Identification and Functional Characterization of Polycomb Group Complexes in *Drosophila*

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Identificatie en Functionele Karakterisering van Polycomb Groep Complexen bij *Drosophila*

Thesis

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by

Anna Lagarou

born in Athens, Greece

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Promotor: Prof.dr. C. P. Verrijzer

Other members: Prof.dr. F. G. Grosveld
Prof.dr. J.N.J. Philipsen
Prof.dr. R. Fodde

to my parents ...

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Chapter 1

Introduction

Polycomb group (PcG) and trithorax group (trxG) proteins were first discovered in *Drosophila* as repressors and activators of homeotic (HOX) genes, a set of transcription factors that specify cell identity along the anteroposterior axis of segmented animals. Subsequent work has shown that PcG and trxG proteins form multimeric complexes that are not required to initiate the regulation of HOX genes, but rather to maintain their expression state after the initial transcriptional regulators disappear from the embryo. The patterns of homeotic gene expression are initially set by the gap and pair-rule gene products. The early expressed gap and pair rule proteins disappear by about four hours of embryogenesis. Then, the PcG proteins maintain transcriptional repression of homeotic genes in cells where the initial expression state is off (McKeon et al., 1994; Simon et al., 1992; Struhl and Akam, 1985). In contrast, the trxG proteins maintain homeotic gene expression in cells where the initial expression state is on (reviewed in Kennison, 1993).

By definition, an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence. Epigenetic regulation of gene expression is necessary for the correct deployment of developmental programs and for the maintenance of cell fates. Since PcG proteins are responsible for the stable propagation of homeotic gene repression, after the initial decision has been made by other factors, they are referred as epigenetic regulators. Recent studies provide evidence that the PcG maintenance system regulates many other target genes in addition to homeotic genes, involved in development, cell proliferation, stem cell identity and cancer (Martinez and Cavalli, 2006; Ringrose and Paro 2004; Schwartz and Pirrotta 2007; Sparmann and van Lohuizen 2006).

The dissection of cis-regulatory sequences of Hox genes in reporter gene assays and the use of chromatin immunoprecipitation (ChIP) assays converged to reveal that PcG proteins associate with specific *cis*-regulatory sequences that are needed for PcG repression, called Polycomb Response Elements (PREs) (Chan et al., 1994; Simon et al., 1993; Strutt et al., 1997). Biochemical purifications and characterization of PcG protein complexes contributed to the understanding of PcG mediated-repression mechanisms. Studies of the physical and regulatory interactions between the components of these complexes revealed that they function through modulation of chromatin structure and covalent post-translational modifications of histones. PcG-mediated repression is counteracted by trxG proteins that also act through chromatin modification. Genome-wide distribution of PcG proteins and studies concerning their recruitment to chromatin and regulation of their target genes has made considerable progress towards the understanding of PcG silencing mechanisms.

Biochemical activities of PcG protein complexes

Understanding the mechanisms by which PcG proteins repress gene expression required the isolation of functional PcG complexes. To date, three distinct *Drosophila* PcG protein complexes have been biochemically purified and characterized, which are working together during PcG silencing, referred as PRC1, PRC2 and PhoRC complexes (Ringrose and Paro 2004; Schwartz and Pirrotta 2007) (Figure 1). At least at HOX genes in *Drosophila*, all three complexes cobind in a localized manner to PREs (Papp and Muller, 2006). The presence of closely related homologues of certain PcG genes in *Drosophila* that substitute for their function in different tissues, developmental stages or even at different target genes, reveal the complexity of the PcG system. Using different conditions or different tissues additional purifications have uncovered the identification of other PcG-related complexes.

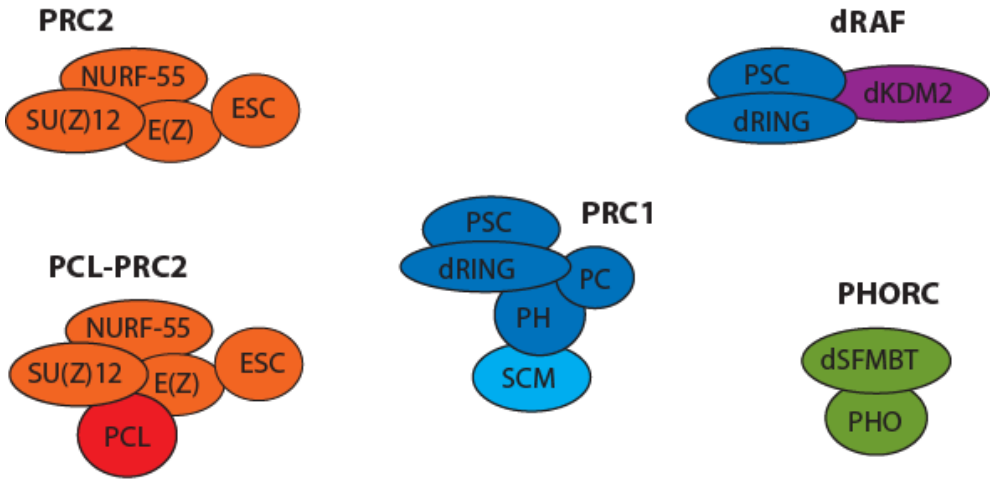

Figure 1. Characterized *Drosophila* PcG complexes. Schematic representation of *Drosophila* PcG complexes discussed in the text.

PcG and trxG proteins are often said to be evolutionary conserved. Indeed most trxG components are found in fungi, animals and plants, consistent with a conserved role in the regulation of global gene expression. Concerning PRC2 components, they are found in animals and plants (Pien and Grossniklaus, 2007), but not in the distantly related fungi *S.cerevisiae* and *S.pombe*. In contrast, the picture is much more complicated for PRC1 subunits. Importantly, there is no expression of genes that encode the core PRC1 subunits in plants and fungi. Blast analysis of several sequenced genomes showed that PRC1 genes originated early in animal evolution. PRC1 genes are complete in several vertebrate and insect species, but they are missing from other ones. These observations indicate that PRC1 genes have been repetitively lost during evolution of the animal kingdom.

PRC1 and H2A ubiquitylation

Drosophila PRC1 contains 4 core PcG proteins Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC) and Sex combs extra (SCE), usually referred to as dRING, which constitutes the PC core complex (PCC). Apart from these proteins, PRC1 complex biochemical purification has revealed the presence of Sex comb on midleg (Scm) in much lower amounts, but also additional proteins namely Zeste, TAFs, and elements of other multiprotein complexes as Mi2, Sin3A, SMRTER (Saurin et al, 2001; Shao et al, 1999). The mammalian PRC1 complex that has been isolated from HeLa cells using exogenously expressed tagged proteins (Levine et al, 2002), contained core components that were similar to *Drosophila* PRC1 (dPRC1), but no TAFs were detected in association with it. Specifically, the purified complex included HPC1-3, HPH1-3, RING1A and RING1B, BMI1 and its potential homolog MEL18, which are correspondingly homologous to the fly PC, PH, dRING and PSC.

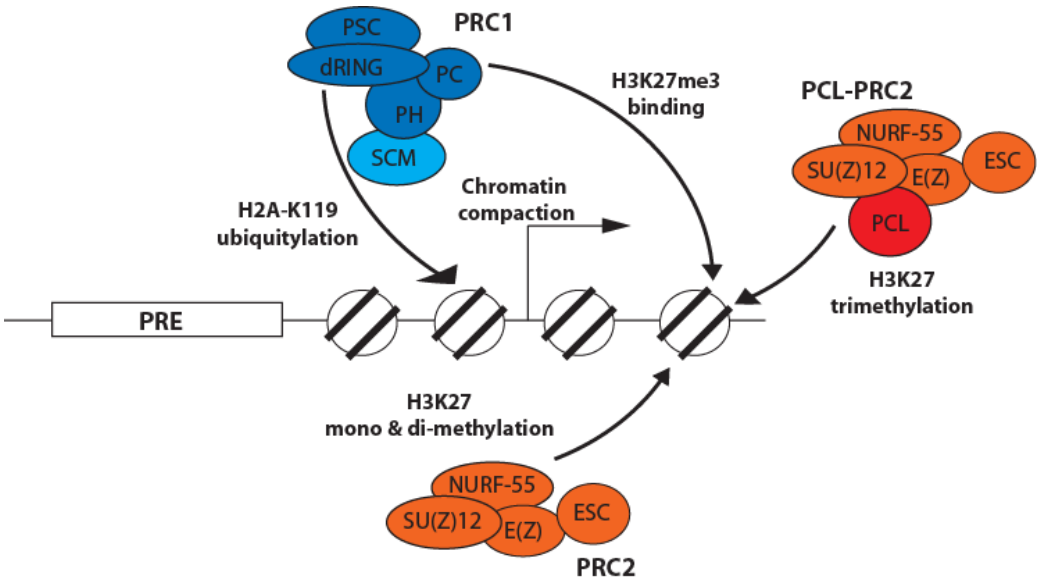

Figure 2. PcG complexes exert their effects in the chromatin flanking PREs. PRC1 targeted to PREs by PHORC, can ubiquitylate H2A at K119 by PSC/dRING and induce chromatin compaction by PSC,counteracting nucleosome remodeling by SWI/SNF remodelers at the flanking chromatin. PCL-PRC2 trimethylates H3K27 in the flanking chromatin whereas PRC2 lacking PCL generates the genome-wide H3K27 mono- and di-methylation. Moreover, PRC1 interacts with H3K27me3 through the PC subunit.

