 1) core components: PC, PH and PSC. This analysis revealed that 41.8% of PRC1 bound TSSs are also associated with FKBP39 (Table 2.1). A given TSS was said to be PRC1 bound if the three core components independently complied with the requirements of association. The promoter proximal binding characteristics of the different proteins are depicted in Figure 2.4.2.2 B. Shown is the position-wise mean fold enrichment of FKBP39 and the PRC1 core components on the set of independent 9193 TSS, where independency is defined as being unique within the interval defined by the 1000 bases upstream as well as downstream the TSS. Interestingly, in the metagene comparison the different proteins exhibit close binding proximity and the mode of the FKBP39 distribution is closest to the TSS.

**Table 2.1 Transcription start sites uniquely associated with FKBP39 and PRC1**

<table>
<thead>
<tr>
<th></th>
<th>FKBP39</th>
<th>PRC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>uniTSS</td>
<td>965</td>
<td>275</td>
</tr>
<tr>
<td>FKBP39 AND PRC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uniTSS</td>
<td>115 (41.8% of PRC1 uniTSS)</td>
<td></td>
</tr>
</tbody>
</table>

The numbers of transcription start sites uniquely associated with FKBP39 and PRC1 are listed. **uniTSS** indicates the transcription start sites that uniquely associated with a certain protein. **FKBP39 AND PRC1** defines the transcription start sites associated with FKBP39 and PRC1. A large proportion of PRC1 targets are also the targets of FKBP39.
A

Scale
chr3R 8850000
distance
100.07
'100

Distance w.r.t TSS [bp]

Fold Enrichment w.r.t input [log2]

PC

PH

PSC

FKBP39

FKBP39 peaks

B

Read Density across 1kb TSS window

Fold Enrichment w.r.t input [log2]

Distance w.r.t TSS [bp]

# indep. TSS: 9193
Results

Figure 2.4.2.2 FKbp39 correlates with PRC1 on TSS
(A) FKBP39 is co-localized with PC, PH and PSC at TSS. Screenshot from UCSC genome browser, which covering two different genomic loci and including the data from FKBP39 (shown in red) and PC, PH, PSC ChIP-seq (shown in blue). The identified peak regions from replicated FKBP39 ChIP-seq are indicated with black rectangles. The Ensemble annotations are indicated in light blue. The orange arrows indicate the direction of transcription. (B) Position-wise mean fold enrichment of FKBP39 and the PRC1 core components on the set of independent 9193 TSS. Here independency is defined as being unique within the interval defined by the 1000 bases upstream as well as downstream the TSS. w.r.t: with respect to.

2.4.3 FKBP39 works as a gene activator and is involved in PcG regulated gene expression
To investigate the function of FKBP39 in gene regulation, we analyzed the gene expression levels of FKBP39 and PRC1 unique target genes using the mRNA-seq data from S2-DRSC cells (Enderle et al., 2011). To further discern the effects of FKBP39 binding, we investigated differences between empirical gene expression distributions of gene sets differing in protein binding composition within the promoter-proximal region. For this purpose we identified different sets of genes bound by “FKBP39”, “PRC1 AND FKBP39” and “PRC1 NOT FKBP39” (here, logical operators were used to define the sets). The comparison of the empirical cumulative gene expression density distribution profiles between different gene sets revealed that the genes associated with FKBP39 were in general more active (Figure 2.4.3.1A). Among the PRC1 target genes, FKBP39 bound genes (n=115) were also more active than the ones without FKBP39 binding (n=155) (Figure 2.4.3.1B). These findings suggest that at target genes bound by PcG, FKBP39 is primarily involved in gene activation.
Results

**Figure 2.4.3.1 FKBP39 binding genes are more active**

(A) The analysis of expression level of total *Drosophila* genes and FKBP39 uniquely bound genes in S2-DRSC cells, the target genes of FKBP39 are more active. The figure shows the empirical cumulative density distribution of the genes according to the RPKM (Reads Per Kilobase of exon model per Million mapped reads) value for each gene. (B) The analysis of expression level of PRC1 targets with and without FKBP39 binding, the ones with FKBP39 binding are more active. (A, B) The p-value of each comparison was obtained using a two-sample Kolmogorov-Smirnov test. X-axis indicates the mRNA expression level (RPKM) of genes; Y-axis indicates the cumulative fraction of the genes in each group. ECDF: empirical cumulative distribution function.

**Figure 2.4.3.2 FKBP39 is downregulated in FKbp39 RNAi S2 cells**

(A) *Drosophila* S2 cells were treated with *FKbp39* dsRNA for 5 days, the mRNA level of *FKbp39* was detected with qRT-PCR. *FKbp39* was robustly down regulated. (B) The protein level of FKBP39 was checked in the 5 days *FKbp39* RNAi S2 cells, showing a clear reduction. Tubulin protein control was not affected.
To confirm this correlation, using RNA interference we investigated the effects of FKBP39 on the previously identified FKBP39 target genes. After 5 days incubation of S2 tissue culture cells with FKbp39-specific double stranded RNA (dsRNA) the expression level of FKbp39 was monitored by real-time quantitative reverse transcription PCR (qRT-PCR) and Western blotting. The mRNA of FKbp39 was found to be substantially down-regulated, as was the protein level (Figure 2.4.3.2A and B).

Purified mRNAs from control (GFP-RNAi-treated) and FKBP39 knock-down S2 cells were used for genome-wide transcriptome profiling (mRNA-seq). To detect significantly misregulated genes, we compared the number of reads within the known Ensemble exon regions for each gene between control and FKBP39 knock-down S2 cells from two biological replicates. The differential expression analysis was done with the algorithm implemented in the DEseq, which is an R package to analyze count data from high-throughput sequencing assays such as RNA-Seq and test for differential expression (Anders and Huber, 2010). Using a fold change cutoff of 1.2 and a p-value cutoff <0.1, we observed a total of 90 down-regulated and 23 up-regulated genes consistently changed in the two replica experiments (14869 genes were tested) (Table 2.2). The mRNA-seq analysis was validated by qPCR, 10 misregulated genes were randomly chosen and all measurements confirmed the corresponding misregulation observed in the mRNA-seq analysis (Figure 2.4.3.3).
### Table 2.2 - Down regulated genes are enriched in FKBP39 targets

<table>
<thead>
<tr>
<th>genes number</th>
<th>Down regulated p&lt;0.1 (FC &gt;1.2)</th>
<th>Up regulated p&lt;0.1 (FC &gt;1.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (14869)</td>
<td>90</td>
<td>23</td>
</tr>
<tr>
<td>FKBP39 allTSS (1638)</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2.32FE p=2.78E-05</td>
<td>0.79FE p=0.47</td>
</tr>
<tr>
<td>FKBP39 uniTSS (904)</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3.11FE p=6.43E-06</td>
<td>0.71FE p=0.41</td>
</tr>
<tr>
<td>PRC1 AND FKBP39 allTSS (214)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.21FE p=0.0096</td>
<td>0</td>
</tr>
<tr>
<td>PRC1 NOT FKBP39 allTSS (162)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.07FE p=0.26</td>
<td>0</td>
</tr>
<tr>
<td>PRC1 AND FKBP39 uniTSS (115)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.42FE p=0.0052</td>
<td>0</td>
</tr>
<tr>
<td>PRC1 NOT FKBP39 uniTSS (155)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.12FE p=0.24</td>
<td>0</td>
</tr>
</tbody>
</table>

mRNA-seq data from FKbp39 RNAi S2 cells are combined with FKBP39 and PRC1 ChIP-seq data. The numbers of significantly misregulated genes are listed. The cut-off is p-value<0.1 and fold change (FC) >1.2. The total numbers of misregulated genes are shown in **bold.** The numbers of the genes in each group are indicated in bracket. The fold enrichments (FE) of down regulated genes or up regulated genes and the p-values are shown in *Italic.* The p-values were calculated according to hypergeometric distribution. The enrichments of down regulated genes are detected in FKBP39 targets and PRC1 AND FKBP39 targets, but not in the PRC1 NOT FKBP39 targets. There is also no enrichment of the up-regulated genes detected. Here “allTSS” indicates the genes bound by FKBP39, PRC1 AND FKBP39 or PRC1 proteins which associated with at least one TSS; “uniTSS” means the genes bound by FKBP39, PRC1 AND FKBP39 or PRC1 proteins which associated uniquely with only one TSS.
Figure 2.4.3.3 Validate mRNA-seq data from *FKbp39* RNAi S2 cells with real time qRT-PCR

Ten misregulated genes from mRNA-seq data were checked with qRT-PCR, the result is consistent with mRNA-seq analysis. FBgn0034282, FBgn0037504, FBgn0085403, FBgn0030469, FBgn0031914 were chosen from the down regulated FKBP39 targets; FBgn0050035 was chosen from the up regulated FKBP39 targets; FBgn0033649, FBgn0041180, FBgn0053197 were chosen from the down regulated FKBP39-PRC1 targets; FBgn0039073 was chosen from the up regulated non-FKBP39 targets. Two replica samples were used in this validation.

Among the FKBP39 targets, down-regulated genes are significantly enriched (Table 2.2 and Figure 2.4.3.4). In the 904 uniquely associated FKBP39 targets, 17 (1.9% of the FKBP39 targets) genes were significantly down-regulated. Compared to the ratio of down-regulated genes in total, which is 0.6%, which shows more than 3-fold enrichment in FKBP39 targets. However, there was no enrichment of up-regulated genes detected. Next, to find out whether FKBP39 plays a role in PcG-regulated gene expression, we checked the significantly misregulated genes in PRC1 targets. We found the down-regulated genes were notably enriched in the PRC1 target genes with FKBP39 binding (“PRC1 AND FKBP39” groups), but not in the PRC1 target genes without FKBP39 binding (“PRC1 NOT FKBP39” groups). There was
also no enrichment of up-regulated genes detected in PRC1 targets. (Table 2.2 and Figure 2.4.3.4). These results suggest that FKBP39 works as a gene activator rather than a suppressor and is involved in PcG regulated gene expression.

Figure 2.4.3.4 FKBP39 works as a gene activator in PcG mediated gene regulation

Fold enrichment of down-, up- regulated genes in the groups of ‘total Drosophila genes’, ‘FKBP39 bound genes (FKBP39)’, ‘PRC1 target genes with FKBP39 binding (PRC1 AND FKBP39)’, ‘PRC1 target genes without FKBP39 binding (PRC1 NOT FKBP39). Here “allTSS” indicates the genes bound by FKBP39, PRC1 AND FKBP39 or PRC1 proteins which associated with at least one TSS; “uniTSS” means the genes bound by FKBP39, PRC1 AND FKBP39 or PRC1 proteins which associated uniquely with only one TSS. Fold enrichment was calculated as (down or up regulated gene number in the group / total gene number in the group) / (total down or up regulated gene number/total Drosophila genes). The p-value of the enrichment is listed in Table 2.2. Down regulated genes are enriched in FKBP39, PRC1 AND FKBP39 target genes in Fkbp39 RNAi treated S2 cells.

2.4.4 Fkbp39 mutant enhances the phenotype of Pc mutants

To investigate whether FKBP39 genetically interacts with the PcG system, fkb39\textsuperscript{HA-2440} and fkb39\textsuperscript{1} mutant alleles were crossed to 2 different alleles of Pc (3, and XT109). Pc heterozygous mutants showed an extra sex comb phenotype, which is a homeotic transformation of the mesothoracic and metathoracic legs into prothoracic legs (Lewis, 1978; Papaceit et al., 1991). This abnormality
observed in Pc males indicates a serious anterior segmental transformation (Lewis, 1978). After crossing to fkbp39<sup>5-HA-2440</sup> and fkbp39<sup>i</sup> mutants and growing in 25°C, the F1 males were scored for enhancement or suppression of extra sex comb phenotype. Interestingly, fkbp39<sup>5-HA-2440</sup> and fkbp39<sup>i</sup> null mutants significantly enhanced the extra sex comb phenotype in both the Pc alleles, more extra sex combs appeared on the 2<sup>nd</sup> /3<sup>rd</sup> pairs of legs of male flies (Figure 2.4.4.1A and B).

**Figure 2.4.4.1** fkbp39 mutants enhance the extra sex comb phenotype of Pc mutants

(A) fkbp39<sup>5-HA-2440</sup> mutant crossed with Pc mutants (alleles Pc<sup>3</sup> and Pc<sup>XT109</sup>). The Pc alleles crossed to wild type flies, Pc<sup>3/+</sup> and Pc<sup>XT109/+</sup> represent the control crosses. fkbp39<sup>5-HA-2440</sup> mutant strongly enhanced the extra sex combs phenotype of Pc mutants. More than 150 male flies were analyzed for each cross and mean values of 3 independent experiments are presented. Error bars represent the standard deviation. (B) fkbp39<sup>i</sup> null mutant crossed with Pc mutants (alleles Pc<sup>3</sup> and Pc<sup>XT109</sup>). fkbp39<sup>i</sup> mutant strongly enhanced the extra sex combs phenotype of Pc mutants. 100 male flies were analyzed for each cross and mean values of 2 independent experiments are presented. (A and B) On the basis of strength of phenotype (number of bristles on second and third legs of males), flies were categorized as follows: 0, no extra sex combs; +, 1–2 bristles on the second leg; ++, 3 or more bristles on the second leg; ++++, 3 or more bristles on the second leg and 1–2 bristles on the third leg; and +++++, 3 or more bristles on both the second and the third pairs of legs.
$P_{c}$ homozygous mutants are lethal at late embryonic stages and the segments of $P_{c}$ mutants showed strong anterior to posterior transformation phenotypes, the segments tend to transform towards the morphological patterns of A8 (Lewis, 1978; Sato and Denell, 1985). Interestingly, $fkbp39^{5\text{-HA}-2440}$ mutants also enhances this phenotype at the embryonic stage. Homozygous $P_{c}^{3}$ or $XT^{109}/fkbp39^{5\text{-HA}-2440}$ double mutants show much stronger A8 transformation phenotype compared to single $P_{c}$ mutants (Figure 2.4.4.2 A and B). These results further suggest a cooperation of FKBP39 with the PcG-system.
Results

Figure 2.4.4.2 *fkbp39* mutants enhance the A8 transformation phenotype of *Pc* mutants

*fkbp39*^{5-HA-2440} mutant recombined with *Pc* mutant (alleles *Pc*$_3$ and *Pc*$_{XT109}$). The cuticles of the homozygous *fkbp39*^{5-HA-2440}, *Pc* embryos were compared to *Pc* embryos. (A) The standard segments pattern of *Drosophila* embryos are shown with wild type embryonic cuticles. The white arrows indicate the segments which show the pattern of A8 in homozygous *Pc* and *fkbp39*^{5-HA-2440}, *Pc* mutants. (B) 150 embryonic cuticles were analyzed for each cross and mean values of 2 independent experiments are presented. In both *Pc*$_3$ and *Pc*$_{XT109}$ alleles, *fkbp39*^{5-HA-2440} mutant strongly increased the number of A8 transformation segments.

2.4.5 FKBP39 regulates the expression of *Psc* and *ph*

Our ChIP-seq and RNAi depletion results correlated FKBP39 with gene activation while the mutant analysis indicated that the *fkbp39* mutant enhances the *Polycomb* phenotype and thus is potentially involved in gene silencing. The puzzling contradictory results made us consider whether FKBP39 might actually control the activity of PcG genes, hence giving the observed Polycomb phenotype when mutated. Indeed, when the expression levels of *polyhomoetic-d* (*ph-d*), *ph-p*, *Posterior sex combs* (*Psc*) and *Polycomb* (*Pc*) were determined in S2 cells down-regulated for FKBP39 by RNAi, their transcript levels were clearly affected. We found the expression of *ph-d*, *ph-p* and *Psc* to be reduced by 31%, 22% and 24%, respectively, while the effect on *Pc* was rather mild (Figure 2.4.5.1).
Results

Figure 2.4.5.1 FKBP39 regulate the expression of Psc and ph

*Drosophila* S2 cells were treated with *FKbp39* dsRNA for 5 days, the mRNA levels of *FKbp39*, *ph-d*, *ph-p*, *Psc*, *Pc* and *trx* were detected with qRT-PCR. *FKbp39* was robustly down regulated. *ph-d*, *ph-p* and *Psc* showed more than 20% down regulation. The mean values of 3 independent experiments are presented. Error bars represent the standard deviation. dsRNA of GFP was used as a control in this experiment.

The ChIP-seq data demonstrated FKBP39 to be enriched at the transcription start sites of *ph-d*, *ph-p* and *Psc* (Figure 2.4.5.2A, B). To further confirm that the regulation of *ph-d*, *ph-p* and *Psc* is due to the enrichment of FKBP39 at the transcription start sites, we checked the binding of FKBP39 at these loci in S2 cells after *FKbp39* down-regulation by RNAi. FKBP39 binding is substantially reduced at the transcription start sites of *ph-d*, *ph-p* and *Psc* genes (Figure 2.4.5.2C), suggesting that the down-regulation of *ph-d*, *ph-p* and *Psc* is also due to the reduction of FKBP39 at their transcription start sites. Thus, the enhancement of the Polycomb phenotype can be explained by a direct down-regulation of some of the PcG genes in the *fkbp39* mutants.
Figure 2.4.5.2 FKBP39 regulate the expression of Psc and ph

(A) Enrichment of FKBP39 at the promoters of ph-d, ph-p and Psc. Screenshot from UCSC genome browser covering the gene regions of ph-d, ph-p and Psc, including the data from FKBP39 ChIP-seq (shown in orange). The identified peak regions from replicated FKBP39 ChIP-seq are indicated with black rectangles. The Ensemble annotations are indicated in red. The blue arrows indicate the direction of transcription. (B) FKBP39 ChIP samples were also analyzed by qPCR, the enrichment of FKBP39 at the promoters of ph-d, ph-p and Psc was detected. The intergenic region closed to ImpL1 was used as a negative control. (C) The binding of FKBP39 at the promoters of ph-d, ph-p and Psc was analyzed by ChIP in FKbp39 dsRNA treated S2 cells. FKBP39 was heavily reduced from the promoters of ph-d, ph-p and Psc. Here we used GFP dsRNA treated S2 cells as a control.