Purification of an H2A-K119 mono-ubiquitin (H2Aub) E3 ligase activity led to the description of a mammalian PRC1-like complex, in which the RING finger proteins RING1B and BMI1 provide the H2A ubiquitylase activity (Buchwald et al., 2006; Wang

et al., 2004; Weake and Workman, 2008). Knockdown of RING1B in human cells largely reduced the level of ubH2A, indicating that this enzyme is mostly responsible for the H2A ubiquitylation in vivo (Wang et al., 2004). Site-directed mutagenesis revealed that both RING1B and dRING contain intrinsic E3 ligase activity and that the conserved residue (Arg70 and Arg65 respectively) in the RING domain is critical for the enzymatic activity. The exact role of H2A ubiquitylation is unknown, but in its absence, PcG-dependent silencing is abrogated. Moreover, structure analysis showed that the RING finger domain of BMI1 is not required for catalytic activity, but strongly stimulates the RING1B E3 ubiquitin ligase activity through RING-RING formation (Ben-Saadon et al., 2006; Buchwald et al.2006; Cao et al. 2005).

Another functional feature of PRC1 apart from the RING domain is the chromodomain of the Polycomb (PC) protein that binds selectively to the repressive mark trimethylated lysine 27 of histone 3 (H3K27me3). Specifically, a crystal structure analysis providing a direct measurement of the affinity of the Polycomb (PC) chromodomain to the amino terminus of trimethylated H3K27, explained the specificity of the interaction (Fischle et al., 2003). As it will be discussed further below this interaction plays essential role during PcG silencing (Figure 2).

PRC2, Pcl-PRC2 and H3K27 methylation

The key component of PRC2 is the SET domain H3 methyltransferase protein Enhancer of zeste (E(Z)). Biochemical purification of PRC2 from *Drosophila* showed, along with the presence of SU(Z)12 (Suppressor of Zeste 12) and ESC (Extra sex combs), NURF55 (RBAP46/RBAP48) (Czermin et al., 2002; Muller et al., 2002), 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). E(Z) has no methyltransferase activity on its own, but only when assembled in the complex, it can methylate H3K27. Analysis of PRC2 subcomplexes showed that the different components provide mechanistically distinct functions within PRC2. Specifically, SU(Z)12 and NURF55 make up the minimal nucleosome-binding module that anchors the E(Z) enzyme on chromatin substrates, whereas ESC is required to enhance the enzymatic activity of PRC2 (Ketel et al., 2005; Nekrasov et al., 2005). Although ESC is strictly required for the HMTase activity of the complex, *esc* homozygous *Drosophila* mutants develop into viable adults with only mild PcG phenotypes (Struhl, 1981). The finding that ESCL (ESC-like), a protein closely related to ESC, encoded by a separate gene, functionally replaces ESC in its absence, resolved the mystery (Kurzhals et al., 2008; Ohno et al., 2008; Wang et al., 2006). However, in mammals the picture is more complicated since PRC2, PRC3 and PRC4 complexes have been biochemically characterized, where the differences depend on the presence of differential isoforms of EED, the mammalian homolog of fly ESC (Kuzmichev et al., 2004; 2005)

High-resolution genome-wide mapping of PcG proteins and the trimethylated H3K27 chromatin mark indicated that all the main morphogenetic pathways in *Drosophila* are controlled by the PcG system (Schwartz et al., 2006), and that PcG

silencing is characterized by formation of broad trimethylated H3K27 domains and localized PcG proteins to PREs. However, it has been shown that apart from trimethylation of H3K27, E(Z) is also responsible for wide-spread mono- and di-methylation of more than 50% of H3K27 in the *Drosophila* genome (Ebert et al., 2004), whose role in PcG silencing remains unknown. It has been also shown that PRC2 is also able to methylate H3K9 in vitro (Czermin et al., 2002; Kuzmichev et al., 2002). Chromatin immunoprecipitation analysis revealed that, in the repressed state, the nucleosomal profile across a Hox gene, namely the *Ultrabithorax* gene, contains three repressive chromatin marks, specifically trimethylated H3K27, H3K9 and H4K20 (Papp and Muller, 2006). However, these results do not show that PRC2 trimethylates H3K9 in vivo. A mammalian complex, that contains the homologous proteins, methylates selectively histone H3K27 on nucleosomes (Cao et al., 2002; Kuzmichev et al., 2002).

A PRC2-related complex has been purified from *Drosophila* embryos containing the PcG protein Polycomb-like (PCL) in association with ESC, E(Z) and SU(Z)12, so called PCL-PRC2 (Nekrasov et al., 2007). PCL is required for Polycomb silencing of homeotic genes and is found at Polycomb sites on polytene chromosomes (Papp and Muller, 2006; Tie et al., 2003). Removal of *Pcl* in *Drosophila* embryos or larvae resulted in reduction of PRC2 binding and of H3K27me3 levels at target genes, and concomitant misexpression (Nekrasov et al., 2007; Savla et al., 2008). Apparently, PCL is required specifically for PRC2 anchoring at PREs in order to achieve high levels of trimethylated H3K27, needed to maintain a PcG-repressed chromatin state (Figure 3). Similarly, mammalian PRC2 complexes that contain the *Pcl* homolog PHF1 (PHD-finger protein) have been described, where PHF1 specifically stimulates the activity of E(Z)H2 to catalyze trimethylated H3K27, playing important role in PcG-mediated gene repression (Cao et al., 2008; Sarma et al., 2008).

The PhoRC complex

In *Drosophila*, PcG protein complexes assemble at specific cis-regulatory DNA sequences called Polycomb Response Elements (PREs) (see further below). PHO and its closely related homolog, PHO-like (PHOL), are the only PcG proteins with sequence-specific DNA binding activity that can initiate the recruitment of PcG silencers on PREs (Brown et al. 1998; Fritsch et al. 1999; Mohd-Sarip et al., 2005, 2006; Wang et al., 2004). PHO and PHOL bind the same DNA sequence, the two proteins act to a large extent redundantly and double mutants show severe loss of HOX gene silencing (Brown et al., 2003). DNA-binding sites are present in all the characterized PREs and mutational analyses of these binding sites have shown that they are essential for PRE-mediated silencing. Their mammalian homolog is the factor YY1 (Yin-Yang 1), whose name comes from its dual function as an activator and repressor (Brown et al., 1998).

Although PHO has been shown to interact with subunits of the PRC1 and PRC2 complexes in flies and mammals (Levine et al., 2002; Satijn et al., 2001; Poux et al., 2001), it is not an important component of either of these two complexes. Instead, PHO has been purified in two different complexes from *Drosophila* embryo nuclear extracts,

which do not contain any of the PRC1 and PRC2 subunits. In one case, PHO associates with the INO80 ATP-dependent chromatin remodeler and other components. The inability of the INO80-containing complex to associate with PREs and to be reconstituted using recombinant proteins at in vitro assays, provided evidence that this complex is not clearly involved in PcG mechanisms (Klymenko et al., 2006). PHO was also purified from embryos in a very stable complex with the PcG protein dSFMBT (Scm-related gene containing Four MBT domains), as PHORC (PHO Repressive Complex) (Klymenko et al., 2006). In vivo binding and targeting of PHORC to PREs requires intact PHO/PHOL binding sites. The characteristic features of the *Drosophila* dSFMBT and its mammalian homolog are four malignant brain tumor (MBT) repeats and a sterile alpha motif (SAM) domain. Knockout studies of *dSfmbt* revealed that dSFMBT functions as a bona fide PcG protein that is critically needed for HOX gene silencing.

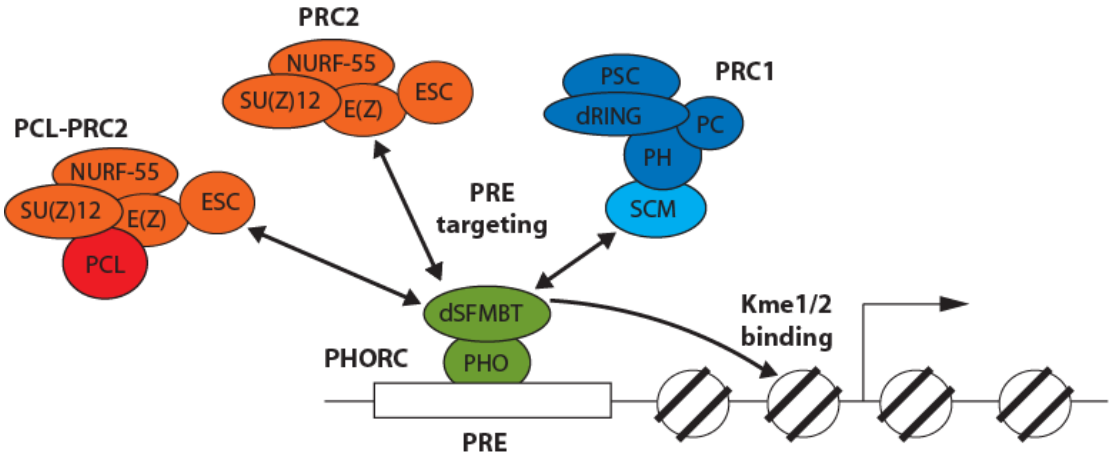

Figure 3. Targeting functions of the PHORC complex. PHORC targets PRC1 and PRC2 to PREs via PHO and interacts with the flanking chromatin via the selective methyl-lysine histone binding activity of dSFMBT.