To investigate whether the down regulation of FKBP39 target genes is due to the increasing of PC at the promoter region, the binding status of PC on some down regulated PRC1/ FKBP39 target genes was analyzed in the FKbp39
Results

RNAi treated S2 cells by real time PCR. However, at the FKBP39 peak region, the reduction of FKBP39 rarely affected the PC binding (Figure 2.4.5.3).

![Figure 2.4.5.3 FKBP39 does not affect the chromatin binding of Polycomb](image)

The promoter binding of PC at some gene loci were checked by ChIP in FKbp39 RNAi treated S2 cells. The FKBP39/PRC1 target genes: FBgn0033649, FBgn0041180, FBgn0053197, ph-d, ph-p and Psc were checked. Here GFP RNAi was used as a control. Y-axis: amount of DNA which has been pulled down. X-axis: gene promoters. (33649: FBgn0033649, 41180: FBgn0041180, 53197: FBgn0053197)

2.5 The link between FKBP39 and Hsp90

Remarkably, with the assessment by genome-wide ChIP-seq data of Heat shock protein 90 (Hsp90) (R. Sawarkar, C. Sievers and R. Paro, submitted 2011), it was found that about 73.5% FKBP39 peaks overlapped with Hsp90 peaks and 97.5% FKBP39 target genes are also the targets of Hsp90 (Figure 2.5.1 C). Moreover, the enrichment of FKBP39 and Hsp90 on chromatin is well correlated (Figure 2.5.1 A). By co-immunoprecipitation, FKBP39 was found to interact with Hsp90 in S2 cells (Figure 2.5.1 B) (unpublished data from R. Sawarkar), suggesting that FKBP39 may directly cooperate with Hsp90 on chromatin.
Results

**Figure 2.5.1 FKBP39 correlate and interact with Hsp90**

(A) A scatter plot showing fold enrichment of Hsp90 and FKBP39 ChIP signal calculated within peak regions co-occupied by both proteins, and plotted against each other. A Pearson correlation coefficient is shown. (B) Nuclear extract (NE) of S2 cells was immunoprecipitated with anti-FKBP39 polyclonal antibody, the Western blot analysis of the immunoprecipitated material with anti-Hsp90 antibody reveals that FKBP39 interacts with Hsp90. The reciprocal immunoprecipitation also indicates an interaction between FKBP39 and Hsp90. IgG was used as a negative control. (C) A venn diagram showing the overlapping of Hsp90 and FKBP39 target genes.

**Figure 2.5.2 Inhibition of Hsp90 lead to reduction of FKBP39 at the promoters**

The binding of FKBP39 at the promoters of *ph-d*, *ph-p* and *Psc* was analyzed in radicicol (Rad) treated S2 cells. FKBP39 was heavily reduced from the promoters of *ph-d*, *ph-p* and *Psc* in 1.5 hours radicicol treated cells.
Additionally, PRC1 genes are also the targets of Hsp90, the binding of FKBP39 on promoters of *ph-d*, *ph-p* and *Psc* was reduced when Hsp90 was inhibited by 1.5 hours radicicol treatment (Figure 2.5.2) (unpublished data from R. Sawarkar). This suggests that Hsp90 may be also involved in the regulation of the expression of PRC1 genes and essential for the stability or chromatin binding of FKBP39. Most interestingly, FKBP39 might act as a co-chaperone to Hsp90 at these target genes.
Discussion
3. Discussion

3.1 The biological function of FKBP39 in *Drosophila* development

3.1.1 FKBP39, a multiple-domain protein localizes in the nucleus and nucleolus

FKBP39 belongs to the class of FK506-binding proteins. Besides the conserved FKBP PPIase domain, it contains a unique and acidic N-terminal non-PPIase domain (Figure 3.1.1). The acidic stretches are reminiscent of the acidic regions that are found in many transcription factors and some nuclear proteins (Theopold et al., 1995). In this work, we confirmed the nuclear localization of FKBP39 and revealed the enrichment of FKBP39 in nucleolus. FKBP39 was found to be abundant in the nucleolus in unfixed salivary glands as well as in polytene squashes. The *S. pombe* yeast homolog of FKBP39, SpFKbp39p and the budding yeast homolog, Fpr4, were found to be histone chaperones and the histone chaperone activity was shown to depend on the acidic N-terminal non-PPIase domain, (Himukai et al., 1999; Kuzuhara and Horikoshi, 2004; Xiao et al., 2006). The PPIase domain of FPR4 is an enzymatic domain, which has a potential capacity to catalyze the *cis/trans* isomerization of prolyl bonds, it has been shown to inhibit the histone chaperone activity and also can regulate gene expression by switching the conformation of histone tail (Nelson et al., 2006; Xiao et al., 2006). Interestingly, Somarelli et al. further predicted as part of a tertiary conformation of FKBP39, a DNA-binding central helix-loop-helix motif (Somarelli et al., 2008), suggesting that FKBP39 could work as a transcription factor (Littlewood and Evan, 1995; Massari and Murre, 2000; Murre et al., 1994). All together, these findings suggest that as a nuclear protein, FKBP39 may also contain the histone chaperone activity and may function in chromatin mediated transcriptional regulation. Moreover, FPR4 was shown to be enriched in nucleolus and essential for silencing at the rDNA locus (Kuzuhara
and Horikoshi, 2004), which suggests that the nucleolus-enriched FKBP39 may also be important in the regulation of rDNA locus. However, the precise molecular function of this multiple-domain protein is still unclear.

![Figure 3.1.1 The domain structure of Drosophila FKBP39.]

The conserved FKBP PPIase domain is shown in green, the Acidic domains are shown in orange and the basic regions are shown in blue.

### 3.1.2 FKBP39 in spermatogenesis

Chromatin reorganization of the complete genome leading to compaction is an essential feature during spermiogenesis (Braun, 2001; Hecht, 1998; Hennig, 2003; Sassone-Corsi, 2002). The switch from the nucleosomal to the condensed conformation in mammals is essential to get a more hydrodynamic sperm head and also may protect the genome from physical and chemical damage (Jayaramaiah Raja and Renkawitz-Pohl, 2005). In mammals, the testis specific linker histones first appear (Catena et al., 2006; Martianov et al., 2005; Tanaka et al., 2005; Yan et al., 2003), and then histones are replaced by transition proteins (major types: TP1 and TP2), which in turn are replaced by highly basic protamines, to ensure the remodeling of chromatin to a typically highly condensed and transcriptionally silent state of mature sperm. These replacements lead to a shift from histone-based nucleosomal conformation to a fundamentally different conformation, resembling stacked doughnut structures containing protamines as major chromatin condensing proteins and DNA (Braun, 2001; Kimmins and Sassone-Corsi, 2005; Sassone-Corsi, 2002). So far, no linker histone variants have been identified in Drosophila, but variants of H2A (H2AvD) and H3 (H3.3) are known (Akhmanova et al., 1997; Swaminathan et al., 2005). Recently, it has been shown that Drosophila sperm also contain protamines as well as a protein
Discussion

termed Mst77F, representing at least one further chromatin component (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The transition protein-like chromosome protein in *Drosophila*, Tpl\textsuperscript{94D} was also discovered (Rathke et al., 2007). In addition, it is expected that histones to be displaced from the chromatin would be marked by characteristic modifications, as is known to be the case for histone H4 that is acetylated in mammals (Meistrich et al., 1992). Furthermore, phosphorylation and ubiquitylation were also suggested to be important during this stage (Braun, 2001; Roest et al., 1996; Wu et al., 2000a). In *Drosophila*, histone H4 hyper-acetylation and H2A mono-ubiquitylation shortly after meiosis are indicative of the switch in chromatin architecture, which might indicate an opening of the chromatin which is accompanied by the expression of a transition protein such as chromatin component (Tpl\textsuperscript{94D}) (Rathke et al., 2007).

As mentioned in the results section, the *fkbp39* null mutant flies were defective in nuclear condensation during spermatogenesis; the development of the spermatids was stopped in early elongating nuclei stage. FKBP39-EGFP was localized on chromosomes and expressed from spermatogonia stage to spermatids with round nuclei stage, which suggests an essential function of FKBP39 in spermatogenesis. A previous study showed that the transition protein-like chromosome protein in *Drosophila*, Tpl\textsuperscript{94D}, replaced the histones on chromatin in canoe-shaped nuclei stage (Rathke et al., 2007). This suggests that missing of the spermatids in canoe-shaped stage in *fkbp39* mutant may due to the defect of Tpl\textsuperscript{94D} replacement, FKBP39 may be required to keep the nucleosome-based chromatin structure in a condition in which the transition proteins can replace the histones and accumulate to chromatin later on. How does FKBP39 play this role is still unclear, whether it is required to maintain some specific histone modifications and/or regulate the expression of Tpl\textsuperscript{94D} before histones displacement still need to be investigated, the expression of Tpl\textsuperscript{94D} in *fkbp39* null mutant testes and the phenotype of Tpl\textsuperscript{94D} mutant should be first analyzed.
3.1.3 FKBP39 in nucleolar structure maintenance

In situ hybridization experiments have revealed that clusters of rDNA repeats, known as nucleolus organizer regions (NORs), are embedded in heterochromatin in most eukaryotes (Henderson et al., 1972). Each rRNA gene encodes a precursor transcript that can be processed into 28S, 18S and 5.8S rRNAs. Transcriptional activation of NORs by RNA polymerase I results in the formation of a membrane-free, well-differentiated intranuclear compartment, known as the nucleolus (Espada et al., 2007). Single rDNA genes inserted into euchromatin are able to form mini-nucleoli through a self-assembly process that is likely to be initiated by rRNA transcription (Karpen et al., 1988). Furthermore, in previous studies, it was observed that human cells lacking DNA methyltransferase 1 (Dnmt1) had a loss of DNA methylation and an increase in the acetylation level of lysine 16 histone H4 at the rRNA genes. The DNA methylation and chromatin changes at ribosomal DNA are associated with a structurally disorganized nucleolus, which is fragmented into small nuclear masses (Espada et al., 2007). In *Drosophila*, the investigations aimed at identifying regulators of nuclear architecture demonstrated that cells lacking H3K9 dimethylation and RNA interference (RNAi) pathway components displayed disorganized nucleoli, multiple nucleoli were present in su(var) or dicer-2 mutant cells in salivary-gland (Peng and Karpen, 2007). Thus, the increasing of the number of nucleoli indicates an active state of rDNA.

In contrast to the salivary gland nuclei, which have one nucleolus, there are multiple nucleoli in the nurse cells of ovary and the vast majority of all nucleolar segments are interconnected. They form a thick network shaped like a spherical shell whose outer boundary lies close to the inner surface of the nuclear envelope (Dapples and King, 1970). In *fkbp39* null allele, the nucleoli of nurse cell in ovary aggregated into one single nucleolus, which implies a relatively silenced epigenetic state of the repeated DNAs in nurse cells during oogenesis. Moreover, the alteration of nucleoli organization was not detected in salivary glands and spermatocytes (data not shown), which indicates an ovary-specific nucleolar structure maintaining function of FKBP39. However,
the mechanism under the morphological maintenance by FKBP39 is still unclear. Whether FKBP39 involves in epigenetic state maintenance need to be further investigated. Fpr4, the budding yeast homolog of FKBP39, localizes in the nucleus and is enriched in nucleolus. It is a histone chaperone and associates with chromatin at the rDNA loci. The histone chaperone domain of Fpr4 is essential for silencing at the rDNA locus and the PPIase domain has a regulatory role in silencing process (Kuzuhara and Horikoshi, 2004). Thus, FKBP39 may also control the structure of nucleoli in nurse cells by regulating the expression of rRNA genes. In this study, it was found that the RNA level of 28S was significantly down regulated in the ovaries of \textit{fkbp39} \textsuperscript{1} mutants (Figure 2.3.9). It is consistent with the observation of the aggregated nucleoli in \textit{fkbp39} \textsuperscript{1} mutant nurse cells, which may indicate a relative silent state. However, to be sure the down regulation of rRNA is due to the alteration of transcription but not some other reason, for example RNA degradation, the transcripts before processing should be evaluated. In \textit{Drosophila}, the first processing site is on the boundary of ETS (external transcribed spacer) and 18S (Schneider et al., 2010), thus this site should be chosen to check the transcriptional level of rRNA.

3.1.4 Chromatin association of FKBP39

The nuclear localization of FKBP39 in unfixed salivary glands encouraged us to detect the chromatin association of FKBP39. Both polytene staining and genome-wide ChIP-seq data showed FKBP39 bound to chromatin. Compared to the polytene staining, where only about 70 bands of FKBP39 were detected, ChIP-seq data gave us a much higher resolution across the genome. With two biological replicates, 3254 intersecting binding sites were identified, which uncovered the binding profile of FKBP39 with an extreme high resolution. Among the 3254 binding sites, 1446 (44.4% from total) are associated with at least one transcription start site of annotated genes, 965 (30% from total) are associated with exactly one transcription start site. Thus, we present here the first genome-wide chromatin profile of FKBP39, better defining its association with the promoter region. It indicates a potential role of FKBP39 in gene regulation at the chromatin level. However, FKBP39 ChIP signals assessed
Discussion

by enrichment of FKBP39 ChIP over input correlated only weakly with the
digital expression data obtained from the same S2-DRSC cell line (Enderle et
al., 2011) (Figure 3.1.2). Together with the finding that most of the FKBP39
peaks are within the fold enrichment 0.5-1.5 (log2 value) (Figure 3.1.2), which
is not very big, it suggests that the binding of FKBP39 could be dynamic
rather than a strong association. FKBP39 may fine-tune the expression of
genes rather than play a dominant decision of active versus inactive state.

Figure 3.1.2 The correlation between RNA expression and FKBP39 ChIP signal
A scatter plot relating gene expression measured by RNA sequencing (Enderle et al.,
2011) and FKBP39 ChIP signal at each of the 904 unique TSS in the genome
targeted by FKBP39. The expression is shown as the number of reads per kilobase
of exons per million mapped reads (RPKM). A Spearman correlation coefficient is
shown.

3.1.5 FKBP39 controls the development of Drosophila
In a previous study, (Theopold et al., 1995) found FKBP39 had the highest
expression in embryos, implying a crucial function in embryonic stage. In this
study, I found around 60% of the embryos from \textit{fkbp39}^{1} \textit{null} mutant were lethal, supporting the indispensability of FKBP39 during embryonic development.

Developmental control genes are genes that are critical for pattern formation and cell fate specification during the development of multicellular organisms and are defined by their specific mutant phenotypes in genetic screens (Dickmeis and Muller, 2005). Transcription factors and signaling molecules are strongly enriched among developmental control genes (Busser et al., 2008; Davidson and Erwin, 2006).

To investigate the function of FKBP39 in embryonic development, I analyzed the patterns of the embryonic ventral epidermis. The \textit{Drosophila} embryonic ventral epidermis has served as a unique tissue for the genetic analysis of patterning. Two types of epidermal cells are easily distinguished: those that secrete short, thick hair-like structures called denticles and cells that only secrete smooth cuticle. Dentine-secreting cells form segmentally repeated belts and the patterns provide a sensitive readout of patterning mechanisms (Alexandre, 2008). With the analysis of the embryonic ventral epidermis from the \textit{fkbp39}^{1} lethal embryos, a large disorder of segment patterning was detected. 63.5% of total lethal embryos showed misregulated segments, including missing segments, segment fusions and deformed segments. Therefore, FKBP39 is involved in embryonic development and to a certain extent control the patterning of embryos. In the adult stage, the deformed segments were shown in 32.09% \textit{fkbp39}^{1} null mutants, indicating that FKBP39 may also affect the patterning of adult cuticles.

The chromatin binding of FKBP39 was detected on squashed polytene chromosomes and in ChIP-seq experiment, which hints towards some effects of FKBP39 on chromatin. To investigate whether FKBP39 could function as a gene-regulator by controlling the gene transcription, the transcription start sites (TSSs) - binding profile of FKBP39 was analyzed from the ChIP-seq data. A big portion of FKBP39 peaks show to be binding at TSSs, which suggests a
potential role of FKBP39 in chromatin-controlled gene regulation. With the Gene Ontology (GO) analysis, the genes involving in “cell differentiation”, “anatomical structure morphogenesis” and “organ development” are enriched in the genes uniquely bound by FKBP39 at TSSs. It suggests a regulation role of FKBP39 in *Drosophila* development control genes. Furthermore, we noticed that some genes involved in patterning of the fly embryo are the targets of FKBP39, they include maternal genes, gap genes, pair rule genes, segment polarity genes and the direct Hox target genes (Table 3.1), which further suggest the function of FKBP39 in the patterning of fly. On the other hand, in *fkbp39* null mutant lethal embryos, there were still more than 35% containing normal segments, which suggests a more comprehensive function of FKBP39 during development rather than only controlling patterning.

3.2 FKBP39 in PcG-mediated gene regulation

3.2.1 The link between FKBP39 and PcG proteins

Polycomb-group (PcG) proteins have been shown to play an important role in the maintenance of gene repression during development. Numerous genome-wide studies have mapped components of PcG complexes by ChIP-chip or Chip-seq in *Drosophila* (Bracken et al., 2006; Enderle et al., 2011; Kwong et al., 2008; Negre et al., 2006a; Oktaba et al., 2008; Schuettengruber et al., 2009; Schwartz et al., 2006; Tolhuis et al., 2006), mouse (Boyer et al., 2006; Squazzo et al., 2006), and human cells (Lee et al., 2006; Squazzo et al., 2006). Many of the PcG target genes are involved in developmental patterning, morphogenesis, and organogenesis. For instance, 55% of the transcription factors known to be involved in segmentation of the fly embryo were scored as PcG targets (Schuettengruber et al., 2007). In this work, we found PRC1 components were also targets of FKBP39. Hence, the disorder of segment patterning observed in *fkbp39* mutants also could due to a misregulation of PcG genes.
Table 3.1 Some FKBP39 target genes involved in patterning of the fly embryo

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List of the FKBP39 target genes which are involved in patterning of the fly embryo, including maternal gene, gap gene, pair rule gene, segment polarity gene and HOX target gene.

Originally mutations were assigned to the Polycomb group because of their phenotypes. In *Drosophila*, ectopic activation of homeotic genes in *PcG* mutants leads to morphological transformations; appearance of additional sex combs on the first tarsal segment of the second and third leg pairs in *Pc* heterozygous males and the posterior transformation of embryonic segments in *Pc* homozygous (Kennison, 1995; Kennison, 2004; Lewis, 1978; Tariq et al., 2009). Multiple combinations of PcG mutant alleles enhance the severity of
the phenotype (Bel et al., 1998; Cheng et al., 1994). The genetic interaction between FKBP39 and PC was studied by crossing \textit{fkbp39}^1 and \textit{fkbp39}^{HA-2440} mutants to \textit{Pc} mutants, the strong enhancement of extra sex combs phenotype in adult flies and posterior transformation in embryos by \textit{fkbp39} mutant illustrates that FKBP39 genetically interacts with PC and works as a PcG protein, further indicating the link between FKBP39 and the PcG system.

With the co-staining of PC and FKBP39-EGFP on polytene chromosomes, many bands of FKBP39 were found overlapping with PC protein. Furthermore, the investigation of biochemical interactions, by co-immunoprecipitation, also revealed a physical interaction between FKBP39 and PC. All these findings highly suggest that FKBP39 may be involved in the gene regulation mechanism controlled by PcG proteins.

Similarly, in the genome-wide ChIP-seq experiments, which gave us a higher resolution picture of the binding profile of FKBP39, 40.6% FKBP39 peaks were shown to be intersecting with PC peaks on chromatin. After taking the core components of PRC1 complex (PH, PSC and PC) into account, it became more promising, in 275 PRC1 bound TSSs, 41.8% were also bound by FKBP39. In a previous study, PhoRC, a PcG complex with DNA binding activity, has been mapped to a region within 500 bp of TSSs (Oktaba et al., 2008). In agreement with this finding, the ChIP-seq experiments in this work showed significant enrichments of PcG proteins in promoters binding preferentially in a 500-bp window upstream of TSSs. Interestingly, FKBP39 exhibit close binding to PcG proteins and the mode of the FKBP39 distribution is closest to the TSS. These genome-wide results further encourage the hypothesis that FKBP39 may function in PcG-controlled gene regulation.

### 3.2.2 Empirical distribution function: measure the difference between two distributions

To gain insight into the function of FKBP39 in gene regulation, a genome-wide analysis of expression level was performed by RNA-seq in S2-DRSC cells. To compare the gene expression between the genes bound and not bound by
FKBP39, the empirical distribution function and Kolmogorov-Smirnov test were used in this work. The empirical distribution function is the cumulative distribution function associated with the empirical measure of the sample. In probability theory, an empirical measure is a random measure arising from a particular realization of a (usually finite) sequence of random variables (Shorack, 1986) and the cumulative distribution functions are used to specify the distribution of random variables. The Kolmogorov–Smirnov test is based on cumulative distribution functions and can be used to test to see whether two empirical distributions are different or whether an empirical distribution is different from an ideal distribution (Gentle, 2009). In this study, the $p$-value from Kolmogorov-Smirnov test indicated that the difference between the empirical distributions of FKBP39 bound and not bound gene groups are specific.

3.2.3 FKBP39, a gene activator and an unusual PcG protein

As a common concept, mutations in PcG genes lead to the ectopic expression of homeotic genes, resulting in posterior transformations of segments and body structures, thus it was suggested that the genes of the PcG are part of a specific repressor system (Lewis, 1978). However, the interaction of FKBP39 with PRC1 at promoter regions, the relative higher expression level of FKBP39 binding genes in PRC1 targets and the enrichment of down regulated genes specifically within “FKBP39” and “PRC1 AND FKBP39” targets in FKBP39 knock-down S2-DRSC cells revealed that FKBP39 is involved in PcG-mediated epigenetic control but works as a gene activator. Moreover, none of the homeotic genes is bound by FKBP39 at their TSS. Therefore, we consider FKBP39 to play the role as a PcG gene in an unusual way. With the expression analysis of the PRC1 components in FKBP39 knock-down S2-DRSC cells, we detected the down regulation of the FKBP39 targets, PRC1 components, $ph-d$, $ph-p$ and $Psc$. In FKBP39 knock-down S2 cells, the binding of FKBP39 at the TSS of $ph-d$, $ph-p$ and $Psc$ was clearly reduced, which indicates that the down regulation of these genes is specifically due to the reduction of FKBP39 at their TSS. All together, it results in the explanation that FKBP39 appeared as a PcG protein at both embryonic and adult stages.
in *Drosophila* because of its activator function on *ph-d*, *ph-p* and *Psc* genes. However, the regulation of *ph-d*, *ph-p* and *Psc* genes by FKBP39 is not prominent; the down-regulation of these genes in FKBP39 knock-down S2 cells is less than 31%. This could be due to the self-regulation and negative feed-back of PcG on *ph-d*, *ph-p* and *Psc* genes (Ali and Bender, 2004).

### 3.2.4 Why does the *fkbp39* null mutant not show homeotic transformation phenotypes?

Although *fkbp39* null mutants heavily enhanced the homeotic transformation phenotype of *Pc* mutants, they did not show such kind of phenotype in either embryonic stage or adult stage as single mutation. Instead, a complete spectrum of the segment morphologies, which include segments missing, segments fusion and deformed segments was found. One explanation could be the rather weak regulation of PRC1 components by FKBP39. As the knock-down of *FKbp39* has almost no effect on the expression of HOX genes in S2 cells, it is possible that the down regulation of PRC1 components in *fkbp39* mutant alone is under the threshold to affect the HOX genes, but can influence some other development control genes. With the GO analysis, the genes involved in embryonic development and tissue morphogenesis were found to be enriched in the 90 down-regulated genes, which validate the conjecture that the defect of FKBP39 affects the gene expression of development control genes. Only when *fkbp39* mutants are combined with *Pc* mutants, the genetic background becomes sensitive, and hence the homeotic transformation phenotypes is enhanced.

### 3.2.5 Why only a small number of misregulated genes were detected in FKBP39 RNAi experiments?

In this mRNA-seq experiment, we faced the problem that the variation of gene expression was mild in FKBP39 RNAi S2-DRSC cells. With a fold change cutoff of 1.2 and a *p*-value cutoff <0.1, only 0.76% of total genes were found to become misregulated, although FKBP39 was 95% down-regulated. The explanation could be (i) probably the role of FKBP39 in gene expression is gentle, it fine-tunes the expression of development essential genes, rather
than being involved in on-off decisions. (ii) there is some unknown member in FKBPs family sharing the function with FKBP39, due to the redundancy and hence only knocking down FKBP39 is possibly not sufficient, (iii) according to the general RNAi protocol, in this work the cells have been treated with dsRNA for 5 days. However this may be already long enough for the cells to partially recover the misregulation of genes by some unknown rescue mechanism.

3.2.6 Conceivable mechanisms of FKBP39 in PcG-mediated gene regulation

3.2.6.1 FKBP39, a PPIase-domain containing protein
To become functionally active, a protein chain must fold into a unique three-dimensional structure, the native fold. However, the peptide bond preceding proline residues realizes a cis/trans conformational switch with high switching resistance in native proteins and folding intermediates, which has been frequently found to be a rate limiting step (Aumuller and Fischer, 2008; Dolinski et al., 1997). Interestingly, the evidence indicating that the conformational switch of the prolyl bond serves as a general mechanism for modulating the bioactivity of proteins in the native state is starting to emerge (Andreotti, 2003; Aumuller and Fischer, 2008) and this principle is implicated in important physiological processes such as ion channel gating (Lummis et al., 2005), cell signaling (Brazin et al., 2002; Sarkar et al., 2007; Wulf et al., 2005), neurodegeneration (Pastorino et al., 2006), and DNA binding (Cui et al., 2003).

As mentioned above, FKBP39 contains a conserved FKB PPIase domain, but its activity has not been validated in vitro yet. PPIase activity is most commonly monitored using the spectrophotometric assay developed by (Fischer et al., 1984), which relies on the ability of a-chymotrypsin to release $p$-nitroaniline from peptides such as succinyl-Ala-Ala-Pro-Phe-$p$-nitroanilide (succ.AAPF.pNA) only if the bond on the N-terminal side of the Proline residue is in the trans conformation (Kay, 1996). However, in this study, we
noticed that FKBP39 is cleaved by a-chymotrypsin, therefore to test the PPIase activity of FKBP39, a protease-free assay for peptidyl prolyl cis/trans isomerases (Dolinski et al., 1997; Janowski et al., 1997) should be employed in the future.

3.2.6.2 FKBP39 and PC protein

_Drosophila_ Pin1 homologue, the Dodo protein is a PPIase and involved in dorso-ventral patterning of the follicular epithelium in the egg chamber. Hsu et al. (2001) found that Dodo facilitates the degradation of the transcription factor Cf2, which requires a prior activated epidermal growth factor receptor-MAPK signaling. Dodo interacts with the phosphorylated Cf2 and facilitates the degradation by inducing a conformation change of Cf2, which is more accessible for ubiquitination (Hsu et al., 2001). Furthermore, recent studies showed that a nuclear immunophillin, Cyclophilin33 (Cyp33) interacts through its RRM domain with the third PHD finger of Trithorax, the interaction is also conserved in human (Anderson et al., 2002; Fair et al., 2001; Mi et al., 1996). Cyp33 was found to be a negative regulator of MLL (the human orthologue of TRX)-induced transcription, the PPIase domain of Cyp33 regulates the conformation of MLL1 through Proline isomerisation within the PHD3-Bromo linker, thereby disrupting the PHD3-Bromo interface and facilitating binding of the MLL1-PHD3 domain to the Cyp33-RRM domain (Hom et al., 2010; Park et al., 2010; Wang et al., 2010). These findings indicate the molecular switch role of PPlase is also essential in regulating the activity of transcription factors.

The recent genome wide mapping of PcG proteins showed that PcG was highly enriched at repressed PREs and the corresponding target gene promoters whereas the binding levels were significantly decreased at active loci (Enderle et al., 2011; Schwartz et al., 2006). This indicates that the epigenetic state of a PRE may primarily be regulated through the modulation of PcG binding activity. To investigate the effect of FKBP39 in PC binding, the correlation between the ChIP signal of PC and FKBP39 was analyzed, however, only weak correlation could be detected (Figure 2.4.1.4 B). Furthermore, the binding status of PC on some down regulated PRC1/FKBP39 target genes was also rarely affected by the reduction of FKBP39.
(Figure 2.4.5.3). All together, it suggests that FKBP39 may regulate the activity of PC without changing its binding activity.

FKBP-family PPlases usually have a strong preference for substrates with a bulky hydrophobic residue preceding the Proline, so the succ.ALPF.pNA or succ.AFPF.pNA is most commonly used to assay these enzymes (Kay, 1996; Stoller et al., 1995). In Polycomb protein, we found there were 2 potential PPlase-recognize sites in front of the repression domain, which contain the amino acids of ALPP and WLPA (Figure 3.2.1).

![Figure 3.2.1 Potential PPlase-recognize sites in Polycomb protein.](image)

The chromodomain (orange) and the C-terminus repression domain (dark blue) are indicated. Close to the repression domain, there are 2 potential PPlase-recognize sites (indicated by red triangles).

The C-terminal part of PC was previously identified as the repression domain. It is necessary for the repression of reporter constructs in transgenic flies (Muller, 1995) or in mammalian tissue culture cells (Bunker and Kingston, 1994). In addition, PC protein interacts with nucleosomal core particles directly and the main nucleosome-binding domain coincides with a region in the C-terminal part of PC (Breiling et al., 1999). Owing to the suggestion that PcG proteins silence genes by creating higher-order chromatin structures at their chromosomal targets, the interaction between C-terminal part of PC and nucleosomal core particles provide a potential function in establishment or regulation of higher-order chromatin structures. Therefore, it will be interesting to investigate the prolyl bond conformational switch function of FKBP39 in modulating the bioactivity of PC protein. FKBP39 may fold the repression domain of PC into a position, which is not able to interact with nucleosome and the higher-order chromatin structure may be disrupted, consequently FKBP39 works as a gene activator in PcG mediated gene regulation. In this
work, FKBP39 could be co-immunoprecipitated with PC, but whether they interact directly is still unknown. Thus, to find out a potential prolyl bond conformational switch function of FKBP39 on PC, the first step will be the detection of the direct interaction between FKBP39 and PC by \textit{in vitro} binding assay.

\textbf{3.2.6.3 FKBP39 may regulate the histone modifications}

On the other hand, the surface of nucleosomes is studded with a multiplicity of modifications, alterations in the histone modifications are also important in regulating the access to DNA of the complex machinery that controls nuclear phenomena such as gene expression control (Workman and Kingston, 1998). The immunophilin Cyp33 shows isomerization activity for H3P30 and H3P16. This activity suggests a mechanism for the Proline isomerase function of Cyp33, which by means of isomerization of Prolines in the histone tail, Cyp33 can effect a change in the epigenetic modification profile on the H3 tail (Park et al., 2010). Furthermore, the budding yeast homolog of FKBP39, Fpr4 was found to be able to regulate the level of H3K36 methylation by adjusting the conformation of Proline-38 residues near Lysine-36. Abrogation of Fpr4 catalytic activity \textit{in vivo} results in increased levels of H3K36 trimethylation by Set2 and delayed transcriptional induction kinetics of specific genes in yeast (Nelson et al., 2006). These evidences suggest the isomerization of one or more prolines may easily alter the activity of histone acetyl transferase, histone deacetylase, histone methyl transferase, or histone demethylase enzymes at specific loci, resulting in an alteration of the epigenetic profile.

To primarily investigate the potential role of FKBP39 on histones, we detected the interaction between FKBP39 and Histones \textit{in vitro} by GST pull-down. In a previous study, it was shown that FPR4, the yeast homolog of FKBP39 was interacting with Histone H3 and H4, but not H2A and H2B (Nelson et al., 2006). We also analyzed the interaction between FKBP39 and Histone H3 and H4 in this study. It was found that FKBP39 interacted with Histone H3 but not Histone H4 (Figure 3.2.2). However, there was no clear interaction detected between FKBP39 and the tail of Histone H3 (1-40) (data not shown).
Figure 3.2.2 FKBP39 interacts with Histone H3 \textit{in vitro}

Recombinant histone H3 and H4 (NEB) (4 µg) were mixed individually with GST or GST-FKBP39. Following recovery with glutathione-sepharose, bound histones were resolved by SDS-PAGE and detected by staining with Coomassie blue. FKBP39 interacts with Histone H3, but not Histone H4. Input: 1 µg Histone.

The \textit{in vitro} interaction between FKBP39 and Histone H3 suggests the potential regulation role of FKBP39 on Histone H3. In a previous study, it has been shown that FPR4 bound region and the catalyzed region were different on Histone H3 (Nelson et al., 2006). Therefore, although there was no clear interaction between the tail of histone H3 and FKBP39, FKBP39 may still control gene expression via modulating the structure of the tail of Histone H3 by prolyl bond conformational switching. However, which proline of Histone H3 is affected by FKBP39 is still unknown. To find out which histone modification may be regulated, the PPlase activity of FKBP39 on the prolines H3P16, H3P30 and H3P38 should be first tested, it would give useful information to predict the FKBP39 regulated histone modifications. To investigate the alteration of the epigenetic profile, the histone modifications closed to the proline residue, which is isomerized should be analyzed in detail.
3.2.6.4 FKBP39 and transcription elongation

In *Drosophila* and mammals, PRC1 has been proposed to mediate gene repression either by regulating the initiation phase of RNA polymerase II (Pol II) or, at a later step, by stalling Pol II elongation (Dellino et al., 2004; Stock et al., 2007). Recently, it was found that PcG proteins preferentially bind stalled promoters, which suggests that PcG proteins may repress their target via modulating the rate of RNA Pol II elongation (Enderle et al., 2011).

Interestingly, the PPIase Ess1 (yeast homolog of Pin1), was found to interact with the C-terminal domain (CTD) of RNA Pol II and inhibit transcription elongation, which suggests that Ess1 may isomerize the CTD of RNA Pol II and coordinate the binding of elongation factors to the CTD (Wu et al., 2003; Wu et al., 2000b). Involvement of PPIases in transcription elongation suggests a possibility that FKBP39 may also regulate the CTD of Pol II via PPIase activity and facilitate the transcription elongation, which are controlled by PcG proteins. To study this hypothesis, the interaction between FKBP39 and the CTD of Pol II should be first investigated. Moreover, promoters occupied by stalled Pol II are characterized by the presence of small promoter proximal RNAs and this class of RNAs has been quantitatively measured by Illumina sequencing in *Drosophila* S2 cells (Nechaev et al., 2010). If FKBP39 bound promoters produce reduced amounts of promoter-proximal RNAs at PRC1 target genes this might suggest a functional interaction.