In addition, the MBT repeats of dSFMBT consist of a novel methyl-lysine recognizing module that selectively binds the N-terminal tails of histones H3 and H4 when they are mono- and di-methylated at K9 and K20, respectively. In conclusion, PHORC consists of a PcG complex with sequence-specific binding activity via PHO and selective methyl-lysine histone binding activity via dSFMBT (Klymenko et al., 2006). Genome-wide binding profiling of PHO and PHORC, in *Drosophila* embryos and imaginal discs, and ChIP analysis at selected target genes, revealed that PHORC is specifically localized at discrete PRE sequences, many of which are co-occupied by PRC1 and PRC2 (Kwong et al., 2008; Oktaba et al., 2008). The observation that the same PRE sites are occupied in both tissue-cultured cells and in developing *Drosophila* implies that PHORC, PRC1 and PRC2 may be constitutively bound to a large fraction of target genes. Moreover, sequence analysis of all known so far PREs, defined an extended PHO-

binding motif that is part of the signature of PHORC-bound PREs. This definition in correlation to the extensive overlap between bound chromosomal regions, suggested that PHORC is a core PRE-binding complex that might be needed for PRC1 and PRC2 anchoring at many PcG target genes (Figure 3).

dRAF versus PRC1

A distinct *Drosophila* PcG complex has been identified which is called dRAF (dRING-associated factors) and shares with PRC1, dRING and PSC as common subunits (Lagarou et al., 2008). dRAF lacks the PRC1 subunits PC, PH or SCM, but contains additional proteins involved in distinct cellular processes. One of the key dRAF signature subunits is dKDM2, an intriguing protein, harboring regulatory motifs such as a JmjC type demethylase domain (Tsukada et al., 2006) and an F-box involved in targeting substrates to ubiquitylation. As it has been described, dKDM2 greatly enhances the H2A ubiquitylase activity of dRING/PSC on nucleosomes in vitro. Depletion studies in *Drosophila* S2 cells revealed that dKDM2, dRING and PSC, but not PC or PH, are required for bulk histone H2A ubiquitylation. This result suggested that at least in *Drosophila*, dRAF is mainly responsible for H2A ubiquitylation. Genetic interaction assays revealed that *dkdm2* enhances homeotic transformations caused by increased HOX gene expression in *polycomb* heterozygotes flies. This observation indicated that dKDM2 participates in PcG-mediated repression.

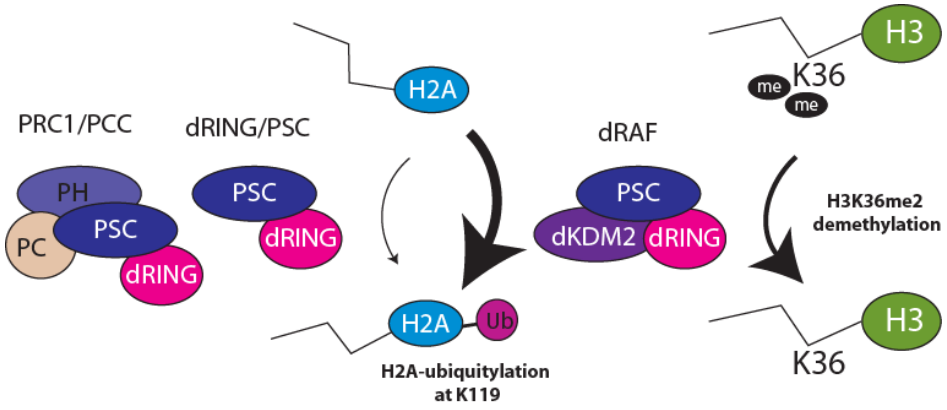

Figure 4. dRAF versus PRC1. dRAF uncovers a novel trans-histone pathway during PcG silencing, where dKDM2 removes the active H3K36me2 mark and strongly enhances establishment of the repressive H2Aub-K119 mark by dRING/PSC.

Another histone modification is mediated by dRAF, since dKDM2 selectively demethylates histone 3 lysine 36 dimethyl (H3K36me2) in vivo and in vitro. In a genome-wide profiling study in *Drosophila* Kc cells, they found that the chromatin mark

H3K36me2, generated mainly by the histone methyltransferase (HMTase) dMES-4, is preferentially enriched in the 5' coding regions (Bell et al., 2007). However, another study provided convincing evidence that mammalian and *Drosophila* Ash1 specifically methylated H3K36 in recombinant histones in vitro (Tanaka et al., 2007). Interestingly, *ash1 dkdm2* double heterozygotes exhibited reduced frequency of homeotic transformations observed in *ash1* heterozygotes caused by reduced HOX gene expression (Lagarou et al. 2008), showing that dKDM2 suppresses Ash1 activity. In conclusion, dRAF uncovers a novel trans-histone pathway acting during PcG silencing, where dKDM2 plays a central role by removing the active H3K36me2 mark and promoting the establishment of the repressive H2Aub mark by dRING/PSC (Figure 4).

PcG-mediated repression by PREs

The target genes of PcG proteins carry *cis*-regulatory elements that enable PcG proteins to bind and maintain the status of their transcriptional activity over many cell generations. They were originally identified in *Drosophila*, where they prevented activation of Hox reporter genes outside of their normal expression domains (Pirrotta et al., 1994; Simon et al., 1993). Since these specific-DNA sequences could direct PcG-mediated silencing of one or more promoters placed in their vicinity, they were called Polycomb Response Elements (PREs) (Francis and Kingston, 2001; Sigrist and Pirrotta, 1997). PREs consist of several elements of 100-300 base pairs that function weakly on their own, but together they work synergistically to maintain expression patterns. Although several PREs have been identified in *Drosophila*, most of the information about their structure and function comes from work on a few specific PREs: the bxd PRE and the Fab-7 PRE from the *Drosophila* Bithorax complex, and the engrailed PRE from the *Drosophila engrailed* (*en*) loci (Figure 5).

All three PcG complexes, as well as the antagonist trxG proteins, bind to PREs. The first chromatin immunoprecipitation experiments (ChIP) in *Drosophila* embryos and tissue-cultured cells, revealed the enrichment of PC and other PRC1 components at PREs (Orlando et al., 1998; Strutt et al., 1997). Lately, it has been confirmed that most of the components of PRC1, PRC2 and PHORC bind specifically at PREs of many other target genes, in addition to HOX genes (Cao et al., 2002; Klymenko et al. 2006; Papp and Muller, 2006). Functional analysis of PRE reporter genes showed that Hox gene PREs can act at a distance to repress transcription from different promoters. It has been observed that the *bxd* PRE from the *Ultrabithorax (Ubx)* gene can mediate PcG silencing, not by preventing binding of transcription factors to the promoter, but via blocking the ability of RNA polymerase to form the initiation complex (Dellino et al., 2004). In this way, *bxd* PRE seems to behave as a strong transcriptional silencer acting in a dominant fashion in order to prevent transcription. It has to be mentioned that, although *bxd* PRE, as well as other PREs, are located dozens of kilobases away from the transcription start site. There are also target genes, like *engrailed* (Kassis, 1994), *hedgehog* (Maurange and Paro, 2002), *polyhomeotic* (Bloyer et al., 2003), where PREs are in promoter-proximal regions.

In order to achieve correct maintenance of cell identity during the establishment of the body plan, PcG-mediated repression has to be counteracted by the action of trxG proteins. Analysis of *trxG* single mutant and *trxG PcG* double mutant flies, provided evidence that Ash1 and Trx HMTases function as anti-repressors that prevent PcG proteins from silencing Hox genes in cells where these genes need to be expressed (Klymenko and Muller, 2004). In addition, ChIP analysis at the *Ubx* gene in wild type and *ash1* mutant larvae, showed that Ash1 prevents PRC2 from depositing the repressive H3K27me3 mark on nucleosomes near the promoter and coding regions of *Ubx* (Papp and Muller, 2006). In conclusion, PRE-tethered PcG proteins appear to silence by default Hox genes that were repressed in the early embryo, but this silencing is prevented by trxG proteins in cells where Hox genes should be expressed, which actively prohibit the formation of PcG-repressed chromatin at promoter and coding regions of these genes.

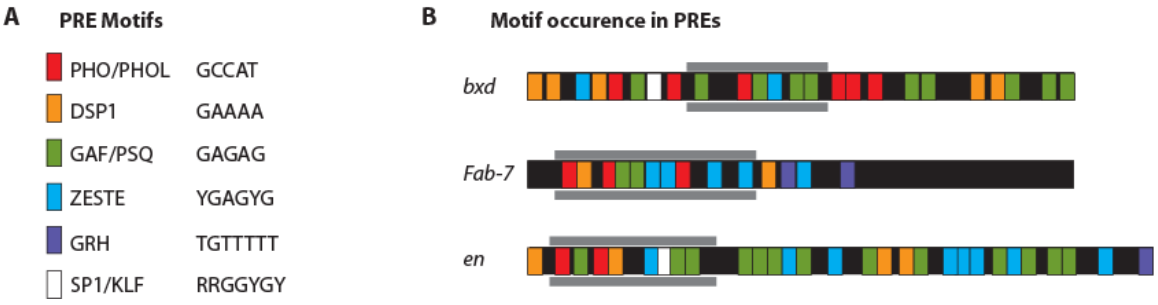

Figure 5. PRE motifs and flexibility of PRE design. A) DNA motifs shown to be important for PRE function. B) PREs have different combinations of motifs, with no preferred order or number. Grey lines show minimal PRE where these have been defined. Flanking sequences contain additional motif clusters, which may contribute to the function of these PRE in their endogenous context.