3.2.7 FKBP39 and Hsp90

At a molecular level, Hsp90 binds to substrate proteins, which are in a near native state and thus at a late stage of folding poised for activation by ligand binding or interaction with other factors (Jakob et al., 1995). In fulfilling its role, Hsp90 operates as part of a multichaperone machinery in the cytosol, which includes Hsp70, PPIases and other cochaperones (Bose et al., 1996; Duina et al., 1996a; Freeman et al., 1996). Hsp90 also maintains several nuclear hormone receptors and the inactive steroid receptor heterocomplexes contain multi-domain PPIase of the Cyp and FKBP families, FKBP51, FKBP52 and Cyp40 are known to bind Hsp90 (Callebaut et al., 1992; Owens-
Discussion

Grillo et al., 1995; Pratt et al., 2004). These finding suggest that molecular chaperones may function in heterocomplexes and PPIases probably function together with Hsp90. Interestingly, according to the ChIP-seq data, it was found that the enrichment of FKBP39 and Hsp90 on chromatin are well co-localized and correlated (Figure 2.5.1 A, C). By co-immunoprecipitation, FKBP39 was found to interact with Hsp90 (Figure 2.5.1 B), which suggest that FKBP39 and Hsp90 may cooperate on chromatin. Sawarkar et al. (2011) found Hsp90 could regulate gene expression by affecting RNA polymerase II pausing, it bound and stabilized Negative Elongation Factor complex at paused promoters. Together with the involvement of PPIase in transcription elongation, this suggests that FKBP39 may be able to cooperate with Hsp90 in regulating gene expression by affecting RNA polymerase II pausing. In addition, Hsp90 was found bound to promoters of some heat-shock loci such as hsp22, hsp26, hsp27, hsp70 and hsp83 (Sawarkar et al., 2011), In this study, we found some of them are also the targets of FKBP39, such as Hsp26, Hsp70 and Hsp83, which suggests that FKBP39 may cooperate with Hsp90 in the regulation of these heat-shock loci.

Furthermore, the binding of FKBP39 on promoters of ph-d, ph-p and Psc were reduced when Hsp90 was inhibited (Figure 2.5.2) (unpublished data from R. Sawarkar), which suggests that Hsp90 may be essential for the stability or chromatin binding of FKBP39 and also regulate the expression of PRC1 genes. Recently, Hsp90 was found to interact with TRX protein and cooperates with TRX at chromatin for maintaining the active expression state of target genes (Tariq et al., 2009). In this study, FKBP39 was found to interact with PC protein and work as a gene activator at PcG targets, this suggests that FKBP39 and Hsp90 may work together and control two aspects of PcG/TrxG regulating system to activate the gene expression: Hsp90 maintains the activity of TRX and FKBP39 may inhibit the activity of PC, However, this hypothesis still needs to be investigated in detail. First, whether the defect of Hsp90 also leads to down regulation of FKBP39 targets should be studied. Second, whether Hsp90 is required for the stability of FKBP39 protein in general should be tested. Third, in cytoplasm, the FKBP proteins bind to Hsp90 via TPR (tetramericopeptide repeat) domain (Jarczowski et al.,
2009; Pratt et al., 2004), however FKBP39 doesn’t contain such a domain, whether and how FKBP39 bind to Hsp90 directly or what are the other factors mediate the binding of FKBP39 and Hsp90 should also be investigated.

3.3 Conclusion and prospects
In this study, we found FKBP39, a *Drosophila* member of a family of proteins that bind the immunosuppressive drug FK506, a nuclear immunophilin, played a comprehensive role in *Drosophila* development; including embryonic stage, adult stage, spermatogenesis and oogenesis. The genome wide overlapping between FKBP39 and PC, the correlation of FKBP39 with PRC1 at promoter regions, suggest the involvement of FKBP39 in PcG mediated gene regulation. The relative higher expression level of FKBP39 binding genes in PRC1 targets and the enrichment of down regulated genes within both FKBP39 and PRC1 AND FKBP39 targets in FKBP39 knock-down S2 cells reveal that FKBP39 works as a gene activator and involves in PcG mediated epigenetic control. On the other hand, FKBP39 was also genetically interacting with PC; *fkbp39* mutants strongly enhanced the phenotype of *Pc* mutants in both embryonic and adult stage. The PRC1 components *ph* and *Psc*, the targets of FKBP39 were down-regulated in FKBP39 knock-down S2 cells. Therefore, FKBP39 showed as a PcG protein at both embryonic and adult stage in *Drosophila* may due to its activator function on *ph-d*, *ph-p* and *Psc* genes. Moreover, FKBP39 was found to physically interact with histone H3 and PC, it suggests that FKBP39 may regulate the gene expression via switching the protein conformation of PC and histone H3.

The germline phenotype of *fkbp39* null mutant suggests that besides the function in PcG mediated gene regulation, FKBP39 is also essential for chromatin compaction in spermatogenesis and nucleolar structure maintenance in oogenesis.
**Open questions**

To understand the precise function of FKBP39 in *Drosophila* spermatogenesis, oogenesis, gene expression and PcG proteins mediated gene regulation, several questions should be addressed in the future:

1. Does the PPIase domain of FKBP39 exert PPIase activity on Histones? Which prolyl bonds are isomerized? Which histone modifications are controlled via this prolyl bond conformational switching? Are these histone modifications affecting the regulation of gene expression? To answer these questions, the PPIase assay should be first performed to study the PPIase activity of FKBP39 on histone H3. Afterwards, the histone modifications closed to the Proline residue, which is isomerized should be analyzed in detail.

2. Does FKBP39 interact with PC directly? Does FKBP39 have PPIase activity on the potential PPIase recognize sites in PC protein? Whether FKBP39 regulates the repression activity of PC? To address these questions, the first step will be the detection of the PPIase activity of FKBP39 on PC and the direct interaction between FKBP39 and PC by *in vitro* binding assay.

3. Does FKBP39 regulate transcription elongation? To find out the answer, the interaction between FKBP39 and the CTD of Pol II should be first investigated. Afterwards, the stalling index and the small promoter-proximal RNAs of FKBP39 targets and non-targets should be compared within Hsp90 target genes and to see whether the FKBP39 targets are significantly enriched with the genes showing lower stalling index and lower amount of promoter-proximal small RNAs.

4. Does Hsp90 work in the same complex as FKBP39 to regulate PcG-mediated gene expression? To study this question, the effect of Hsp90 on PcG targets and the effect of Hsp90 inhibition on FKBP39/Hsp90 target genes should be first analyzed in a genome-wide scale.
5. During spermatogenesis, does FKBP39 affect histone displacement? Does FKBP39 control the expression of transition protein-like chromosome protein? To answer these questions, the expression of Tpl^D in *fkbp39* null mutant testes and the phenotype of Tpl^D mutant should be first analyzed.

6. During oogenesis, does FKBP39 regulate the expression of rRNA genes? Whether FKBP39 involves in epigenetic state maintenance by regulating histone modifications? To study on these questions, the transcription level of rDNA in *fkbp39* null mutant should be analyzed. The level of H3K9 dimethylation could be first checked in the ovary of *fkbp39* null mutant, the histone modifications closed to the Proline residues, which are isomerized should be also analyzed.

Finding answers to these questions will provide a more detailed picture of the mechanism underlying the function of FKBP39 in spermatogenesis, nucleoli structure maintenance, gene expression control and PcG proteins-mediated gene regulation.
Materials
4. Materials

4.1 Antibodies

<table>
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<tr>
<th>Antibody</th>
<th>Source</th>
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<th>Dilution</th>
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<td>rabbit</td>
<td>Paro lab</td>
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<td>donkey</td>
<td>Jackson Immuno Research</td>
<td>1:200</td>
</tr>
</tbody>
</table>

4.2 Molecular weight markers

- 1kb DNA ladder     NEB (New England Biolabs)
- 100bp DNA ladder   NEB
- SeaBlue plus 2 Protein ladder Invitrogen

4.3 Enzymes

- Benzonase          Merck
- DNase (RNase-free)  Roche
- Restriction enzymes NEB
- RNase (DNase-free)  Roche
- SuperscriptIII™ reverse transcriptase Invitrogen
4.4 Oligonucleotides

All primers were purchased from SIGMA

4.4.1 Primers used for pENTR cloning

Amplification of the full length FKbp39 containing no stop code
FK-GFP-GW-F
5'-CACCATGTCGATGTTTTGGGGTTTGAACA-3'
FK-GFP-GW-R
5'-ATGCACAGCTTTCAGTTCCACCTTCG-3'

Amplification of the full length FKbp39 containing stop code
FK-GFP-GW-F
5'-CACCATGTCGATGTTTTGGGGTTTGAACA-3'
FKRstop-GW for N term fusions
5'-CTAATGCACAGCTTTCAGTTCCACCTTCG-3'

4.4.2 Primers used for GST cloning

FK CDS 5' EcoRI
5'- GGCCGAATTCATGTCGATGTTTTGGGGTTTGAACA-3'
FK CDS 3' Xhol with stop
5'-GCACAGCTTTCAGTTCCACCTTCG-3'

4.4.3 Primers used for semi-quantitative RT-PCR

Detection of the FKbp39 transcription level
FK-GFP-GW-F
5'-CACCATGTCGATGTTTTGGGGTTTGAACA-3'
FKRstop-GW for N term fusions
5'-CTAATGCACAGCTTTCAGTTCCACCTTCG-3'

Detection of the Sra-1 transcription level
Sra-F 3292 5'-AGAATGAAAAACCGGAGGCT-3'
Sra-R 3736 5'-TGTACCCGCAGAATGTGGA-3'
Detection of the alpha-Tubulin transcription level as an internal control

**Tubulin F**
5’-TTGTCAAGCCTCATAGCCGGC-3’

**Tubulin R**
5’-TGACAACACTGAATCTGGCCGA-3’

### 4.4.4 Primers used for real-time qRT-PCR

#### Detection of the FKbp39 transcription level

**FKbp39-1F**
5’-CCAGCTTCTTAAGGATCCACG-3’

**FKbp39-1R**
5’-GAGGCTGTCAGGGAAGTCTTG-3’

#### Detection of the Sra-1 transcription level

**Sra-qPCR-1F**
5’-TACCACATTCTGCGGGTACA-3’

**Sra-qPCR-1R**
5’-ATCGCCCGCCCTTTAGATAC-3’

#### Detection of the ATPase cf6 transcription level as an internal control

**ATPcf6-F**
5’-AGAAGAGCGCCGGTGCAA-3’

**ATPcf6-R**
5’-AACATCGGGGAACTGAATTC-3’

#### Detection of the PRC1 genes transcription level

**Pc-F**
5’-ACTCCATGCGAAACCGAAG-3’

**Pc-R**
5’-GTCGGCGCGGAGGAATATA-3’

**ph-d-s**
5’-ACCAGACAGTATCGGACGT-3’

**ph-d-r**
5’-ATTGGCAAGGGCAATGTC-3’

**ph-p-s**
5’-GATGATCTCTAACCGGAGCAG-3’

**ph-p-r**
5’-TGGGTTAAGACCTGATGGC-3’

**Psc-s**
5’-AGGAGTGGAATTCCGACTG-3’

**Psc-r**
5’-GTGGGTTGAAACCAACGAAG-3’

#### Detection of the Trx transcription level

**Trx-rt-1f**
5’-ATTGTGAACAGGCAACCACA-3’

**Trx-rt-1r**
5’-GCTTGAGCTTCAATCAAGGC-3’

#### Detection of the 28S rRNA level

**28Sa-q1F**
5’- CAGGTTGAAGTGCGGGAAA-3’

**28Sa-q1R**
5’- CACCAGCTATCGAGGGAA-3’
28Sb-q1F 5’- AGACCGTCGTGAGACAGGTT-3’
28Sb-q1R 5’- AACGGACGTAGCGTCATACC-3’

4.4.5 Primers used for dsRNA generation
T7-EGFP-F 5’-taatacgactcactatagggACGTAACCGCCACAATGTT-3’
T7-EGFP-R 5’-taatacgactcactatagggTGTCTGGTAAATGGTCG-3’
T7 FKbp39-F 5’- taatacgactcactatagggTCAAATGACGATCGAGAATTT-3’
T7 FKbp39-R 5’- taatacgactcactatagggACGACCTGATCCACAATCTT-3’

4.4.6 Primers used for enrichment detection in ChIP
FK-X peak 1f 5’-GCGGTTCGAAAATCAAGAGT-3’
FK-X peak 1r 5’-CCGTTCTACACACCTACGCA-3’
AbdB-pk03-F 5’-TCATGGATTTAGACTCCGCC-3’
AbdB-pk03-R 5’-TGTTCTGCTGGTAGTGGTCG-3’
ImpL1 5’-s 5’-GATTGGACGTGGATTTCCTG-3’
ImpL1 5’-r 5’-GGTTCGGTGATCTGGTTGTT-3’
ph-d FKpeak-s 5’-GTATGCGACACATCGACACC-3’
ph-d FKpeak-r 5’-TTGAAACTTAACTTGGCGGC-3’
ph-p FKpeak-s 5’-AGAGCGCAAGAACCGTACAC-3’
ph-p FKpeak-r 5’-CCGATCCGAACGAAAAGTAG-3’
Psc FKpeak-s 5’-GCTCTGGGCGAGTATCTTTG-3’
Psc FKpeak-r 5’-ACTTGTTGCCCAGTGAGAGG-3’
33649 FKpeak-s 5’-ATGTTCGACGAGGCTCAGAT-3’
33649 FKpeak-r 5’-ATTTCCAAGGGCTTGGTG-3’
41180 FKpeak-s 5’-AGTCGCTAACCAACTTCCG-3’
41180 FKpeak-r 5’-GATTGGTGCTAATGAGGG-3’
53197 FKpeak-s 5’-ATACCAGAGCCTGCTGAGA-3’
53197 FKpeak-r 5’-TTCTGTTTGCCTGACTGAG-3’

4.4.7 Primers used for mRNA-seq validation
FBgn0034282-s 5’-TAACCTCGATTGGGAAGGCG-3’
FBgn0034282-r 5’-TCTGACTTGGGAGGCG-3’
FBgn0037504-s  5’-CACAGAGATCACCGACGAGA-3’
FBgn0037504-r  5’-TGCTAGTGATCCAAATGCGA-3’
FBgn0085403-s  5’-GAATAGAATTTCATGCAGC-3’
FBgn0085403-r  5’-GTGGGTTCATTTGTCCGTT-3’
FBgn0030469-s  5’-CTTCAGGGAGATTCAACGAG-3’
FBgn0030469-r  5’-GATTTCCTGATTTGTGACG-3’
FBgn0031914-s  5’-GCTCTTTGCCCCGATTCAAGC-3’
FBgn0031914-r  5’-CAGTTCCCATGTGCCAGAC-3’
FBgn0050035-s  5’-TGACCCAGACCTCCAGGAC-3’
FBgn0050035-r  5’-ATCATCTTTGCTCGATGTC-3’
FBgn0033649-s  5’-GAAACTTCTCTGCTGGAC-3’
FBgn0033649-r  5’-TCAGCAGACCACATGTTA-3’
FBgn0041180-s  5’-CTGATGAGGATAGCTTCGC-3’
FBgn0041180-r  5’-TGTACGCCTCTATGCGAATG-3’
FBgn0053197-s  5’-GGCCAGAGAAAGAAACGAAG-3’
FBgn0053197-r  5’-AATATGCGCATTAAGCACCAG-3’
FBgn0039073-s  5’-TGCACAGCTACTCCCAGATG-3’
FBgn0039073-r  5’-ATGGTCTCTCAGATGCTGCC-3’

4.5 Vectors

pAWG The destination vector containing actin promoter, gateway cassette and EGFP-tag (Invitrogen)
pCasper4-Tubulin pCasper4 containing the 2.4kb alpha tubulin promoter (from C.Beisel)
pCasper4-Tubulin-Flag-W The destination vector containing alpha tubulin promoter, Flag-tag and gateway-cassette (W) (from C. Beisel)
pCasper4-Tubulin-W-EGFP The destination vector containing alpha tubulin promoter, gateway-cassette (W) and EGFP-tag.(this work)
pENTR The vector containing gateway cassette and TOPO cloning site (Invitrogen)
pGEX-4T-1 A tac promoter for chemically inducible, high-level expression of GST-tagged
recombinant proteins, containing Thrombin recognition site for cleaving the desired protein from the fusion product.

### 4.6 Cell culture

**Kc**

Origin: Disaggregated young embryos (8 to 12h old) of *Drosophila melanogaster*. Karyotypes: Kc is fundamentally diploid, with a female chromosomal set (XX), but one single IVth punctiform chromosome.

**S2-DRSC**

S2 cells from Drosophila RNAi Screening Center. Origin: The S2 cell line was derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos. Karyotypes: The cells of this line have eight metacentric / submetantric chromosomes; four are probably third chromosomes and four are second chromosomes. In addition, these S2 cells have two heteromorphic X chromosomes, a small acrocentric chromosome and one or two dot (fourth) chromosomes.

### 4.7 Bacterial strains

- **Top10**
  - *E. coli* F- *mcrA Δ(mrr-hsdRMS-mcrBC)*
  - φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 gaiU galK rpsL (StrR) endA1 nupG

- **DB3.1™ Cells**
  - Competent Cells suitable for propagation of plasmids containing the ccd>B gene (Invitrogen)

- **BL21(DE3) codon + RP**
  - Rare codon translation AT rich genomes (argU, proL); (Stratagene)

### 4.8 Fly lines

#### 4.8.1 General fly lines

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>w¹¹¹⁸</td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>yw, <em>BcGla/CyO</em></td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>yw, TM3Sb/Dr</td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>yw-p&gt; ; TM3Sb/Dr</td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>yw, Δ2-3TM3Sb/Δ2-3Dr</td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>w, <em>Ifa/CyO; MKRS/TM6Tb</em></td>
<td>Paro lab stock collection</td>
</tr>
<tr>
<td>w, TM3,P(Actin-GFP,w⁺),Ser/Sb</td>
<td>Paro lab stock collection</td>
</tr>
</tbody>
</table>
4.8.2 Transgenic flies generated

- Tu-Flag-FKbp39-3rd: homozygotes, carry tubulin promoter and Flag-FKbp39 on 3rd chromosome, this work
- Tu-FKbp39-EGFP-2nd: homozygotes, carry tubulin promoter and FKbp39-EGFP on 2nd chromosome
- Tu-FKbp39-EGFP-3rd: homozygotes, carry tubulin promoter and FKbp39-EGFP on 3rd chromosome

4.8.3 Mutant flies

- $Pc^3$: $Pc$ null mutant, Paro lab stock collection
- $Pc^{TT109}$: $Pc$ null mutant, Paro lab stock collection
- $fkbp39^{5-HA-2440}$: $fkbp39$ P-element mutant, (from Gunter Reuter)
- $fkbp39^1$: $fkbp39$ null mutant, generated in this work

4.9 Technical devices

4.9.1 Microscopy

- Microscopes: Leica DMI 6000B
- Stereomicroscopes: Leica MZ16FA
- Camera: DFC 350X
- LAS AF
- KL 1500 LCD (Lamp)

4.9.2 Spectrometers

- SmartSpec 3000
- Nanodrop ND-1000

4.9.3 Agarose Gel Electrophoresis

- MUPID gel chamber and power supply

4.9.4 SDS – Gel Electrophoresis

- Novex Mini Cell

4.9.5 Western blotting

- TE Series Transfer chamber
4.9.6 Detection of Western blots and DNA Gels
ECI Western blot detection reagents GE
SuperSignal West Femto substrate Pierce
Hyperfilm ECL GE
Fixer FX-31 Typoon
Entwicklerkonzentrat Ref 00176 Adefo

4.9.7 Mechanic Homogenizer
Glass Dounce homogenizer (12ml) Wheaton

4.9.8 PCR Machines
Gene Amp PCR System 9700 Applied Biosystems
Light Cycler 480 Roche

4.9.9 Beads
Ni-NTA superflow QIAGEN
Streptavidin Sepharose GE
Protein A Agarose GE
Protein G Agarose GE

4.9.10 Sonifier
Bioruptor Diagenode

4.9.11 Centrifuges
5415-R Eppendorf
Sorvall RC 6 PLUS THERMO
Sorvall WX Ultra Series THERMO
Sorvall SS-34 Rotor THERMO
Sorvall TH641 Rotor THERMO
Fiberlite F13B rotor Piramoon
4.9.12 Microinjection

Femtotips          Eppendorf
Microloader tips    Eppendorf
Microinjector      FemtoJet®, Eppendorf
Micromanipulator   Leitz
Microscope         Leitz Labovert

4.9.13 Further devices and materials

Coverslips           Menzel
DAPI (4’,6-diamidino-2-phenylindole) Sigma
dNTPs                Peqlab
Double-sided sticky tape Scotch 3M
Filters (0.2μm, 0.45μm) Schleicher & Schuell
Filter paper         Whatman, 3mm
Fluoromount-G        SouthernBiotech
Fly cages            ZMBH fine mechanics workshop

Fly vials small (Ø 2.7cm, height 6.4cm) Greiner

Fly vials middle (Ø 3.5cm, height 8.1cm) Greiner

Fly vials large (Ø 4.9cm, height 10cm) Greiner
Forceps              A. Dumont & Fils
Highspeed Plasmid Midi Kit Qiagen
Horizontal shaker    GFL-Gesellschaft für Labortechnik

Hybond ECL Nitrocellulose Membranes Amersham

Magnetic stirrer     Ikamag
MEGAscript ® T7 Kit  Ambion
Micropetle           Eppendorf
Microwave            Panasonic Dimension
Nylon membrane       Roche
Paper filters        Sartorius

Pipetman             IBS Integra Biosciences
Pipettes (1ml, 200μl, 20μl) Gilson
Pipette (2μl)        Eppendorf
Petri dishes         Greiner
pH meter 766         Knick
QIAquick Gel Extraction Kit Qiagen
QIAquick PCR Purification Kit Qiagen
Rotator              Heidolph
RNeasy Minelute cleanup Kit Qiagen
Sieve with steel mash (400μM pore size) Roth

Sieve with steel mesh (200μM pore size) Roth
Slides               Menzel
### Materials

- Slide-A-Lyzer Dialysis Cassette, 0.5-3ml, 7,000 MWCO
- Snake skin Pleated Dialysis tubing
- Syringes
- Thermomixer
- Tubing (10kDa)
- Tubing (12-14kDa)
- Vortex Genie
- Waterbath

#### 4.10 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>Applichem</td>
</tr>
<tr>
<td>Acrylamid/Bisacrylamid (37.5:1)</td>
<td>Roch</td>
</tr>
<tr>
<td>Agar</td>
<td>Serva</td>
</tr>
<tr>
<td>Agarose Ultra Pure</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>Ammonium Sulfate (NH₄)₂SO₄</td>
<td>Applichem</td>
</tr>
<tr>
<td>Ammonium persulfate (NH₄)₂S₂O₈</td>
<td>Applichem</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Applichem</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Serva</td>
</tr>
<tr>
<td>Bradford Staining solution</td>
<td>BioRad</td>
</tr>
<tr>
<td>BSA (Bovine Serum Albumin)</td>
<td>Serva</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Applichem</td>
</tr>
<tr>
<td>DABCO</td>
<td>Merck</td>
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<tr>
<td>DAPI</td>
<td>Sigma</td>
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<tr>
<td>DEPC</td>
<td>Sigma</td>
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<td>Applichem</td>
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<td>Applichem</td>
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<td>EGTA</td>
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<td>Ethanol</td>
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<tr>
<td>Ethidium bromide</td>
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<td>Formaldehyde solution 37%</td>
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<td>Gel Code Coomassie stain</td>
<td>Pierce</td>
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<td>Gel Red</td>
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<td>Fermentas</td>
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<td>Heptane</td>
<td>Merck</td>
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<td>Waterbath</td>
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<td>Material</td>
<td>Supplier</td>
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<td>----------------------------------------------</td>
<td>---------------------------</td>
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<td>Hepes</td>
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<td>Insulin</td>
<td>Sigma</td>
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<td>KH$_2$PO$_4$</td>
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<td>KOH</td>
<td>Applichem</td>
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<tr>
<td>LDS Sample Buffer</td>
<td>Invitrogen</td>
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<td>Leupeptin Hemisulfate</td>
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<td>LiCl</td>
<td>Applichem</td>
</tr>
<tr>
<td>Methanol</td>
<td>Applichem</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Applichem</td>
</tr>
<tr>
<td>Milk Powder</td>
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</tr>
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<td>NP-40</td>
<td>Sigma</td>
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<tr>
<td>Orange G</td>
<td>Sigma</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma</td>
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<tr>
<td>Phenol/Chloroform/Isoamylalkohol (25:24:1)</td>
<td>Applichem</td>
</tr>
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<td>Pepstatin</td>
<td>Applichem</td>
</tr>
<tr>
<td>PMSF</td>
<td>Applichem</td>
</tr>
<tr>
<td>Poly(L)lysine</td>
<td>Sigma</td>
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<tr>
<td>Roche Complete protease inhibitors</td>
<td>Roche</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma</td>
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<tr>
<td>Sodium chloride (NaCl)</td>
<td>Applichem</td>
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<tr>
<td>Sodium citrate</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>Sigma</td>
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<tr>
<td>Sodium hydrogenphosphate (Na$_2$HPO$_4$)</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium hypochlorite (NaClO)</td>
<td>Roth</td>
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<td>Sodium phosphate</td>
<td>Applichem</td>
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<td>Applichem</td>
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<tr>
<td>SilverQuest Silver Staining Kit</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SYBR Green 1 Master Mix</td>
<td>Roche</td>
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<td>TEMED</td>
<td>Applichem</td>
</tr>
<tr>
<td>Tris</td>
<td>Applichem</td>
</tr>
</tbody>
</table>
Triton X-100       Merck
TRIzol Reagent   Invitrogen
Tween 20         Sigma
tRNA (Yeast)     Invitrogen
Tween 20         Sigma
Voltalef-10S oil Lehmann & Voss & Co.
Western blot Stripping Buffer Pierce
Yeast (instant)  S.I.Lesaffre
Methods
5. Methods

5.1 Molecular methods

5.1.1 Molecular cloning procedures

5.1.1.1 Polymerase Chain Reaction (PCR)

The template DNA was amplified by PCR using Taq polymerase (Qiagen) or High Fidelity Taq polymerase (Roche) as appropriate. Specific sense and antisense oligonucleotide primers flanking the desired target sequence were used.

A typical PCR reaction was prepared as following:

- 0.1-100ng DNA
- 5µl 10x appropriate reaction buffer
- 2.5µl 10µM sense primer
- 2.5µl 10µM antisense primer
- 4µl 2.5mM dNTP-mix (dATP, dTTP, dGTP, dCTP; Peqlab)
- 1U DNA polymerase
- add to 50µl ddH₂O

For PCR reactions using cDNA library, 20ng were used as a template. The following parameters were adjusted for each PCR reaction, depending on the template, the size of the fragment to be amplified, and the melting temperatures of the primer:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5min</td>
<td>1x</td>
</tr>
<tr>
<td>94°C</td>
<td>15s</td>
<td>1x</td>
</tr>
<tr>
<td>56°C-63°C</td>
<td>30-45s</td>
<td>25-35x</td>
</tr>
<tr>
<td>72°C</td>
<td>0.5-3min</td>
<td>35x</td>
</tr>
<tr>
<td>72°C</td>
<td>10min</td>
<td>1x</td>
</tr>
</tbody>
</table>

5.1.1.2 Analysis of DNA fragments by agarose gel electrophoresis

Depending on the size of the DNA fragments to be separated, 0.3-2% (w/v) agarose gels were used. Ethidium bromide was added to a final concentration of 0.5µg/ml. Samples supplemented with DNA sample buffer were loaded and electrophoresis was conducted at 50-150V for 30min-1hour. After separation, the gel was analyzed by a transilluminator with UV light, photographed, and printed with a gel documentation device. For preparative gels, the fragment of interest was cut out under 70% UV light and the DNA was purified.
**Methods**

**DNA electrophoresis buffers:**
1 x TAE buffer: 40mM Tris-acetate  
1mM EDTA pH8.0  
Ethidium bromide stock: 10mg/ml  
6 x DNA sample buffer: 0.25% (w/v) Bromophenol-blue or Orange G  
30% (w/v) glycerol  
0.1mM EDTA

5.1.1.3 *PCR purification/ gel extraction*

According to the manufacturer’s protocol, the QIAquick PCR purification kit (Qiagen) was used for the purification of DNA following PCR reaction or prior to restriction endonuclease digestion. The DNA was eluted in 30-50µl ddH₂O.

To purify DNA from agarose gels, the QIAquick gel extraction kit (Qiagen) was applied as described in the manufacturer’s manual. The DNA was also eluted in 30-50µl ddH₂O.

5.1.1.4 *Restriction endonuclease digestion of DNA*

For analytical digests, 100-300 ng DNA were digested with 1-10U of restriction enzyme (New England Biolabs) with its appropriate buffer according to the manufacturer’s protocol in a volume of 20µl. Generally, the digests were incubated at 37ºC for 1hour.

For preparative digests, 5-10µg DNA were used with 10-40U of restriction enzyme. Digests were incubated for 3hours at 37ºC. For some reactions, a phosphatase treatment step was necessary to prevent re-ligation of vectors. Afterwards, the digested DNA was purified with the QIAquick PCR purification kit or isolated by agarose gel electrophoresis.

5.1.1.5 *Dephosphorylation of the digested vector*

To prevent re-circularization of a digested vector DNA in a ligation reaction, the DNA was treated with Antarctic Phosphatase (New England Biolabs) to remove the 5’ end phosphate group of the DNA. Phosphatase treatment was typically performed directly following a restriction digestion. For the Antarctic reaction, 1/10 volume of 10 x Antarctic Phosphatase Reaction
Buffer and 5 Units Antarctic Phosphatase were added to 1µg of DNA cut with any restriction endonuclease in any buffer, incubated for 15 min at 37°C for 5’ extensions or blunt-ends and 60 min for 3’ extensions. Afterwards, the reaction was incubated at 65°C for 5min to inactivate the phosphatase and the DNA was isolated by agarose gel electrophoresis or spin column purification using the PCR purification kit.

5.1.1.6 **Ligation of DNA fragments**

Ligation reactions were prepared with 100ng vector DNA and a 3-fold molar excess of insert DNA in 10µl with 1 U T4 DNA ligase (Roche) according to the manufacturer’s instructions. The reaction was incubated at 16°C overnight.

5.1.1.7 **Production of chemical-competent TOP10 and BL21 CodonPlus E.coli cells**

A single colony of TOP10 or BL21 CodonPlus *E.coli* cells was inoculated into 3ml LB medium and incubated overnight at 37°C under vigorous shaking. The next day, 0.5ml of this culture was transferred into 20ml LB medium and incubated overnight at 37°C under vigorous shaking. The third day, 1ml of this culture was transferred into 100ml LB medium and incubated at 37°C until a density of OD600=0.7-0.8 was reached. Afterwards, this culture was separated into two 50ml falcon tubes and the following steps were conducted at 4°C using pre-chilled materials. The culture was incubated on ice for 15min and centrifuged for 15min at 5000rpm. The sedimented cells were resuspended in half volume (25ml for 50ml *E.coli* cells) pre-cold 0.1M CaCl₂ and incubated on ice for 30min. The resuspended cells were centrifuged at 5000rpm for 15min again and resuspended in 1/20 volume pre-cold 0.1M CaCl₂/10% Glycerin. Finally, cells were aliquoted into sterile 1.5ml Eppendorf tubes and shock-frozen in liquid nitrogen. Competent cells were stored at -80°C.

**LB (Luria-Bertani) medium:**

- 5g Bactotryptone
- 2.5g Yeast extract
- 5g NaCl
Add H₂O up to 500ml

5.1.1.8 Transformation of E.coli

Competent cells were thawed on ice. 1µl plasmid DNA or 10µl of a ligation reaction was added to a 100µl cell aliquot and incubated on ice for 30min. Afterwards, cells-DNA mixture was heat shocked at 42°C for 90s and 200µl LB medium was added. Subsequently, transformation mix (cells plus DNA) was incubated for 1 hour at 37°C on a roller shaker. Transformed cells were plated on LB agar plates supplemented with the appropriate antibiotic and incubated overnight at 37°C.

Preparation of LB agar plates:

For agar plates, 1.5% Bacto-Agar was added to the LB medium. After boiling in a microwave, the appropriate antibiotic was supplemented to a final concentration of 50-100µg/ml for Ampicillin, 170µg/ml for Chloramphenicol and 50µg/ml for Kanamycin. When the solution was cooled down to 60-70°C, this LB-agar was poured into Petri-dishes until the thickness of the LB-amp plates were around 0.8cm (500ml=22-25plates).

Antibiotic stocks:
- **Ampicillin stock:** 100mg/ml
- **Chloramphenicol stock:** 34mg/ml
- **Kanamycin stock:** 10mg/ml

5.1.1.9 Isolation of Plasmid DNA from bacteria

For DNA isolation in general, alkaline lysis was applied using the buffers supplied by Qiagen.

Small scale DNA preparation (Miniprep)

A single colony was inoculated into 3 ml LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C under vigorous shaking. A 1.5ml of the overnight culture was transferred into an Eppendorf tube and centrifuged for 3min at 5000rpm (Eppendorf table top centrifuge). The supernatant was discarded and the cell pellet was resuspended in
300µl P1-Buffer. Then 300µl P2-Buffer was added. After gentle mixing, cell lysis reaction was incubated at room temperature for less than 5min. Afterwards, 300µl P3-Buffer was added and after gentle mixing, the tubes were incubated on ice for 10min. The bacterial lysate was centrifuged for 30min at 13,000rpm at 4ºC. The supernatant was transferred into a new tube and the DNA was precipitated by adding 0.7 volume of 100% isopropanol (Applichem). The precipitated DNA was pelleted by centrifugation at 13,000rpm for 30min at 4ºC. The DNA pellet was washed with 500µl 70% Ethanol and centrifuged at 13,000rpm for 10min at 4ºC. The pellet was air dried and resuspended in 20µl Sterile H₂O.

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

Large scale DNA preparation (Midiprep)
The High speed Midi kit from Qiagen was used to isolate larger quantities of pure DNA according to the manufacturer’s protocol. The DNA was eluted in 500µl ddH₂O.

5.1.1.10 pENTR TOPO cloning reaction and Gateway reaction

pENTR TOPO cloning

pENTR TOPO cloning (Invitrogen) allows efficient cloning of High fidelity Taq polymerase-amplified PCR products into the TOPO vector. Usually, the ligation reaction was set up with half the volume, which was recommended:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>1.5ng</td>
</tr>
<tr>
<td>Salt solution (TOPO kit)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>pENTR TOPO vector</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Sterile H₂O</td>
<td>Add to 3µl</td>
</tr>
</tbody>
</table>

The ligation reaction was incubated at RT for 30min and 3µl of the ligation product was added to an aliquot of “One Shot Top10 chemically competent E.coli” (Invitrogen) cells followed by gently mixing by tapping. After 10-20
min incubation on ice, the cells were transformed by a 30 sec heat-shock in a 42°C water bath without shaking. The transformed cells were immediately transferred onto ice. A 250µl room temperature S.O.C medium was added and the reaction tubes were incubated on a horizontal shaker (200rpm) at 37°C for 1 hour. Finally 50µl and 200µl aliquots from each transformation were spread onto prewarmed LB agar plates containing the appropriate antibiotic and incubated at 37°C overnight.

S.O.B medium:  
2% (w/v) Bacto-tryptone  
0.5% (w/v) yeast extract  
0.05% (w/v) NaCl  
2.5mM KCl  
10mM MgCl₂  
pH7.5

S.O.C medium  
S.O.B medium  
20mM glucose (sterile)

**Gateway LR in vitro recombination**

Gateway cloning technique (Invitrogen) allows transfer of DNA fragments between different cloning vectors while maintaining the reading frame. It has effectively replaced the use of restriction endonucleases and ligases. The ORF, which is cloned in pENTR TOPO vector, can be transferred to any destination vectors. The LR recombination reaction was set up at room temperature as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry clone 50ng/reaction</td>
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</tr>
<tr>
<td>Destination vector 75ng/reaction</td>
<td>1µl</td>
</tr>
<tr>
<td>5 x LR Clonase Reaction Buffer</td>
<td>1µl</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>1µl</td>
</tr>
</tbody>
</table>

The LR clonase enzyme mix was moved out from -80°C, thawed on ice for about 2minutes and vortexed briefly twice. Then 1 µl of LR clonase enzyme mix was added to each reaction and vortexed briefly twice. The reactions were incubated at 25°C for 1 hour and 0.5µl of the Proteinase K solution was added into each reaction, incubated at 37°C for 10 minutes. 1µl LR
recombination reaction was added into 100µl TOP10 competent cell and mixed gently. The reactions were incubated on ice for 30min and the cells were transformed by a 30 sec heat shock in a 42°C water bath. Afterwards, the cells were put on ice immediately followed by addition of 450µl room temperature S.O.C into each reaction. The transformation mix was incubated at 37°C for 1 hour on a shaker (200rpm). Finally 20µl and 100µl aliquots from each transformation were spread onto prewarmed LB agar plates containing the appropriate antibiotic for selection and incubated at 37°C overnight.