DNA binding proteins at PREs

Most PcG proteins do not have sequence-specific DNA-binding activity, so the simplest model for PRE function was that recruitment factors exist to recognize and bind specific sequences in PREs and interact with PcG proteins. So far, PHO and PHOL are the only sequence specific DNA-binding proteins that bind to PREs (Brown et al., 1998; 2003). The DNA-binding domain of these factors is highly conserved, so there is significant redundancy and removal of both proteins causes severe loss of Hox gene silencing (Klymenko et al., 2007). However, genome-wide binding profiles of PHORC, PRC1 and PRC2 complexes in embryo and larvae chromatin, revealed that PRC1 and PRC2 were also bound at genomic regions where PHORC was not detected, suggesting that they are targeted there by other factors (Oktaba et al., 2008). Moreover, reporter assays have identified additional sequences in PREs and a number of other proteins have been reported to bind to PREs. PREs are not defined by a conserved sequence, instead they include many conserved short motifs, several of which are recognized by known-DNA binding proteins. These proteins include GAGA factor, also known as Trx-like or

GAF (Horard et al., 2000), Pipsqueak (PSQ) (Schwendemann and Lehmann, 2002), Zeste (Mahmoudi et al., 2003), Grainyhead (Grh), also known as neuronal transcription factors 1–NTF-1 (Blastyak et al., 2006), Dsp1 (Dejardin et al., 2005) and Sp1/KLF family members (Figure 5). Mutant flies for these genes do not show any classic PcG phenotypes, so that the role of these factors in PRE function is poorly understood.

The idea that clusters of such motifs is a characteristic feature of PREs was exploited by Ringrose et al. (2003) to produce an algorithm searching for putative PREs through the *Drosophila* genome. This algorithm in combination with genome-wide mapping of PcG proteins by ChIP and DamID approach, suggested that PHO, as well Zeste and GAGA, are not the only DNA-binding proteins associated with PREs, since their sites cannot make up a functional PRE. Thus, it is possible that other unidentified DNA binding factors that recognize PREs exist that play pivotal roles during PcG silencing.

It is currently unknown how PcG proteins are specifically recruited to target genes in mammals. Although the mammalian PcG proteins are highly conserved, and known to bind and regulate the chromatin of homeotic and other genes bearing the repressive H3K27me3 mark, no PREs have been identified to this point. In *Drosophila*, even though the H3K27me3 mark covers large domains (Papp and Muller, 2006), PcG proteins bind to restricted regions, presumably PREs. In contrast, mammalian PcG proteins bind throughout the H3K27me3 regions which are much broader than in *Drosophila* (Bracken et al., 2006; Lee et al., 2006), so that it is much more difficult to identify PRE candidates. Whether it is difficult because of possible existence of many scattered sequences within mammalian PREs, or just because of resolution problems, remains to be clarified in future work.

Modulation of chromatin structure by PRC1

Apart H2A ubiquitylation, PRC1 is involved in other mechanisms of PcG-mediated repression. It has been shown that in vitro PRC1 functions by interacting with nucleosomal arrays to stabilize them for subsequent remodeling by the SWI/SNF-class ATP-dependent chromatin remodelers (Shao et al., 1999). Later, it was demonstrated by electron microscopy that PRC1 induces compaction of nucleosomal arrays (Francis et al., 2004). Structural analysis of the core PRC1 subunit PSC revealed that its C-terminal domain is required for chromatin compaction, inhibition of chromatin remodeling and in vivo gene silencing (King et al., 2005). SUZ(2) was considered to be a functional homolog of PSC, since *Psc-Suz(2)* double mutants showed much more severe misexpression than *Psc* single mutants (Beuchle et al., 2001). Recently it was observed that SU(Z)2 inhibits SWI/SNF-mediated chromatin remodeling on nucleosomal arrays and compacts chromatin in vitro, similarly to PSC (Lo et al., 2009). Several subunits of the *Drosophila* SWI/SNF-class remodelers, PBAP and BAP, are encoded by genes classified originally as trxG members, since their heterozygous mutants suppress the HOX misexpression phenotypes of PC heterozygous flies (Mohrmann and Verrijzer,

2005; Tamkun et al., 1992). In conclusion, according to the chromatin compaction model, PcG proteins antagonize SWI/SNF/trxG remodelers in order to silence gene expression.

Table 1. **Main components of the Polycomb/Trithorax maintenance system**

Drosophila protein	Complex	Protein domains	Biochemical activity	Mouse protein homologues
Polycomb group				
PC	PRC1	Chromodomain	Binding to trimethyl H3K27	NPCD, M33 (CBX2), CBX4 CBX6, CBX7, CBX8
PH	PRC1	SAM	?	PHC1, PHC2, PHC3
SCE (dRING)	PRC1	RING	E3 ubiquitin ligase specific to H2AK119	RING1A, RING1B
PSC	PRC1	RING	Cofactor for dRING	BMI1, MEL18
SCM	PRC1?	SAM, MBT, Zn-finger	?	SCMH1, SCML2
E(Z)	PRC2	SET	Methylation of H3K27, H3K9	EZH2, EZH1
ESC	PRC2	WD40	Cofactor for E(Z)	EED
ESCL	PRC2	WD40	Cofactor for E(Z)	EED
SU(Z)12	PRC2	Zn-finger	?	SU(Z)12
PCL	PRC2	PHD, Tudor	Anchoring of PRC2 at PREs	PHF1, MTF2 (M96)
PHO	PHORC	Zn-finger	DNA binding, PcG recruiter	YY1, YY2
PHOL	?	Zn-finger	DNA binding	YY1, YY2
dSFMBT	PHORC	MBT, SAM	Binding to mono- and dimethyl-H3K9, H4K20	L3MBTL2, MBTD1
Trithorax group				
TRX	TAC1	PHD, SET	Methylation of H3K4	MLL1, WBP7
ASH1	?	SET, PHD, BAH	Methylation of H3K4, H3K9, H4K20	ASH1L
BRM	SWI/SNF	SNF2, HELICc, BROMO	ATP-dependent nucleosome sliding	SMARCA4
MOR	SWI/SNF	SWIRM, SAINT	Cofactor for BRM	SMARCC1, SMARCC2
OSA	SWI/SNF	BRIGHT	Tethering SWI/SNF to target genes	ARID1B

However, results from many studies do not favor the chromatin compaction model. Nuclease mapping and ChIP analysis revealed that PRC1 as well as other PcG complexes are bound to nucleosome-depleted PREs (Mohd-Sarip et al., 2006; Papp and Muller, 2006; Schwartz et al., 2006). In addition, pairing sensitivity transgene assays showed that PREs contain nuclease hypersensitivity regions within which PcG DNA-binding proteins like GAGA and PHO are bound, exerting gene silencing in vivo (Mishra et al., 2001). Moreover, measuring histone replacement and nucleosome occupancy at *Drosophila* homeotic gene clusters at high resolution revealed that PcG and trxG binding sites are characterized by high histone replacement, nuclease-hypersensitivity and nucleosome depletion (Mito et al., 2007). This suggests that there is a continuous disruption of nucleosomes within PREs, resulting in a dynamic process that allows to antagonistic factors to exert their function at these sites. The model of PcG-repression by blocking chromatin access was also ruled out by the kinetic properties of PcG proteins observed in *Drosophila* embryos and tissues (Ficz et al., 2005). Specifically, FRAP microscopy analysis revealed the high exchangeability of PcG complexes throughout all

developmental stages and the relatively short residence times of proteins in the repression complex. This suggested that the PcG system is much more dynamic and flexible than previously anticipated. In correlation with these observations in living *Drosophila*, in vivo studies in the mammalian system found that the antagonistic interactions between SWI/SNF remodelers and PcG silencers involve a dynamic equilibrium rather than a static chromatin state (Kia et al., 2008). This study provides evidence that PcG binding does not necessarily block SWI/SNF function in vivo, since SWI/SNF can effectively counteract prebound PRC1 and PRC2 complexes by displacing them during the regulation of endogenous target genes. In conclusion, it is more likely that PRC1 does not compact nucleosomes at the PREs, but when targeted at PREs through interactions with the key tethering factor PHO and/or other DNA-binding proteins, it forms a repressive, non-accessible chromatin environment at the promoters or the coding regions of target genes (Figure 2).