5.1.2 RNA purification and quantification

5.1.2.1 RNA isolation and purification from cultured cells

For isolation of RNA from *Drosophila* tissue culture cells, 3 ml of a well-grown culture (5x10^5- 1x10^7 cells /ml) were collected in 1.5 ml reaction tubes and settled during a 3 min spin at 1500 rpm on a desktop centrifuge. After adding 1000 µl Trizol the cells were quickly homogenized by repeated pipetting. A 10 min spin at full speed on a desktop centrifuge in the cold was used to pellet bulk genomic DNA and tissue fragments and the supernatant was further incubated for 5 min at RT. The supernatant was mixed with 200 µl chloroform by vortexing for 15 sec, followed by an incubation of 3 min at RT. After a 15 min full speed spin at 4°C, the upper aqueous phase was transferred to new tube and supplemented with 2 µl glycogen (5 mg/ml) for very small RNA quantities. An equal volume of 500 µl ice-cold isopropanol was used to precipitate the RNA by 10 min incubation at room temperature. The precipitate was settled by a 10 min full-speed spin at 4°C and washed twice with 1 ml of ice-cold 75% ethanol. The pellet was air-dried for 5 min or until the color of the pellet changed from whitish to clear. RNA was dissolved in 25 µl of nuclease free water by pipetting.
5.1.2.2 RNA Isolation from adult flies

For isolation of total RNA from adult *Drosophila*, 100mg flies were collected into a 15ml falcon tube. For each 50mg of flies, 1ml TRIzol Reagent was added and the flies were homogenized mechanically using a Polytron Ultra Turrax homogenizer, generally 60-90s is enough. (The lysate can be stored in -80°C.) The lysate was transferred into 1.5ml Eppendorf tubes, incubated at room temperature (RT) for 5min and centrifuged at 4°C for 10min at 13,000rpm. Supernatant was transferred into new tubes and incubated at RT for 5min. Afterwards, for each 1ml of Trizol, 0.2ml chloroform was added followed by vigorous mixing for 15s by vortex. This mix was incubated at RT for 2-3min followed by centrifugation at 13,000rpm at 4°C for 15min. The aqueous supernatant containing the RNA was transferred into a new tube. RNA was precipitated by adding 1µl 35mg/ml glycogen and 0.5ml Isopropanol for each 1ml of Trizol used and incubating at RT for 10min. Finally the RNA was precipitated by centrifugation at 13,000rpm at 4°C for 10min. The RNA pellet was washed twice with 75% EtOH (1ml EtOH for 1ml Trizol) and between the washing steps, the samples were centrifuged at 9000rpm 4°C for 5min. The RNA pellets were air dried for about 1min and excess liquid was removed using a pipet tip. RNA was resuspended with 25μl DEPC-treated ddH₂O and the concentration was measured by OD260. Subsequently, RNA was flash-frozen in liquid nitrogen and stored at -80°C.

5.1.2.3 RNA isolation and purification from ovary, testes or fly heads

25 pairs of ovaries or testes, or 50 fly heads were collected in 50µl of TRIZOL reagent, after homogenized with a pestle, another 750µl of TRIZOL reagent was added. The sample can be stored in -80°C or proceeded according to method 5.1.2.2

5.1.2.4 DNase treatment of purified RNA

To get rid of DNA contamination from RNA preparations, the samples were treated with TURBO DNase kit (Ambion). Up to 20µg of total RNA was digested using the DNA-free™ kit (Ambion) according to the manufacturer’s
instructions. DNase-treated RNA samples were flash-frozen in liquid nitrogen and stored until use at -80ºC.

**5.1.2.5 Reverse transcription PCR (RT-PCR)**

c**DNA synthesis**
The cDNA was synthesized using the Super Script III™ first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Briefly, the following reactions were set up first in 1.5ml eppendorf tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA mix: 2μg total DNase treated RNA</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>2μl Primer oligo dT</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>2μl 10mM dNTP mix (dATP, dTTP, dCTP, dGTP; Peqlab)</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>add DEPC-treated ddH2O to 20μl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The samples were denatured at 65ºC for 5min followed by quickly placing on ice for at least 1min. The cDNA mix was divided into 2 equal parts into 250μl PCR tubes to make RT plus and RT minus reactions as follows:

The cDNA synthesis reaction was carried out in a thermocycler using the following conditions:

- 50min 55ºC
- 5min 85ºC

Finally, 1μl *E.coli* RNase H (2U/μl) was added and the samples were incubated for 20min at 37ºC to remove RNA templates. The synthesized cDNA samples were stored at -20ºC.
5.1.2.6 Semi-quantitative PCR
To semi-quantify the transcription level of certain genes, different amount of cDNAs were used as templates in the polymerase chain reaction. In this work, 5μl, 2.5μl and 1μl of 1:5 diluted cDNA were used.

5.1.2.7 Quantitative PCR

For quantitative real-time PCR measurements, the DNA purified from ChIP experiment was diluted 1:20; the cDNA input was freshly diluted 1:100. For each of the 10 μl qPCR reactions in a 96 well plate format 4μl of template DNA, 5μl of 2x Lightcycler 480 SYBR Green I master mix and 1μl of 5 μM primer mix (final concentration in reaction: 0.5 μM for each primer) were combined into a single well.

Thermocycle as follows: target temp. hold ramp rate
1x 95°C 10 min 4.4°C/s
45x 95°C 10 sec 4.4°C/s
60°C 10 sec 2.2°C/s
72°C 10 sec 4.4°C/s
1x 95°C 5 sec 4.4°C/s
65°C 1 min 2.2°C/s
97°C 5 sec 0.11°C/s
1x 40°C 30 sec 1.5°C/s

The TM calling function of the Lightcycler 480 software (Release 1.5.0 SP3) was used to analyze integrity of the PCR reaction and experiments with more than one product were dismissed. Further, the absolute Quant/2nd-derivative function was employed to calculate crossing points and used in relative quantification.

5.1.3 Chromatin Immunoprecipitations

Chromatin fixation and immunoprecipitation were performed essentially as described in (Orlando et al., 1997). S2-DRSC tissue culture cells (5x10^8) were fixed in 100 ml medium with 1% formaldehyde for 10 min at room
temperature. Cross-linked cells were sonicated to produce chromatin fragments of an average size of 200–400 bp. Soluble chromatin was separated from insoluble material by centrifugation. The supernatant containing chromatin of 5x10^7 cells was used for immunoprecipitation by anti-FKBP39. All ChIPs were quantified by using a Light cycler 480 real-time PCR systems (Roche) and SYBR Green was used to monitor the accumulation of double-stranded DNA in the PCR mixtures at the end of each cycle to produce amplification profiles. Finally, percentage inputs in each ChIP reaction were calculated as described (Dellino et al., 2004).

5.1.4 Preparation of ChIP-seq and mRNA-seq libraries

Sequencing libraries were prepared with the Illumina mRNA-Seq 8-Sample Prep Kit (Cat# RS-100-0801) and ChIP-Seq DNA Sample Prep Kit (Cat# IP-102-1001) according to Illumina's instructions. After adapter ligation library fragments of ~250 bp were isolated from the agarose gel. The DNA was PCR amplified with Illumina primers for 15 (RNA-seq) and 18 (ChIP-seq) cycles, purified and loaded on an Illumina flow cell for cluster generation. Libraries were sequenced on the Genome Analyzer II and Genome Analyzer IIx following the manufacturer's protocols.

5.2 Drosophila handling and genetic methods

5.2.1 Drosophila handling

Fly stocks were raised on standard fly food 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:

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

5.2.2 Preparation of acetic acid agar plates for embryo collection

22.5g Agar in 1L dH₂O was boiled in a microwave and allowed to cool down to approximately 60°C. Subsequently, 25g sucrose and 3.5ml 100% Acetic acid were added before the mixture was poured into 9cm diameter Petri dishes. After solidification, acetic agar plates were stored at 4 ºC. For embryo collection, adult flies were transferred into medium-size (9 cm diameter, 12 cm height) cages, which were closed at the bottom with acetic acid agar plates. Fresh yeast paste was added to feed the flies.

5.2.3 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 to be injected were prepared as follows:

Plasmid DNA from midiprep was repurified with PCR purification kit (Qiagen) and eluted in ddH₂O. A 9µg of plasmid DNA together with 3µg of pUCΔ2-3 helper DNA encoding the P transposase were mixed in 30µl injection buffer. Prior to injection, the DNA mixture was centrifuged at 4°C for 30min at 13,000rpm (Eppendorf table top centrifuge) to pellet any dust particles or undissolved material. Finally, 3µl of the supernatant were loaded into Femtotip needle (Eppendorf). The injection needle was submerged in oil to avoid clogging. Adult 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 30min at 25°C. Using a brush and tap water, embryos were transferred into a sieve and washed extensively. After dechorionation by 1-3min incubation in 3% bleach and extensive washing in tap water, the floating 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 and an Eppendorf FemtoJet Microinjector at 200-1500 hPa injection pressure. In general, about 300-400 embryos were injected for every construct. After injections, embryos were allowed to develop in oil in a humid chamber at 18°C for 2 days. Importantly, only embryos in which the pole cells were not yet visible were injected, all embryos older than stage 2 were discarded. Finally with the help of a brush, freshly hatched first instar larvae were transferred into a small vial containing standard fly food and were kept at 25°C until the founder G₀ generation hatched.

1x Injection buffer: 5mM KCl
0.1mM phosphate buffer (pH6.8)

0.1M Sodium phosphate buffer, pH6.8:
46.3µl 1M Na₂HPO₄
53.7µl 1M NaH₂PO₄
Add ddH₂O to total 1 ml.

Bleach: 3% Na-hypochlorite (Roth) in PBS

PBS: 137mM NaCl
2.7mM KCl
10mM Na₂HPO₄
2mM KH₂PO₄
pH 7.4

5.2.4 Establishing transgenic lines and mapping of the integration site

The hatched founder G₀ generation flies were crossed to yw virgins or males (every 2 G₀ males were crossed to 10 yw virgins and every 4 G₀ females were crossed to 3 yw males) and progenies were then screened for the transformation marker miniwhite or yellow. The F1 generation
transformants were then crossed to *yw; Bcg/CyO* flies to map the chromosome with transgene. Afterwards, the P/CyO male flies were collected and single male was back-crossed to *yw; Bcg/CyO* to map the chromosome on which transgene is inserted:

<table>
<thead>
<tr>
<th>p-element inserted flies</th>
<th>without p-element</th>
</tr>
</thead>
<tbody>
<tr>
<td>X chromosome</td>
<td>only female flies contain p-element transformation marker</td>
</tr>
<tr>
<td>2nd chromosome</td>
<td><em>yw; P/Bc Gla</em></td>
</tr>
<tr>
<td></td>
<td><em>yw; P/CyO</em></td>
</tr>
<tr>
<td>3rd chromosome</td>
<td><em>yw; Bc Gla/+; P/+</em></td>
</tr>
<tr>
<td></td>
<td><em>yw; CyO/+; P/+</em></td>
</tr>
<tr>
<td></td>
<td><em>yw; Bc Gla/CyO; P/+</em></td>
</tr>
</tbody>
</table>

After mapping the transgene on a specific chromosome, homozygous fly lines were established. Heterozygous flies were first crossed to appropriate Balancer flies and homozygous flies then identified by the absence of the dominant marker of the respective Balancer.

**5.2.5 Generation of *fkbp39* null mutant from P-element mutant by imprecise excision**

Female *fkbp39*5-HA-2440 homozygous mutants (*w+*) were crossed to male flies with a transposase Δ 2-3, flies were growing at 25°C, the optimum temperature for Δ2-3 activity. The recombination is to occur especially in the generation of germline. To remove the transposase, the F1 generation *fkbp39*5-HA-2440/Δ2-3 *TM3Sb* male flies were crossed to *TM3Sb/Dr* female flies, the *w-* recombinants with imprecise excision among the progeny were screened by single fly PCR.

**Single Fly PCR**

Single Fly PCRs were routinely performed to check if transgenic inserts for driver, ligase and fusion proteins were inherited in large scale fly cultures. Single flies were squashed in 50μl Gloor and Engel's extraction buffer (Gloor et al, 1993) in a 1.5ml Eppendorf tube using a 200μl pipette tip. The homogenate was incubated for 20 min at 37°C, and then for 2 min at 80°C in order to inactivate proteinase K. DNA was stored at 4°C. Typically, 4μl of
DNA isolated from whole flies, or 5μl 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)

5.2.6 Genetic interaction experiments

The Pc alleles (Pc3, PcxT109) were crossed with fkbp395-HA-2440 and fkbp391 mutant at 25°C, males in the progeny were scored for extra sex combs. On the basis of strength of phenotype (number of bristles on second and third legs of males), flies were categorized as follows: 0, no extra sex combs; +, 1–2 bristles on the second leg; ++, 3 or more bristles on the second leg; ++++, 3 or more bristles on the second leg and 1–2 bristles on the third leg; and +++++, 3 or more bristles on both the second and the third pairs of legs. The cuticles of lethal embryos from homozygous Pc,fkbp395-HA-2440 mutants and Pc mutants were prepared according to the standard protocol (Alexandre, 2008), the segmental pattern of ventral epidermis was analyzed. To obtain Pc,fkbp395-HA-2440 homozygous mutant, fkbp395-HA-2440 homozygous mutants (w+) were crossed to Pc alleles, the virgin flies of Pc/ fkbp395-HA-2440 were selected out (recombination only happens in female flies) and crossed to w; TM3Sb/TM6Tb male flies. In the progeny, the flies with red eyes were collected and crossed back to Pc alleles, the ones couldn’t generate progeny without Sb phenotype are the flies contain Pc and fkbp395-HA-2440 mutant on the same chromosome.

5.3 Drosophila histological methods

5.3.1 Cuticle preparation of lethal embryos

Embryos were collected on acetic acid agar plates at 12 hours intervals and developed an additional 24 hours at 25°C. (For WT embryos, collect at 4
hours intervals and developed an additional 18 hours). The fresh yeast with the larvae was removed and the plate was rinsed with PBS. The embryos expressed GFP (heterozygous mutant embryos) were picked out by hand. The non-GFP embryos (homozygous mutant embryos) were left on the plate. Afterward, the embryos were washed through a 600µm filter into a 100µm Nylon mesh and dechorionated with 3% NaClO for 3 min. These embryos need to be washed extensively with water and with 0.7% NaCl/0.03% Triton-X 100. The embryos were transferred into an Eppendorf tube which containing 700µl Methanol and 700µl Heptane, after shaking intensively for 5 min, the vitelline membrane of embryo was removed by osmotic shock. And then the embryos were shortly washed 3 times in Methanol and transferred onto slides with small volume of liquid. The Methanol should be removed as much as possible and 400µl Hoyer's/lactic acid 1:1 was added. The slides were mounted and put in the 58°C oven for 24 hours. The remaining cuticles were flattened for 12 hours with 2 pieces of 35g metal weights at room temperature.

**Hoyer's medium**

30g gum Arabic to 50ml distilled water, stir overnight until completely dissolved.
Under continuous stirring add:
200g chloral hydrate slowly in small quantities to avoid clumping.
20g Glycerol and mix well.
Centrifuge the mixture until the mountant is clear (overnight in Corex tubes at 12'000g). Store Hoyer's at room temperature for centuries. It is also possible to premix with lactic acid, spin down and store in 1ml aliquotes for several month.

**5.3.2 Immunostaining of polytene chromosomes**

**Chromosome squashes**

Larvae were grown on standard Drosophila medium supplemented with yeast paste. Female wandering third instar larvae were collected in PBS on ice (generally, the salivary glands of females are bigger than those of males). Two pairs of salivary glands were dissected in PBS, and fat body cells were removed as much as possible. Using forceps, the two pairs of salivary
glands were transferred into a drop (approximately 40μl) of fixation buffer on a poly (L) lysine coated slide and covered with 18mm x 18mm coverslip. After fixation time of 10-20min, the salivary glands were squashed by tapping a sharp pencil with constant moderate force onto the coverslip without holding the coverslip (1-2mm). 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 microscope. Using a diamond pencil, the position of the coverslip was marked on the slide before it was flash-frozen in liquid nitrogen. Using a razorblade, the coverslip was removed and the slide was then stored in PBS on ice (for storage up to one week, slides were washed 2x 15min in PBS by slowly shaking and transferred into a jar containing 100% methanol at 4°C).

PBS ( pH 7.4 ):

<table>
<thead>
<tr>
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<th>IL</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>10 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2 mM</td>
</tr>
</tbody>
</table>

The PBS was autoclaved before use.

37% PFA stock:
1.85g PFA was weighed into a final volume of 5ml PBS. The powder was dissolved by heating in a 60-65°C water bath. 100μl aliquots were stored at -20°C. Before use, aliquots were thawed by heating at 95°C for 5 minutes. If precipitates formed, the solution was discarded.

Fixation buffer:
500μl ddH₂O
450μl 100% acetic acid
50μl 37% PFA
This solution was always prepared fresh, as it is stable for 2hours when kept on ice.

**Immunostaining**

Stored slides were washed twice for 10min in PBS on a shaker, and then blocked for 1hour in blocking solution with vigorous shaking. Primary antibodies (rabbit anti-PC 1:20; mouse anti-GFP1:50) were diluted in PBS/1%BSA and 40μl were pipetted onto a slide. The slides were carefully covered with a 20mm x 46mm coverslip avoiding air bubbles. The slides
were incubated in a humid chamber for 1 hour at RT and then overnight at 4°C. The coverslips were removed by immersing the slides in PBS and placed in a Coplin Jar and washed for 3 x 5 min in PBS/5% non-fat milk. Secondary antibodies (preabsorbed goat anti-rabbit-Alexa488 1:200, preabsorbed goat anti-mouse-Cy3 1:200 or preabsorbed goat anti-mouse-Alexa488 1:200) were diluted in PBS/2% goat serum and 40μl were pipetted onto a slide. The slides with secondary antibodies were covered carefully with a 20mm x 46mm coverslip avoiding coverslips. The slides were incubated in a humid chamber for 1 hour at RT. The coverslips were removed by immersing the slides in PBS, followed by washing with Wash buffers 1, 2 and 3 for 15 min each wash in a Coplin Jar with rocking (200rpm). Finally the slides were rinsed once in PBS.

**Blocking solution:**
- PBS
- 3% (w/v) BSA
- 10% (w/v) non-fat dry milk powder
- 0.2% (v/v) NP-40
- 0.2% (v/v) Tween-20
- prepare 1 L, store 100 ml aliquots at -20°C

**Wash 1 buffer:**
- PBS/300 mM NaCl
- 0.2% (v/v) NP-40
- 0.2% (v/v) Tween-20

**Wash 2 buffer:**
- PBS/400 mM NaCl
- 0.2% (v/v) NP-40
- 0.2% (v/v) Tween-20

**Wash 3 buffer:**
- PBS/500 mM NaCl
- 0.2% (v/v) NP-40
- 0.2% (v/v) Tween-20

To counterstain the DNA, the slides were incubated for 10 min in 100 ng/ml DAPI / PBS, followed by a 5 min wash in PBS. The slides were mounted with 40μl Mowiol or “Fluoromount-G” and examined under an epifluorescence microscope. (The slides also could be mounted with 40μl Mowiol/ 100 ng/ml DAPI directly without staining with DAPI/PBS.)

**Preparation of Mowiol Mounting Medium**
To prepare Mowiol Mounting Medium, following components were mixed:
Mowiol 4-88  2.4g  
Glycerol  6g  
ddH₂O   6ml  
The mixture was mixed for 3 hours at RT followed by addition of 12ml 0.2M Tris-HCl pH8.5 and incubation at 50-53°C until the Mowiol was dissolved. During incubation, the mixture was stirred occasionally. To clarify the dissolved Mowiol, it was centrifuged at 4000-5000rpm for 15-20min. Supernatant was transferred into new 50ml falcon tube and DABCO (Diazabicyclo (2.2.2) octane Merck #803456) was added to final 2.5% as antibleaching agent. Mowiol was aliquoted into 500μl and stored at -20°C. The solution is stable at this temperature for up to 12 months. Once defrosted, it can be stored at RT and can be used for several days before solidification.

5.3.3 Immunostaining of *Drosophila* ovaries

2-3 days old female flies were feed with fresh yeast to fatten up the ovaries for 2-4 days. Ovaries were dissected in PBS and the ovarioles should be teased apart. Ovaries were kept in 100μl ice-cold PBS while dissecting the next fly. Ovaries were fixed with 400μl 4% paraformaldehyde (PFA)/PBS/0.1%Tween20 for 20min. After rinse twice with 1ml PBST, the ovaries were incubate in 1ml PBST for 30min in room temperature with gently rock. Follow twice rinse with 1ml PBST the egg chambers were blocked in PBST/BSA for 1 hour with gently rock. Afterward, the egg chambers were rinsed 5 times with PBST/BSA and incubate with 100μl primary antibody, which diluted in PBST/BSA overnight at 4 °C and 1 hour at room temperature next day. After washing with 1ml PBST/BSA 5x 5min on a rotating platform, the egg chambers were incubated in 100μl fluorescently labeled secondary antibody, which diluted in PBST/BSA and 0.1μg/ml DAPI for 2 hours at room temperature in dark. Egg chambers were washed 5 x 5min with 1ml PBST on a rotating platform, rinsed 3 times with 1ml PBS and washed twice with 1ml PBS for 5min on a rotating platform (to dilute Triton X-100, Triton is a fluorescence quencher). Most of PBS was removed and the egg chambers were transferred onto a slide with a cut tip, the rest PBS
was removed from the slide and 40µl “Fluoromount-G” was used to mount the slide. The slides were stored at 4 °C in dark.

PBST: 1xPBS+0.3%Triton-X100

PBST/BSA: 1%BSA in PBST

5.3.4 Immunostaining of *Drosophila* testes

*Drosophila* testes were dissected in TB from 1 day old male flies. 4-5 pairs testes were transferred into drops of TB (total 40µl) on a clean, non-siliconized 22x22mm coverslip. A clean poly-L-Lysine covered slide was placed over the cover slip without pressing and the “sandwich” was inverted. If desired, a very mild squashing can be obtained by removing excessive buffer from the edges of the coverslip with a piece of blotting paper. To fix the testes, the slide was frozen in liquid nitrogen and the coverslip was removed with a razor blade. The slide was immersed in to methanol immediately at -20 °C and left for 5min. Afterwards, the slide was transferred into methanol/acetone 1:1 and then into acetone at -20 °C for 5min. After washes with PBT 3x5min at room temperature, the testes were blocked in PBT/BSA for 45min at room temperature. The testes were incubated with 20µl primary antibody, which were diluted in PBT/BSA, overnight at 4 °C and 1 hour at room temperature the next day. The slides were washed 2x10min in PBT and 1x5min in PBS. Testes were incubated with 20µl secondary antibody, which was diluted in PBS, for 2 hours in a dark and humid chamber. The slides were washed 2x10min with PBS and stained with 30µl 0.2µg/ml DAPI/PBS for 25min. Finally, the slides were rinsed for 5min in PBS and mounted with 40µl “Fluoromount-G”. The slides were stored at 4 °C in dark.

**Testis Buffer (TB)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount to make 100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>183mM KCl</td>
<td>1.364g</td>
</tr>
<tr>
<td>47mM NaCl</td>
<td>0.274g</td>
</tr>
<tr>
<td>10mM Tris-HCl</td>
<td>0.121g</td>
</tr>
<tr>
<td>1mM EDTA</td>
<td>0.037g</td>
</tr>
<tr>
<td>1mM PMSF</td>
<td>add before using</td>
</tr>
</tbody>
</table>

Dissolve in 80ml ddH$_2$O, adjust to pH6.8 and bring to final volume of 100ml with ddH$_2$O. Sterilize by filtration.
**Methods**

PBT: 1xPBS+0.1% TritonX-100

PBT/BSA: PBT+1% BSA

### 5.3.5 Immunostaining of *Drosophila* salivary glands

Salivary glands were dissected in PBS, for each sample 5-6 pairs were prepared. Salivary glands were fixed in 400µl 4% paraformaldehyde (PFA)/PBS for 25min. After rinse twice and wash 1x5min with 1ml PBS, the salivary glands were blocked in 1ml PBS/0.3% TritonX-100/5%BSA for 3x10min in room temperature with gently rock. Salivary glands were incubated in 100µl primary antibody, which was diluted in PBS/0.1% TritonX-100/1%BSA, overnight at 4 °C. After rinsing twice with 1ml PBS/0.1% TritonX-100, salivary glands were incubated in PBS/0.1% TritonX-100/1%BSA for 3x15min. Afterwards, the salivary glands were incubated in 100µl fluorescently labeled secondary antibody, which was diluted in PBS/0.1% TritonX-100/1%BSA/2% goat serum and 0.1µg/ml DAPI, for 2 hours at room temperature in the dark. Salivary glands were rinsed once in PBS/0.1% TritonX-100 and washed 3 x 15min with 1ml PBS/0.1% TritonX-100 on a rotating platform. After rinsing once with 1ml PBS, salivary glands were transferred onto a slide with a cut tip, the rest PBS was removed from the slide and 40µl “Fluoromount-G” was used to mount the slide. The slides were stored at 4 °C in the dark.

### 5.3.6 DAPI staining of *Drosophila* embryos

**Collection and fixation of embryos**

Embryos of desired age were collected (to detect fertilization, 0-5 hours old embryos were collected) from apple juice agar plates and washed with tap water to remove yeast. The embryos were dechorionated in 3% Na-Hypochlorite (Bleach) for 2.5min at room temperature (or under the microscope). The embryos were washed extensively with tap water to remove traces of bleach and were transferred into 2ml eppendorf tube which containing 0.6ml n-Heptane. 0.2ml 4%PFA/PBS was added into the tube
and the embryos were fixed for 20min at room temperature on shaker. To remove vitelline membrane, 0.7ml methanol was added and the tube was vortexed for 1min. The devitellinized embryos will sink to the bottom. Therefore, liquid and unsinkable embryos could be removed. The devitellinized embryos were washed 3x in 1ml methanol, they can be stored in methanol at -20°C for several months.

**Rehydration of embryos**
Embryos were taken out from -20°C and warmed up to room temperature. Embryos were rehydrated by washing 5min in 3:1 methanol: PBT and 5min in 1:3 methanol: PBT on rotator at room temperature. After rinsed with 1ml PBT twice, embryos were incubated in 1ml PBT for 30min at room temperature on a rotator.

**DAPI staining**
Allow the rehydrated embryos to settle to the bottom of the tube and PBT was removed as much as possible. To stain the nuclei of embryos, embryos were incubated for 1min at room temperature in 0.1μg/ml DAPI/PBT (1ml) on a rotator. After rinsed once with 1ml PBT, embryos were washed with 1ml PBT for 10min at room temperature on a rotator.

**Mounting the embryos**
PBT should be removed as much as possible, embryos were transferred onto a glass microscope slide with a yellow tip cut at an angle, the rest PBT was removed from the glass slide, add 40μl of “Fluoromount-G” was added onto the embryos (for 22x22mm coverslip), the embryos were distributed a little bit in the mounting solution and a coverslip was placed over the embryos. Slides were stored at 4°C in the dark.

**PBT**: 1xPBS+0.1%TritonX-100

**5.4 Cell culture methods**

**5.4.1 Cultivation of S2 and Kc cells**
S2-DRSC cells (obtained from the Drosophila Genomics Resource Center) were cultured in Schneider’s *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Hyclone) according to modENCODE standards.

Kc167 cells were cultivated in 50ml cell culture flasks (CellStar) with 5ml growth medium. At 80-90% confluence, the semi-adherent Kc cells detach and proliferate in suspension. Cells were resuspended and 1:10 diluted in fresh medium approximately every 4 days. Cells were cultivated at 25°C under normal atmospheric pressure.

Culture medium: Schneider’s medium (GibcoBRL)
1% Penicillin/ Streptomycin
10% fetal calf serum (FCS)

### 5.4.2 Freezing and thawing of tissue culture cells

Confluent cell cultures were transferred into 15 ml Falcons, settled at 300 g for 5 min and resuspended in 5 ml in Schneider’s medium with 20% FCS and 10% (v/v) DMSO. 500µl aliquots in Cryotubes were stored over night at -80°C and subsequently transferred into a liquid nitrogen tank. For re-establishing cell lines, 500µl aliquots were quickly thawed using hand temperature and heavy flicking. The whole aliquot was transferred into 10 ml growth medium and passaged 1:10 after 7 days.

### 5.4.3 Transient transfection in Kc cells

One day before transfection, 50-100µl of well-grown cell suspension and 1ml culture medium were added into each well for 12- well plates. The next day, all old medium was removed and 800µl fresh culture medium was added into each well 2-3 hours before transfection. Afterwards, the transfection reaction was set up as follows:

Per well: 1µg DNA (incase of single plasmid, 0.5µg each incase of 2 plasmids or co-transfection)
100µl of effectene buffer
The reaction was vortexed for 1s and incubated at RT for 5 minutes. Next, 12.5μl of effectene (QIAGEN) were added and mixed by pipetting up and down 5 times. The reaction was incubated at RT for 10 minutes and 290μl fresh culture medium was added into this DNA effectene mix. Finally, the transfection reaction was added drop-wise to the well and assay was performed 3-5 days after transfection.

5.4.4. dsRNA treatment of tissue culture cells

To generate template PCR product for *in vitro* transcription of dsRNAs, primers containing the T7 promoter sequence were used to amplify a fragment from genomic DNA or the plasmid containing the CDS of the target gene. The fragment was analyzed by gel electrophoresis and the purified 5 μg of PCR product was used in a 100 μl MEGAscript ® T7 reaction, incubated over night at 37°C and digested with TURBO DNase. Finally, the RNA was purified with RNeasy Minelute cleanup Kit (Qiagen) and resuspended in 100 μl dH2O. Confluent S2 cells, passaged not more than 15 times in regular intervals, were centrifuged in a 15 ml Falcon tube at 1000 rpm for 5 min. The cells were gently resuspended into serum-free medium at a concentration of 1.5 x 10^6 per ml. For 6-well plate, in each well, 30μg of dsRNA per target were added after seeding 1ml cells per well (6-well). After serum starvation for 45-60 minutes, 3ml of serum containing medium were added and RNA was isolated after five days of incubation.

5.5 Protein analysis methods

5.5.1 Preparation of 2x SDS loading buffer, Coomassie staining buffer and SDS-PAGE

2x SDS loading buffer:
120mM Tris-Cl (pH 6.8)
10% Glycerol
4% SDS
4% β-mercaptoethanol
Bromphenolblue
Aliquoted in 2ml Eppendorf tubes and stored in -20ºC.
900μl 2x SDS loading buffer was mixed with 100μl 1M DTT before using.

**Coomassie staining buffer: (500ml)**
1.25g Coomassie Brilliant Blue R250 was weighted and dissolved in methanol 225ml
   Acetic acid 50ml
   ddH₂O 225ml

**Preparation of SDS-PAGE:**
To prepare SDS-PAGE, the solution was prepared as follow:
For 15% Resolving gel: (50ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>11.5ml</td>
</tr>
<tr>
<td>30% acrylamide mix (Rotiphorese gel 30% (Roth)</td>
<td>25ml</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>12.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.5ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02ml</td>
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</table>

The solution was poured into the Gel preparation apparatus and isopropanol was added on the top to replenish the resolving gel. Afterwards, the stacking gel was prepared:

5% Stacking gel (30ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
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</tr>
<tr>
<td>30% acrylamide mix (Rotiphorese gel 30% (Roth)</td>
<td>5.1ml</td>
</tr>
<tr>
<td>1M Tris (pH 6.8)</td>
<td>3.75ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>300μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>300μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>30μl</td>
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</tbody>
</table>

After the resolving gel polymerized, isopropanol was removed and the resolving gel was washed with dH₂O and excess H₂O was removed by inverting the gel on a tissue paper. Finally the stacking gel was poured followed by quickly placing the comb before it polymerizes. After polymerization, gels were stored in 4ºC with wet tissue packed.

### 5.5.2 Precast SDS gradient gels and western blot
For the electrophoresis of the SDS gradient gels, special SDS-running buffer was prepared according to manufacturers instructions. A 50ml of 20x MES buffer (for Bis-Tris Gels) or 20x Tris-Acetate SDS running buffer (for Tris-
Acetate gels) (Invitrogen) was added in 950ml ddH₂O. In 200ml of 1x SDS running buffer, 500μl of NuPAGE Antioxident was added and this buffer was added into upper buffer chamber. The lower buffer chamber was filled with 600ml of 1x SDS running buffer.

A maximum of 30μl of each sample was loaded in SDS gels while only 8 μl of broad range prestained marker were used. The gels were run on recommended voltage (200V 35min for Bis-Tris 4-12% Gels, 150V 1hour for Tris-Acetate 3-8% Gels.

**Western blot**

The SDS gel was equilibrated in transfer buffer for 10-20minutes. In the mean time, 6 Whatman filters as well as a piece of ECL nylon membrane were cut into certain size (according to size of the gel to be transferred and immersed in ddH₂O for 5 minutes. Next, the Whatman filters and the nylon membrane were immersed into transfer buffer. While setting up a Western blot, air bubbles were avoided and the direction of cathode core and anode core were taken care of. The chamber was filled with transfer buffer and transfer was carried out in cold room at 200mA for 2hours. Afterwards, the membrane was stained by Ponceau S and destained with PBS. Before incubating with the primary antibody which was diluted in 5% milk in PBS/0.1% Tween20 at 4ºC overnight, the membrane was blocked in 5% milk in PBS/0.1% Tween20 for 1-2hours. The next day, the membrane was washed 3x 10min with PBS/ 0.1% Tween 20 and incubated with secondary antibody for 1hour at room temperature. After being washed 3 x 10min in PBS/0.1% Tween20, the membrane was rinsed in PBS and signal was detected with ECL kit (Amersham).

10x Transfer buffer: 144g Glycin  
30.3g Tris  
add ddH₂O to 1L

1x Transfer buffer: 1/10 10x Transfer buffer  
1/10 methanol
5.5.3 Protein isolation from flies and tissue culture cells

Extracts from adult fly heads were used to detect specific proteins on western blot. Adult flies (10 flies) for each fly line were put in an Eppendorf tubes and were flash frozen. The frozen flies were shaken vigorously and strongly to separate heads from rest of bodies. The flies were transferred into a Petri-dish and heads were collected into 10μl PBS containing 1μl Benzonase (DNase) in an eppendorf tube. The heads were homogenized with a pestle. For tissue culture cells, 0.5ml well-grown cells are enough for the protein isolation. A 30μl 2x SDS loading buffer was added for every 10 heads and samples were boiled at 95°C for 10min. Samples were transferred on ice immediately after boiling and stored at -20°C. Before loading on SDS-PAGE, samples were centrifuged at 13,000rpm for 5min.