The role of H2Aub and H3K27me3 in PcG silencing

To date the two main repressive marks involved in PcG-mediated repression are H2A ubiquitylation at K119 and trimethylation of H3K27. H2Aub at lysine 119 has been implicated in PcG transcription silencing, human X-chromosome inactivation (de Napoles et al., 2004) and heterochromatin formation. A series of genome-wide mapping analyses showed that PcG silencing is characterized by formation of broad trimethylated H3K27 domains and localized PcG proteins to PREs. A simple pathway for PcG protein recruitment has been suggested based on a stepwise model where PRC2 components are recruited by PHO, then generate the H3K27me3 mark at PRE nucleosomes, creating binding sites for the PC chromodomain, so that in the end PRC1 is recruited to PREs (Wang et al., 2004). However, there is strong evidence that PRC1 binding to PREs is not directly dependent on PRC2-mediated H3K27me3. First, polytene chromosome analysis on larvae missing the ESC and ESCL PRC2 components, revealed that the absence of H3K27me3 mark does not affect binding of the PRC1 component PSC to its in vivo targets (Ohno et al., 2008). Then, it was shown that in *Pcl* mutant embryos the significant reduction of H3K27me3 levels at target genes and their misexpression, does not interfere with the binding of PRC1 and PHORC at PREs (Nekrasov et al., 2007). Moreover, many studies have shown that PcG complexes are bound to nucleosome-depleted and hypersensitive sites-containing PREs (Mohd-Sarip et al., 2006; Papp and Muller, 2006; Schwartz et al., 2006), suggesting that the H3K27me3 mark is not the recruiter of PcG proteins at core PREs. These and other observations, support that both PRC1 and PRC2 exert their effects in the chromatin flanking PREs, rather than PREs themselves, in order to achieve PcG-mediated repression (Figure 2).

Recent studies have provided evidence that the repressive marks H2AubK119 and H3K27me3 appear to function during PcG silencing by blocking an early step of transcriptional elongation. ChIP analysis was performed in murine ES cells to determine the binding of PcG proteins and RNA PolII, and the histone modification status on specific developmental regulator genes (Stock et al., 2007). There is a specific group of

genes in ES cells that although bound by PcG proteins and enriched for the H2A-K119ub and H3K27me3 marks, they are transcribed at low levels by the phosphorylated at Ser5-form RNA PolII. In addition, knockdown experiments revealed that RING1B-mediated H2Aub is required for the repression of developmental regulator genes via blocking the release of Ser5-phosphorylated PolII at their promoters (Stock et al., 2007). Other studies in mammalian genes showed that H2Aub inhibits recruitment of the histone chaperone FACT and transcriptional elongation at a subset of target genes (Weake and Workman, 2008). In contrast, the active mark H2B mono-ubiquitylation (H2Bub) at lysine 120, mediated by the E3-ligase BRE1, appears to assist FACT in stimulating RNA polymerase during transcriptional elongation. Although, H2Aub and H2Bub seem to be antagonistic histone modifications, the mechanism of their opposite functions on FACT is unclear.

Concerning the H3K27me3 mark, it is removed selectively by the histone demethylase dUTX. Interestingly, it has been proposed that UTX associates with the elongating form of RNA polymerase II and in this way removes the repressive H3K27me3 mark during transcriptional elongation (Smith et al., 2008). At the mammalian system, UTX and JMJD3 were reported to associate directly with the trxG-related MLL3 and MLL4 complexes H3K4 methyltransferases, suggesting cooperation between H3K4 methylation and H3K27 demethylation (Swigut and Wysocka, 2007). Intriguingly, in *Drosophila* the trxG proteins Ash1 and Kismet reduce H3K27me3 levels in an indirect way (Papp and Muller, 2006; Srinivasan et al., 2008). Kismet consists a member of CHD families of ATP-dependent chromatin remodeling factors and facilitates an early step in transcriptional elongation by RNA PolII (Srinivasan et al., 2005). Polytene chromosome analysis revealed that Kismet is required for the association of ASH1 and TRX with many of their chromatin binding sites (Srinivasan et al., 2008), which are both histone methyltransferases generating active chromatin marks (H3K36me2 and H3K4me3, respectively). This finding could correlate the ability of Kismet to negatively regulate H3K27me3 levels with its role during early stages of transcriptional elongation. More studies in future will be needed to unravel the mechanisms by which H2AubK119 and H3K27me3 marks are implicated in PcG silencing.

Genome-wide mapping studies of PcG proteins

In addition to be essential regulators of embryonic development, certain mammalian PcG proteins have also emerged as key players in the maintenance of adult stem cell populations (Valk-lingbeek et al., 2004). For example, Bmi-1 is required for the self-renewal of hematopoietic and neural stem cells (Lessard and Sauvageau, 2003). Consistent with their critical role in development, several PcGs behave as oncogenes since they are overexpressed in certain types of cancer. Although considerable progress has been made towards the understanding of PcG function biochemically and biologically, there are still a lot of questions to be answered concerning the precise mechanisms of PcG control on developmental and cell fate decisions. Global identification of genomic locations to which PcG protein complexes bind and unraveling

how expression of target genes is regulated by these complexes was carried out in mammals and *Drosophila*, in order to understand how the PcG system controls transcription of the genome.

Genome-wide analysis in human and murine embryonic stem (ES) cells revealed an extremely significant enrichment for genes connected to transcription and development hierarchies, including organogenesis, morphogenesis, pattern specification, neurogenesis, cell differentiation, embryonic development and cell-fate commitment (Boyer et al., 2006; Lee et al., 2006). Further analysis showed that the target genes within the development and transcription functional groups overlap significantly, indicating that PcG complexes target transcription factors that have key roles in a variety of developmental processes. Moreover, the association of PcG components with repressive chromatin structure and developmental regulators suggested that genes targeted by PRC2 are globally repressed in ES cells but must be activated during differentiation. Specifically, genes repressed by PcG proteins in ES cells maintain the potential to become activated on lineage commitment, revealing a dynamic role for PcG complexes and their chromatin modifications during differentiation. Another study showed a strong and significant binding of PcG proteins together with the presence of the repressive H3K27me3 mark on tissue-specific target genes in undifferentiated cells (Bracken et al., 2006). Interestingly, this binding progressively decreases when PcG proteins receive dif-

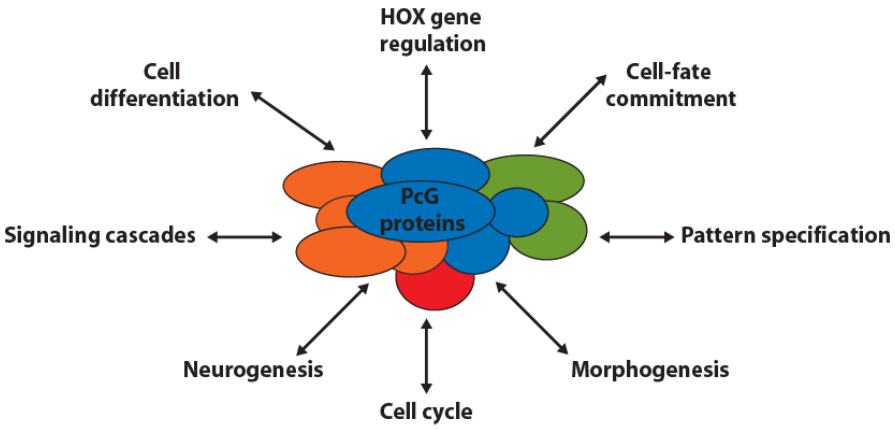

Figure 6. PcG proteins are implicated in many cellular processes. Genome-wide binding studies in *Drosophila* and mammals showed that PcG proteins regulate many target genes apart from HOX genes, involved in development and differentiation.

ferentiation signals, which activate tissue-specific genes, leading to displacement of PcG proteins from these genes (Bracken et al., 2006). One more suggestion from this study is that although the association of PcG complexes with their target genes can change dramatically upon differentiation, the presence of PcG complexes does not always signify transcriptional repression.

In the *Drosophila* system, genome-wide mapping of PcG binding in tissue-cultured cells (Schwartz et al., 2006; Tolhuis et al., 2006) and in different developmental stages in vivo (Negre et al., 2006; Oktaba et al., 2008), were generated in order to gain comprehensive insight into the nature and regulation of PcG target genes. As in mammals, the PcG target genes identified in *Drosophila* represent an assembly of key regulators that generate cellular diversity and patterning in the developing organism. These include growth factors and their receptors, signaling proteins, morphogens, differentiation factors and cell cycle regulators. Additionally, the genes that encode these factors become misexpressed in PcG mutants (Negre et al., 2008; Oktaba et al., 2008). PcG complexes are constitutively bound on most of their targets. Several PcG target genes, that are inactive in particular tissues early in development, are subsequently activated in response to extracellular signals. In this case, PcG proteins show reduced binding and high rate of rearrangement on these targets, suggesting a dynamic interaction between silencing and gene transcription. This is in agreement with the observation that PcG complexes exchange rapidly on chromatin during development (Ficz et al., 2005).

To summarize, the PcG system is required to maintain an unexpectedly large number of cell fate decisions during development in mammals and flies (Figure 6). This observation in association with the role of PcG proteins at the maintenance and stable transmission of gene expression though cell division, provides a system for the stability of differentiated cell states and the stable inheritance of cell fate decisions. In future, it will be of great interest to investigate how PcG complexes control these key developmental target genes, and to analyze the dynamic changes of their chromatin during their repressed or activated expression state.