5.5.4 Purification of His-tagged Protein

An overnight culture of BL21 codon containing specific plasmid was diluted (1:100) in 1 liter LB medium+Ampicilin (Amp) and incubated at 37°C with vigorous shaking until OD600=0.6-0.8. The expression of the fusion proteins was induced with 1mM IPTG and the culture was incubated for another 3hours at 37°C. Finally the bacteria were centrifuged at 6000rpm with JA-10 rotor for 15min and the pellet was frozen in -80°C or resuspended in lysis buffer (2-5ml per gram wet weight). Afterwards, lysozyme was added to 1mg/ml and incubated on ice for 30min. For 15ml lysis buffer-bacterial suspension, 100μl of 500μg/ml RNAse and 20μl of 10U/μl Dnase I were added and incubated on ice for 15min. For efficient and complete lysis, bacteria were subjected to 1000psi pressure (3 times) using a French press. The bacterial lysates were centrifuged at 10,000g (JA20rotor Beckmann) for 30min at 4°C. Before purification, Ni-NTA beads were equilibrated with 5 volumes lysis buffer in a falcon tube on a rotator for 10min. Supernatant was removed and cleared lysate was added to the Ni-NTA slurry and mixed gently by shaking for 60min. The lysate-Ni-NTA mixture was added into column and the lysate flow-through was collected. The beads were washed
with 250ml wash buffer and the wash flow-through was also collected to inspect the purification process. Subsequently, proteins were eluted 7x with 2ml elution buffer.

**Lysis buffer:**
- 50mM NaH$_2$PO$_4$
- 300mM NaCl
- 10mM Imidazole
Add sterile H$_2$O to 500ml and adjust pH to 7.0
Add protease inhibitor and 1mM PMSF before use.

**Wash buffer:**
- 50mM NaH$_2$PO$_4$
- 500mM NaCl
- 20mM Imidazole
Add sterile H$_2$O to 500ml and adjust pH to 7.0

**Elution buffer:**
- 50mM NaH$_2$PO$_4$
- 300mM NaCl
- 250mM Imidazole
Add sterile H$_2$O to 500ml and adjust pH to 7.0

### 5.5.5 Protein dialysis and concentration (for antibody generation)

**Preparation of dialysis tubing**

The tubing was cut into pieces of convenient length (25cm for 15ml protein) and boiled for 10 minutes in 700ml 2% sodium bicarbonate (NaHCO$_3$) and 1mM EDTA (pH8.0) at 80°C. The tubing was thoroughly rinsed in ddH$_2$O and then boiled for 10 minutes in 1mM EDTA (pH8.0) at 80°C. Finally, were stored at 4°C by submerging in 1mM EDTA. Before use, the tubings were washed thoroughly with ddH$_2$O.

A dialysis tubing-specific clamp was used to close one end of the tubing. The protein sample was pipetted into the tubing and bubbles were avoided. Afterwards, the other end of the tubing was closed with one or two clamps and the tubing was inserted into 5L Dialysis Buffer with a magnet stirrer inside. Dialysis buffer was prechilled before use and the volume of the buffer...
was at least 100 times the original volume of the protein. Proteins were
diaiyed against PBS for 3 x 6hours at 4°C.

Dialysis buffer:
0.1-1 x PBS (depend on the concentration of the protein)

After dialysis, the protein was concentrated. Proteins were equally distributed
into 1.5 ml Eppendorf tubes and spun in a speedvac until the volume was
reduced to final volume (depend on the dialysis buffer which was used, after
speedvac the final solvent should be 1x PBS). Subsequently, protein was
pooled together and the concentration was measured by Bradford analysis
and the purity and integrity of the protein was also detected by SDS-PAGE.

Bradford analysis (595nm):
The samples for setting the Standard curve were prepared as follow:

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<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (1μg/μl)</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PBS</td>
<td>800</td>
<td>798</td>
<td>796</td>
<td>792</td>
<td>790</td>
<td>788</td>
<td>786</td>
<td>784</td>
<td>782</td>
<td>780</td>
</tr>
<tr>
<td>Bradford</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<td>200</td>
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</tr>
</tbody>
</table>

The cuvettes were closed with parafilms and the solution was well mixed and
incubated for minimum 8 min before extinction measurements were performed
with an OD of 595 nm using a BioRad spectrophotometer.

The protein samples, which need to be measured, were 1:100-1:10,000
diluted into PBS/Bradford reagent. The dilution factor depends on the
concentration of the protein.

5.5.6 Antigen affinity purification for antibody
The antigen was dialyzed for 3x 6hours in 1L Coupling buffer at 4°C.
Amersham’s HiTrap NHS-activated HP, pre-packed 1ml column was used to
crosslink the antigen to sepharose beads. The NHS resin reacts with
primary amino groups. The coupling is done exactly as described in the
manual and 5mg antigen was used in this purification. Before purification,
5ml serum was dialyzed 3x 6 hours at 4°C towards binding buffer. The
Methods

5.5.7 Purification of GST (Glutathione S-transferase) -tagged Protein

An overnight culture of BL21(DE3) containing specific plasmid was diluted (1:100) in 100 ml LB medium+Amp and incubated at 37°C with vigorous shaking until OD600=0.6-0.8. The expression of the fusion proteins was induced with 1mM IPTG and the culture was incubated for another 3 hours at 37°C. Finally the bacteria were centrifuged at 4000rpm for 10min and the pellet was frozen in -80°C or resuspended in lysis buffer (50ml culture in 10ml lysis buffer). Lysozyme was added to a final concentration of 0.5mg/ml, and the bacteria were incubated on ice for 30min-1hour. The bacteria were broken by sonication with Bioruptor for 2x15min (0.5min ON, 0.5min Off, “H”). The

Coupling buffer: 0.2M NaHCO₃, 0.5M NaCl, pH8.3
Binding buffer: 10mM Tris, pH7.5
Elution buffer: 100mM glycine, pH2.5
Washing buffer: 10mM Tris, 500mM NaCl, pH7.5
Neutralization buffer: 1M Tris, pH8.0
bacteria were centrifuged at 4000rpm for 10min at 4°C, the rest over pellet was stored in liquid nitrogen then -80°C. The supernatant was centrifuged at 16,000 rpm (30000g) with SS34 rotor for 30min at 4°C, the supernatant was transferred to new tube. Glutathione (GSH)-sepharose was equilibrated by 5x1min washes in Lysis buffer. 50µl (100% vol) beads were added to each (50ml culture) sample. (Before adding beads, 50µl sample was kept as input). The mixture was incubated in 4°C for 1hour by rotating and centrifuged at 1500g for 2min. The beads were transferred to a fresh Eppendorf tube and washed 3x1min with Wash II buffer, 3x1min with Wash I buffer and 3x1min with Wash II buffer again. The beads could be stored in 4°C.

**Lysis Buffer:**
50mM Tris-Cl (pH8.2)  
100mM NaCl  
10% glycerol  
2mM EDTA  
*1mM DTT  
*1mM benzamidine  
*2mM PMSF

*each time add freshly

**Wash I buffer:**
50mM Tris-Cl (pH8.2)  
100mM NaCl  
10% glycerol  
5mM EDTA  
0.5% Triton X-100  
*5mM DTT  
*1mM benzamidine  
*2mM PMSF

**Wash II buffer:**
50mM Tris-Cl (pH8.2)  
100mM NaCl  
10% glycerol  
5mM EDTA  
*5mM DTT
*1mM benzamidine  
*2mM PMSF  

(If need to cleave the protein off from glutathione-sepharose by thrombin, the 
beads should wash with the buffer without any protease inhibitor several times. 
If there’s DTT in the solution, 2-5x more enzyme is required.)

**GSH Elution:**  
The protein was eluted by incubation 2x 30min at 4°C in GSH elution buffer by 
rotation.  
**GSH elution buffer:**  
30mM GSH  
50mM Tris-Cl pH8  
150mM NaCl  
1mM DTT  
0.1% Triton-X100  
1x protease inhibitor  
Adjust pH to 8 by adding about 5µl 5M NaOH to 1ml buffer.  
To remove the GSH from the eluted protein, the mixture was dialyzed in 
dialysis buffer (50mM Tris-Cl (pH=8 in 4 °C)/ 100mM NaCl/ 10% glycerol/ 
1mM DTT) at 4°C for 3 times.  
1) 4 hours in 800ml dialysis buffer  
2) Overnight in 800ml dialysis buffer  
3) 4 hours in 800ml dialysis buffer

Afterwards, the protein was aliquoted into 1.5ml Eppendorf tubes and shock- 
frozen in liquid nitrogen. Protein was stored at -80°C.

**5.5.8 Histone binding assay**

4µg Histone was incubated with target protein in IPH300 buffer for 1hour. 10µl 
(100% vol) GSH beads were added into each reaction and incubated another 
40 min. The beads were washed 3x1min with 1ml IPH300 buffer and the 
proteins pull-down were detected on SDS gradient gel.

**IPH300 buffer:**  
50mM Tris-Cl (pH8.0)  
300mM NaCl  
0.5% Tween 20  
*1mM DTT, 1x protease inhibitor  
* add freshly each time
5.5.9 Generation of embryonic nuclear extracts (NE)

Embryos collection:
Flies were put in the cages (50-70 big vials for 4 cages), yeast was applied on the apple juice plates. The plates were changed every 12 hours and the plated with collected embryos were stored in cold room. After 2 days, the embryos were washed off from the plates with the paintbrush and poured into a basket with a mesh bottom. The embryos were washed several times with tap water and afterwards dechorionated with 3% NaClO for 2.5min. The dechorionated embryos were rinsed with tap water for several times, after weighing, the embryos were wrapped with foil and snap-frozen in liquid nitrogen and stored in -80ºC.

Homogenize the embryos
1-1.5g embryos were transferred into a glass homogenizer, 5ml buffer B were used homogenize the embryos. After 10-15 strokes, the homogenates were filtered through 2 layers of Miracloth and collected into a 50ml tube. Each time 5g embryos were used.

Nuclear extraction
Whole cell homogenates were centrifuged using an SS-34 rotor at 2000g for 10 min. The cytosolic supernatant was discarded and the remaining lipids on the tube walls were removed with Kimwipe wrapped around fingers. This is a critical step because lipids interfere with Ammonium Sulfate precipitation and can lead to protein aggregation. Pelleted nuclei were resuspended in 5ml Buffer B (for 2-2.5g embryos). The nuclei were further cleaned by a cushion of 5ml 0.8M Sucrose/Buffer B. The suspension of nuclei was loaded carefully upon the cusion (vol 1:1) and was centrifuged with a swing out rotor (Sorvall TH641) at 10000g for 10min. The pellet was resuspended in 6ml 150mM KCl/Buffer B and 700µl 4M Ammonium Sulfate pH 7.6 was added, the nuclear proteins were extracted for 15 min by incubation on ice. After being centrifugated at 24,000rpm in a TH641 rotor for 1 h, the clear supernatant was collected omitting the top lipid layer and chromatin debris from the bottom. It is very important to not aspirate anything from the chromatin pellets. If clouds of colorless nucleic acids emerge from the bottom, they should not be aspirated. The nuclear extract was precipitated with 0.3g/ml of freshly grinded
Ammonium Sulfate (grinding helps to quickly and evenly dissolve Ammonium Sulfate), which was added slowly while stirring over a period of 8 min in order to assure full precipitation of proteins with different biochemical properties. The solution was left to stir for another 8 min thereafter. The solution was centrifuged at 15,000rpm in an TH641 rotor for 15 min. The pellet was resuspended in 300µl dialysis buffer and was dialyzed in snakeskin tubing with 7 kDa molecular weight cutoff for 2x 4 h in order to remove any remaining Ammonium Sulfate. Finally, nuclear extract was sedimented for 15 min in a SS34 rotor at 12,000rpm and the supernatant was aliquoted to 25µl. The nuclear extracts were frozen in liquid nitrogen and thereafter stored in -80°C. The protein concentration of the nuclear extract was determined by Bradford assay (normally the concentration is 14µg/µl).

2x Buffer B (35ml for 5g embryos)

5.25ml 0.2M HEPES
700µl 1M KCl
350µl 1M MgCl2
14µl 0.5M EDTA
70µl 0.5M EGTA
28.6ml H2O

Before using, it should be diluted into 1x and 1mM DTT, 1mM PMSF and 1x protease inhibitor should be added freshly.

Dialysis buffer:

50mM Tris-Cl (pH=8 in 4 ºC)
100mM NaCl
0.1mM EDTA
10% glycerol
*1mM DTT
*1mM PMSF
*Add freshly before use.

5.5.10 Crosslink antibody onto Protein-A-agarose via DMP

200µg of antibody was used per 100µl wet beads. The antibody was mixed with Protein-A-agarose on a roller at room temperature for at least 1 hour (normally 3 hours). After binding, the beads were washed 2x 10min with 10 volumes borate buffer (0.2M Na-borate pH9.0), each time spin 3 min at 4000
rpm. The beads were resuspended in 10 volumes borate buffer and solid DMP (dimethylpimeliminate.2HCl) was added to a final concentration of 20mM (5.2mg for 1ml). The beads were mixed with DMP on a roller for 45-60min and the reaction was stopped by washing the beads 2x 10min in 0.2M ethanolamine (pH8.0). To completely block the imido ester groups, the beads were incubated in 0.2M ethanolamine (pH8.0) for 2hours at room temperature on a roller. Finally, the beads were washed 2x 10min with PBS and the beads can be stored in PBS at 4ºC.

5.5.11 Co-immunoprecipitation from transiently transfected cells and embryonic nuclear extracts

*From the cells transiently transfected with Flag-FKBP39*

From a well-grown Kc cell suspension, 200μl of cells were seeded in each well of 6-well plates and 2ml culture medium was added into each well. The next day, appropriate plasmids were transfected into Kc cells following the transient transfection protocol. As a control a mock transfection (without DNA) of Kc cells was performed. After 72-96hours of transfection, cells (about 2.5ml) were harvested by pipetting up and down several times and transferred into 15ml falcon tubes.

An aliquot of 200μl was collected as whole cell lysate or input control. The rest of cells were centrifuged at 2000rpm for 10 minutes at RT and washed with 5ml 1x PBS. The washed cells were lysed by adding 1ml cold IP buffer and incubated for 30min on ice. The lysate was transferred into Eppendorf tubes and centrifuged for 30 min at full speed at 4ºC. Afterwards, the supernatant was transferred into new tubes and 40μl (50% vol) anti-Flag M2-agaroses were added, after an overnight incubation at 4ºC with rotation the beads were centrifuged at 2,500rpm for 1minute and washed with 1ml IP buffer for 3 x 10 minutes. Afterwards, the entire IP buffer was removed and 50μl 2 x SDS loading buffer was added. The samples were boiled at 95ºC for 5minutes and put on ice immediately. Samples were resolved on SDS-PAGE followed by western blotting.
From the embryonic nuclear extracts

25 μl nuclear extract was used for each reaction and diluted into 1 ml cold IP buffer. The nuclear extract was pre-cleared with 50 μl (50% vol) blocked (protein A or protein G Sepharoses were blocked with 1 mg/ml insulin or BSA) protein G sepharose (for monoclonal antibody) or protein A Sepharose (for polyclonal antibody) for 1 hour to reduce non-specific binding to beads in IP reaction. After that, 5% were stored as Input control and antibody was added as appropriate and incubated over night. The next morning, 40 μl of 50% blocked Protein G or Protein A Sepharose was added and incubated for 4 h. After that, a minimum of 5 x 10 min washes with IP buffer was applied to wash off non-specific interactions.

IP buffer:
50 mM Tris-Cl pH 8.0
150 mM NaCl
0.1 mM EDTA
1% Triton X-100
add ddH₂O to 50 ml

The buffer need to be filtered and 1 mM DTT, 1 mM PMSF and 1 x protease inhibitors should be added into it before use.

5.6 Bioinformatic procedures

Genomic coordinates
The Apr. 2006 D. melanogaster genome assembly (dm3, BDGP Release 5) provided by the Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org/) was used as a basis for all analyses. The Drosophila melanogaster Ensemble annotation BDGP5.25 was used for this analysis.

Read filtering and alignment
Low-complexity reads were filtered out based on their dinucleotide entropy (removing <1% of the reads). The Alignment of all quality filtered sequence reads to the D. melanogaster genome were performed by the software bowtie (version 0.9.9.1) (Langmead et al., 2009) with parameters --best --chunkmbs 512 -S -M 100 -p 8 -k 1, tracking up to 100 best alignment positions per query with 28 bp seed length and allowing at most two mismatches.
**Peak finding**
The enriched ChIP regions in the genome were detected by MACS (version 1.4.0beta)(Zhang et al., 2008), with parameters: gsize=121000000 -tsize=36 – mfold 3 and default values for all other parameters. Short reads coming from either replicate were processed in the same way. For a more precise characterization of the genomic binding specificity of the protein, the intersection of all peaks coming from the two replicates were identified as truly enriched regions, resulting in 3254, 3244, 5399 and 1517 peaks for FKBP39, PC, PH and PSC proteins, respectively. A peak is associated with a particular TSS (transcription start site) if it overlaps with the genomic interval, defined by the TSS and the 100 bases upstream, by at least one base.

**Calculation of RNA expression levels and misregulated genes detection**
In mRNA-seq, the expression levels of Ensemble genes were calculated as RPKM (Reads Per Kilobase of exon model per Million mapped reads). The exon model is the sum of all the exons of different isoforms of one gene. The reads belonging to the exons of each gene were counted by HTseq (Anders, 2010).The misregulated genes in FKBP39 RNAi treated S2 cells were detected by DEseq (Anders and Huber, 2010) with the control of GFP RNAi treated S2 cells.

**GO term enrichment analysis**
Functional classifications of FKBP39 target genes were assessed using the Gene Set Analysis ToolKit2(http://bioinfo.vanderbilt.edu/webgestalt/option.php) (Duncan, 2010) with standard settings. Within 904 FKBP39 uniquely bound genes, 871 were unambiguously mapped to Entrez IDs.
References
6. References


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Appendix
7. Appendix

Publications


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Basel, 11.10.2011
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Curriculum Vitae

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