Concluding remarks

The combination of recent fundamental discoveries and the development of analytical tools have resulted in a lot of progress towards the understanding of the biological roles of PcG proteins. According to the mechanisms of PcG silencing, it is quite obvious that these proteins act within complexes in an interdependent and combinatorial way in order to create a PcG-repressive chromatin state on their target genes. This chromatin state is the outcome of distinct histone modification patterns executed by all the different enzymatic activities within PcG complexes, and of histone post-translational modifications recognized by specific PcG-protein domains. Many models have been proposed for PcG-mediated repression. However it seems likely that PcG complexes assemble at PREs, but are working across many kilobases of DNA, in order to interact with and modify PRE flanking chromatin in a more dynamic way than previously anticipated. For this reason, it will be of great interest to understand how locally targeted PcG complexes can act over a distance to maintain their target genes in a PcG-repressed state. Genome-wide binding profiling of PcG complexes in different organisms and tissues has provided outstanding information about their biological roles on cell fate maintenance during development. Although these studies revealed a large number of new targets in addition to homeotic genes, the precise mechanistic

contributions of PcG proteins to the regulation of these genes is not known yet. Further analysis will be required to investigate to what extent these PcG targets provide a stable genetic address specifying cell differentiation. Moreover, in a recent systematic genetic screen where *Drosophila* mutants exhibiting PcG phenotypes were isolated, multiple alleles were identified in most of the known PcG loci (Gaytan de Ayala Alonso et al., 2007). Interestingly, at this screen alleles of genes, not known previously to be involved in PcG repression, were also found. The molecular and biochemical characterization of these genes and the identification of potential novel protein complexes will provide further insights into the mechanisms of gene regulation by the PcG system.

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Outline of the Thesis

Understanding the mechanisms by which PcG proteins repress gene expression required the identification and functional characterization of Polycomb Group (PcG) complexes. Studies of the physical and regulatory interactions between the components of these complexes revealed that PcG proteins function through modulation of chromatin structure and covalent post-translational modifications of histones.

In Chapter 2, we identified *Drosophila* PRC1 related complexes, by undertaking an unbiased proteomics approach, in order to gain more insight into the mechanisms of PcG-mediated repression. We came to realize that apart from the PC core complex (PCC), there is variability of the proteins associated with the individual PCC subunits. Moreover, we performed RNAi experiments in *Drosophila* S2 cells, followed by microarray expression analysis, to determine the individual functions of each PCC subunit (PCCs) in gene expression control. We demonstrated that there is a functional cooperation during PCC-driven gene regulation in a wide range of cellular processes. Apart from the overlapping target genes of PCCs, their expression profiles revealed a significant number of unique targets, suggesting that each subunit has additional functions apart from PCC.

Modulation of chromatin structure has emerged as a key molecular mechanism through which PcG proteins control gene expression. In Chapter 3, we characterized dRING and its associated factors (dRAF), aiming to unravel the enzymatic network underpinning PcG silencing. We found that a significant fraction of dRING is part of an assemblage separate from core PRC1 (PCC), which we named dRAF. Interestingly, we identified the histone demethylase dKDM2 as a key dRAF subunit. We described a novel mode of histone crosstalk during gene silencing, in which dKDM2 plays a pivotal role involving the removal of an active histone H3 methyl mark and the formation of the repressive H2Aub mark during PcG silencing.

Mammalian PcG members have been implicated in cell proliferation control, by acting as proto-oncogenes or as tumor suppressors. In *Drosophila* it has long been known that PcG mutations also cause cell cycle defects but it remains unclear how PcG complexes may control cell cycle progression. In Chapter 4, we addressed the role of PSC in cell cycle regulation. Knockdown studies in *Drosophila* flies and cells showed that PSC is essential during development and is required for cell proliferation and normal cell cycle progression, respectively. Moreover, we identified several key cell cycle regulators as PSC-associated factors in *Drosophila* embryo nuclear extracts by mass-spectrometry analysis. Taken together, we suggest a model where PSC plays a differential role apart from PCC in cell cycle regulation.

Chapter 2

Identification of PRC1-related complexes and exploration of core PRC1-regulated transcriptional control

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Summary

The Polycomb group (PcG) proteins are required for the maintenance of homeotic gene repression during development. Understanding the mechanism of PcG-mediated repression requires detailed understanding of the composition of PcG complexes, and how these complexes interact with each other and with chromatin. PcG silencing involves the activity of at least two major types of complexes, PRC1 and PRC2. *Drosophila* PRC1 contains 4 core PcG proteins Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC) and Sex combs extra (SCE), which form the functional Polycomb core complex (PCC). In order to characterize potentially novel PRC1 related complexes, we identified differential PRC1 associated factors from partially purified *Drosophila* embryo nuclear extracts, using an unbiased proteomics approach.

Introduction

Many cell fate decisions are made at the transcriptional level in response to developmental cues. Although some essential regulatory factors are expressed transiently, they establish expression patterns that persist during development. Maintenance of these transcriptional states is achieved by mechanistically distinct factors. In *Drosophila*, the patterns of homeotic gene expression are initially set in early embryos by the gap and pair-rule (segmentation genes) products. When the segmentation proteins decay, PcG and trithorax group (trxG) proteins assume Hox control for the remainder of development. In general, PcG proteins are repressors that maintain the off state and trxG proteins are activators that maintain transcription. The PcG/trxG system is evolutionary conserved from *Drosophila* to mammals. Although primarily known for their role in maintaining cell identity during the establishment of the body plan (Kennison, 1995; Pirrotta, 1998; Simon, 1995), several mammalian PcG members have now been implicated in the control of cellular proliferation and neoplastic development (Gil et al., 2005; Martinez and Cavalli, 2006; Sparmann and van Lohuizen, 2006; Valk-Lingbeek et al., 2004).

PcG proteins were originally identified in *Drosophila* as repressors of Hox genes based on mutant phenotypes involving posterior transformations of body segments (Ringrose and Paro, 2004). At the molecular level, PcG proteins are classified into two groups on the basis of their association with distinct classes of multimeric complexes, which are called Polycomb Repressive Complexes (PRCs), PRC1 and PRC2. The PRC1 complex biochemically purified from *Drosophila*, contained four core PcG proteins: Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC) and Sex combs extra (SCE) (Fritsch et al., 2003), usually referred to as dRING. Another protein, Sex comb on midleg (SCM) has also been purified with PRC1 but in much lower amounts. Many additional proteins co-purified with these core components namely ZESTE, TBP (TATA-Box binding Protein)-Associated Factors (TAFII250, TAFII110, TAFII85 and TAFII62), and components of other multiprotein complexes, such as MI2, SIN3A, SMRTER (Saurin et al., 2001; Shao et al., 1999). The mammalian PRC1 that has been isolated from

HeLa cells using exogenously expressed tagged proteins (Levine et al., 2002), contained core components that were similar to the *Drosophila* PRC1 (dPRC1), but no TAFs were detected in association with it. These findings suggest that purified PRC1 is a mixture of slightly different complexes, depending on the conditions of purification. Polycomb (PC) contains a chromodomain, one of the functional features of PCC that binds specifically to the repressive mark trimethylated lysine 27 of histone 3 (H3K27me3) (Fischle et al., 2003). dRING and the mammalian RING1B, contain a RING domain, and have been shown to function as E3 ubiquitin ligases, responsible for mono-ubiquitylation of lysine 119 of histone H2A, another histone modification associated with transcriptional silencing (Cao et al., 2005; de Napoles et al., 2004; Wang et al., 2004; Weake and Workman, 2008). The catalytic activities of dRING and RING1B are enhanced by PSC (Lagarou et al., 2008) and its mammalian ortholog BMI1 (Buchwald et al., 2006; Li et al., 2006), respectively, that also contain a RING domain.

The key component of PRC2 is the SET domain methyltransferase protein Enhancer of Zeste (E(Z)). Biochemical purification of *Drosophila* PRC2 revealed the presence of SUZ(12), Extra sex combs (ESC) or its homolog ESCL, and p55 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002), a histone binding protein that is also associated with the chromatin assembly factor CAF1, and other remodeling complexes (Polo and Almouzni, 2006; Taylor-Harding et al., 2004). Each of these components contributes to the ability of the complex to bind and methylate nucleosomes (Ketel et al., 2005). A PRC2-related complex containing the PcG protein PC-like (PCL) has also been described and appears to be particularly important for H3K27 trimethylation (Nekrasov et al., 2007; Sarma et al., 2008). One of the poorly understood issues is the mechanisms of recruitment of PcG proteins onto chromatin in order to silence specific genes. In *Drosophila*, a key-tethering factor is the sequence-specific DNA binding PcG protein PHO that binds PcG response elements (PREs) and can initiate the recruitment of PcG silencers (Brown et al., 1998; Fritsch et al., 1999; Mohd-Sarip et al., 2005; 2006). PHO has also been purified in complex with the INO80 ATP-dependent chromatin remodeler and also as PHORC (PHO Repressive Complex) in complex with the PcG protein SFMBT (Klymenko et al., 2006).

In order to gain more insight into the mechanisms of PcG-mediated repression, we identified PRC1 related complexes, by undertaking an unbiased proteomics approach. We used highly specific affinity purified antibodies against the core PRC1 subunits, PC, PH, PSC and dRING, which constitute the PC core complex (PCC), to purify their interactors from *Drosophila* embryo nuclear extracts. We came to realize that apart from PCC, there is variability of the proteins associated with the individual PCC subunits. Moreover, we investigated the relative roles of the individual PCC subunits in gene expression control by performing RNAi experiments in *Drosophila* S2 cells, followed by microarray expression analysis. Although PCC subunits share a large set of target genes, their expression profiles reveal a significant number of unique targets.

Materials and methods

Mass spectrometric analysis

1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm *et al.* (1996). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (0.1 % formic acid; 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBInr database (release NCBInr_20090222; taxonomy: *Drosophila melanogaster*). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table.

Biochemical purification procedures

Protein A Sepharose beads coated with either control anti-GST antibodies (Mock IP) or affinity-purified antibodies directed against PC (rabbit PV69), dRING (guinea pig SN12), PSC (guinea pig GR463/464) and PH (rabbit PV86) were used to identify PRC1 and associated factors from partially purified and concentrated embryo nuclear extracts (H0.4) (described in Lagarou et al., 2008, Mohd-Sarip et al., 2002). After extensive washes with a buffer containing 600mM KCl and 0.1% NP-40, bound proteins were resolved by SDS-PAGE and visualized by silver staining. Proteins present in bands excised from a gel run in parallel were identified by nanoflow LC-MS/MS.

RNAi-mediated knockdowns and genome-wide expression analysis

RNAi-mediated depletion and expression profiling and statistical analysis were performed as described (Moshkin et al., 2007). Briefly, dsRNA targeting the various subunits was synthesized using an Ambion Megascript T7 kit according to the manufacturer's protocol. Primers used for RNAi of Pc, Psc, Ph, dRing in S2 cells are:

PC F' ACCGACGATCCAGTCGATCTAGTG
R' GAAGGGCTGCTGCTGGCTGGGAGT
PSC F' GCGGCCAAAAGCGTTGTCAGCAAT
R' CGGGCAGAATCGCTCCTTTCGCAG
PH F' CAATCCCTACGCCATTCAGGTGAA
R' CGCATCCACTCCCACCTTGGCCAG
dRING F' TACGAGCTGCAGCGCAAGCCGCAG
R' GGATGTGCTGGCGCCCGAGTTTGC

Each oligo incorporates a 5' T7 RNA polymerase binding site (TTAATACGACTCACTATA GGGAGA**)**, resulting in a PCR product of approximately 700 bp. Cells were incubated in the presence of dsRNA for 4 days. The primary antibodies used to test the efficiency of RNAi in RIPA cell extracts were: anti-dRING SN12 (described in Lagarou et al., 2008), anti-PSC monoclonal hybridoma supernatant 6E8 (Martin and Adler, 1993), anti-PC SN965 and anti-PH SN964 (described in Mohd-Sarip, et al., 2002).
RNA samples from three independent biological replicates were isolated, prepared, and hybridized with Affymetrix microarrays. PCA was performed as described (Moshkin et al., 2007). Venn Diagrams were constructed using t-test, assigning 1 for significantly up-regulated genes ($P < 0.05$) and -1 for significantly down-regulated genes ($P < 0.05$). All statistical analysis was performed using R and Bioconductor packages.

Results

Purification of PRC1 related complexes from *Drosophila* embryo nuclear extracts

We sought to elucidate the molecular mechanisms of PRC1 function by isolating the endogenous proteins and their associated partners. For this reason, we analyzed H0.4 fractions from partially purified and concentrated *Drosophila* embryo nuclear extracts by SDS-PAGE, followed by immunoblotting, using highly specific antibodies against PCC subunits (Lagarou et al., 2008). We selected the H0.4 fraction in which these proteins

were highly abundant and we performed stringent immunopurifications by using protein A beads coated with affinity-purified antibodies against PC, dRING, PH and PSC. After extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, we resolved PCC and associated proteins by SDS-PAGE followed by Coomassie staining (data not shown). Mass spectrometric analysis revealed the presence of PCC subunits in all four immunopurifications, and a range of associated proteins indicated on Figure 1. In fact, only a few of these proteins overlapped, and the majority were unique for each PCC subunit.

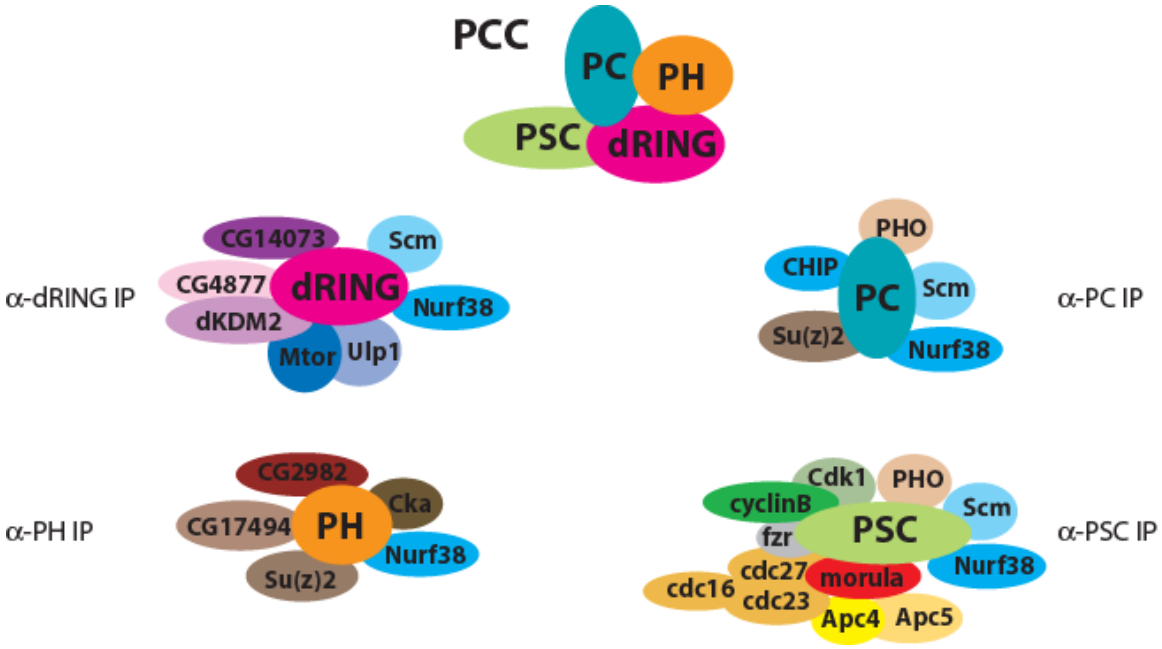


Figure 1. Purification of PRC1 related complexes from *Drosophila* embryo nuclear extracts. PCC and proteins complexes identified by LTQ-Orbitrap mass spectrometer specifically with anti-PC, anti-PSC, anti-PH and anti-dRING are shown clockwise from the top. Only the high Mascot Score proteins are mentioned, and background proteins that we routinely observe in our immunopurifications, like Heat shock cognate 4, γ-tubulin, RanGap, β-tubulin, yolk protein 1 and 2 and Replication Factors (RFCs), were not considered as specific associated proteins.

Polycomb associated factors. Polycomb (PC)-directed immunopurification resulted in the identification of interactors involved in chromatin silencing (Table 1). The most abundant PC-associated factors were Suppressor of zeste 2 (Su(z)2), Pleiohomeotic (PHO), Nucleosome remodeling factor 38 (Nurf-38), CHIP and Sex comb on midleg (Scm). Su(z)2, also identified as a PH-associated factor, is a functional homolog of Posterior sex combs (PSC), that similarly to PSC, binds DNA, compacts chromatin, inhibits chromatin remodeling and can replace PSC in a functional complex with other PcG proteins (Lo et al., 2009). PHO, which was also purified as a PSC-associated factor, binds PcG response elements (PREs) and can initiate the recruitment of PcG complexes (Brown et al., 1998; Fritsch et al., 1999; Mohd-Sarip et al., 2005; 2006). We also detected

the protein dSFMBT (Scm-related gene containing Four MBT domains), but in very low amounts. Nurf-38, which was identified in all four separate immunopurifications, is a subunit of the NURF complex that contains an inorganic pyrophosphatase domain and is involved in chromatin remodeling, nucleosome mobilization and regulation of transcription (Gdula et al., 1998). Another PC-associated protein is CHIP, an Hsp70-interacting E3-ubiquitin ligase that has been implicated in the decision as to whether a target protein enters the refolding or the degradation pathway (Murata et al., 2001). Dimerization of human CHIP is essential for its activity in a reconstituted ubiquitination assay. Sex comb on midleg (Scm), which was also detected in the PSC- and dRING-directed immunopurification, is a substoichiometric component of PRC1 complex (Saurin et al., 2001). Scm is a critical PcG repressor since its complete loss from embryos results in the deregulation of Hox genes and phenotypes that are as severe as loss of PC or PH (Bornemann et al., 1998). Functional studies (described in Peterson et al., 2004) suggest that it plays a noncatalytic role in binding and packaging chromatin in concert with PRC1.

TABLE 1. List of proteins identified in PC IP from Drosophila nuclear extracts using the LTQ Orbi-Trap MS

Name	Mascot Score (Number of unique peptides)	Domains	Functions
PSC (Posterior sex combs;170 kDa)	2503 (38)	Zn finger RING-type; coiled-coil	PCC; chromatin silencing
Sce/dRING (Sex combs extra; 48 kDa)	1836 (19)	Zn finger RING-type	PCC; chromatin silencing; E3 Ub ligase
PH (Polyhomeotic;168 kDa)	1135 (12)	Sterile alpha motif (SAM); coiled coil	PCC; chromatin silencing
PC (Polycomb; 44 kDa)	1003 (14)	Chromodomain	PCC; chromatin silencing
Su(z)2 (Suppressor of zeste 2; 147 kDa)	745 (12)	Zn finger RING-type	functional homolog of PSC; chromatin silencing
PHO (Pleiohomeotic; 58 kDa)	547 (7)	Zinc finger C2H2-type	DNA binding; chromatin silencing
Nurf-38 (38 kDa)	518 (7)	Inorganic pyrophosphatase domain	chromatin remodelling
CHIP (34 kDa)	427 (6)	TPR; Zinc finger RING-type	E3 Ub ligase
Scm (Sex comb on midleg; 96 kDa)	299 (6)	MBT repeat ; Sterile alpha motif (SAM)	PRC1 ; Transcriptional repression

Polyhomeotic-associated factors. Using a highly specific antibody against Polyhomeotic (PH) we detected two proteins of unknown function, CG17494 and CG2982, Connector of kinase to AP1 (Cka), Nurf-38 and Su(z)2 (Table 2) with a high mascot score. Although CG17494 and CG2982 are of unknown function, they contain domains of potentially interesting functions. CG17494 is a member of the Forkhead-associated domain (FHA) family, a putative nuclear signaling domain found in eukaryotic and prokaryotic proteins, that can bind phospho-threonine, -serine, or -tyrosine (Durocher and Jackson, 2002). In eukaryotes, many FHA domain-containing proteins localize to the nucleus where they participate in establishing or maintaining cell cycle checkpoints, cell proliferation, DNA repair, or transcriptional regulation. A well-known example of FHA-protein is Rad53, an essential kinase for stabilization of DNA replication forks during replication stress in *S. cerevisiae*. CG2982 belongs to the MINA53/NO66 group of the

JmjC-domain-only family, conserved from yeast to humans, which all are known to localize to the cytoplasm and might have diverged to carry out functions independent of histone demethylation (Tsukada et al, 2006). Its orthologue in higher eukaryotes NO66 was identified as a component of the nucleoli, where it interacts with components of pre-ribosomal complexes (Eilbracht et al., 2004). NO66 also localizes to some nuclear heterochromatic foci during the late stages of S-phase, indicating that it might have a role in replication or remodeling of certain heterochromatic regions. Connector of kinase to AP1 (Cka) is a novel multidomain protein, involved in several biological processes as JNK (JUN N-terminal kinase) signaling, dorsal closure, compound eye photoreceptor development and phagocytosis. In *Drosophila*, Cka positively regulates the JNK signal transduction pathway by organizing a molecular complex of kinases and transcription factors, leading to coordination of the spatiotemporal expression of AP-1-regulated genes (Chen et al., 2002). Moreover, it has been shown that activation of the JNK pathway causes downregulation of some PcG genes, including *polyhomeotic-p* (proximal) (Lee et al., 2005). Activation of the JNK cascade in mouse embryonic fibroblasts by exposure to ultraviolet light, results in the decrease of *polyhomeotic2* expression, indicating that downregulation of *polyhomeotic* by JNK activation is an evolutionary conserved mechanism of cell fate change.

TABLE 2. List of proteins identified in PH IP from Drosophila nuclear extracts using the LTQ Orbi-Trap MS

Name	Mascot Score (Number of unique peptides)	Domains	Functions
PSC (Posterior sex combs;170 kDa)	3398 (54)	Zn finger RING-type; coiled-coil	PCC; chromatin silencing
Sce/dRING (Sex combs extra; 48 kDa)	1581 (16)	Zn finger RING-type	PCC; chromatin silencing; E3 Ub ligase
PH (Polyhomeotic; 168 kDa)	1538 (18)	Sterile alpha motif (SAM); coiled coil	PCC; chromatin silencing
CG17494 (105 kDa)	1495 (23)	Forkhead-associated (FHA)	Putative nuclear signaling activity
Cka (Connector of kinase to AP-1; 105 kDa)	1005 (13)	WD40 repeat; striatin	JNK cascade ; signaling
CG2982 (73 kDa)	946 (14)	JmjC	ribosome biogenesis; heterochromatin formation
Nurf-38 (38 kDa)	862 (12)	Inorganic pyrophosphatase	chromatin remodelling
PC (Polycomb; 44 kDa)	719 (10)	Chromodomain	PCC; chromatin silencing
Su(z)2 (Suppressor of zeste 2; 147 kDa)	391 (8)	Zn finger RING-type	functional homolog of PSC; chromatin silencing

dRING-associated factors. Mass-spectrometry analysis revealed a range of dRING-associated factors, involved in several cellular processes (Table 3). Apart from Scm and Nurf38, we identified Megator, dKDM2 (CG11033), two proteins of unknown function, CG4877 and CG14073, and Ulp1. Megator is a dynamic subunit of the nucleopore complex that also exists separately in the nucleoplasm, interacts functionally with the MSL dosage compensation complex and has been implicated in mitotic spindle assembly (Mendjan et al., 2006; Qi et al., 2004). dKDM2 is a JmjC-domain containing histone demethylase, member of the JHDM1 group, harboring a series of other domains with interesting functions (Frescas et al., 2007; Tsukada et al., 2006). These include an F-box, found in proteins that associate with SKP1 (S-phase Kinase-associated Protein 1A) to form the SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complex, and a CXXC

zinc-finger domain, a DNA-binding domain that recognizes non-methylated CpG DNA and is involved in epigenetic regulation. The presence of these domains might link histone demethylation to protein ubiquitylation and the DNA methylation status. CG4877 contains a MYND zinc finger, a motif that in certain mammalian proteins constitutes a protein-protein interaction domain functioning as a co-repressor-recruiting interface. CG14073 has an ankyrin repeat, one of the most common protein-protein interaction motifs in nature (Mosavi et al., 2002). This repeat has been found in proteins of diverse function such as transcriptional initiators, cell-cycle regulators, cytoskeletal, ion transporters and signal transducers. Finally, Ulp1 is a nucleopore-associated SUMO-protease (Hay, 2007), expressed in *Drosophila* germline. Ulp1 belongs to a family of proteases that control SUMO function positively, by catalyzing the proteolytic processing of SUMO to its mature form, and negatively, by catalyzing SUMO deconjugation. It is suggested that as a component of the nuclear pore complex, Ulp1 may prevent proteins from leaving the nucleus with SUMO still attached.

TABLE 3. List of proteins identified in dRING IP from Drosophla nuclear extract using the LTQ Orbi-Trap MS

Name	Mascot Score (Number of unique peptides)	Domains	Functions
Mtor (Megator; 263 kDa)	5634 (82)	Coiled-coil; TPR; MLP1/MLP2-like	nuclear pore; mitotic spindle assembly
Psc (Posterior sex combs; 170 kDa)	3036 (48)	Zn finger RING-type; coiled-oil	PCC ; chromatin silencing
CG4877 (126 kDa)	1342 (19)	Zn finger MYND-type; coiled-coil	protein-protein interaction; corepressor recruitment
CG11033 (dKDM2; 151 kDa)	1263 (17)	JmjC; F-box; Zn finger CXXC-type; PHD finger ; LRR repeat	histone demethylation; ubiquitylation; CpG binding
Sce/dRING (Sex combs extra; 48 kDa)	1217 (14)	Zn finger RING-type	PCC; chromatin silencing; E3 ligase
PH (Polyhomeotic; 168 kDa)	902 (10)	Sterile alpha motif (SAM); coiled-coil	PCC ; chromatin silencing
CG14073 (235 kDa)	881 (17)	Ankyrin repeat	protein-protein interaction
Ulp1 (167 kDa)	822 (16)	Peptidase C48, SUMO/Sentrin/Ubl1; coiled-coil	nucleopore-associated SUMO-protease
Nurf-38 (38 kDa)	722 (10)	Inorganic pyrophosphatase domain	chromatin remodelling
Pc (Polycomb; 44 kDa)	716 (7)	Chromodomain	PCC ; chromatin silencing
Scm (Sex comb on midleg; 96 kDa)	495 (6)	MBT repeat ; Sterile alpha motif (SAM)	PRC1 ; Transcriptional repression

PSC-associated factors. Apart from Pleiohomeotic (PHO), Sex comb on midleg (Scm) and Nurf38, which are involved in chromatin silencing and remodeling, most of the PSC-associated factors are implicated in cell cycle regulation (Table 4). These are cdk1, cyclin B and many subunits of the anaphase promoting complex like morula (APC2), Cdc23 (APC8), Cdc27 (APC3), Cdc16 (APC6), APC5, APC4 and the late activator of APC/C, fizzy-related protein (fzr). Cyclin B is a G2/M cyclin that forms a complex with its partner cyclin-dependent kinase 1 (cdk1), leading to activation of its kinase function. Cyclin B/cdk1, which is also called Maturation Promoting Factor (MPF), controls the cell cycle at the G2/M transition stage (Ohi and Gould, 1999). Cyclin B, the levels of which rise in G2, promotes the completion of chromosome condensation and spindle assembly, thereby driving cell-cycle progression into metaphase. The anaphase

promoting complex (APC), also called cyclosome, is a multisubunit E3 ubiquitin-protein ligase of the RING-domain family (van Leuken et al., 2008). Like other members of this family, it binds to an E2 ubiquitin-conjugating enzyme that is covalently linked to the C terminus of ubiquitin and transfers the ubiquitin moiety to its substrates (e.g. securin or cyclins), marking them for degradation by the 26S proteasome. The APC contains 12 or 13 subunits, and in yeast, nine of them are essential for normal activity and are thought to interact (Sullivan and Morgan, 2007). The APC11 subunit contains a RING domain and probably binds the E2–ubiquitin conjugate. Protein targets are recruited by the activator subunits fizzy (Cdc20) or fizzy-related (fzr or Cdh1), which interact with the APC3 and APC6 core subunits. APC-fizzy triggers indirectly the degradation of cohesin, the protein complex that binds sister chromatids together, allowing anaphase onset. APC-fizzy also targets the mitotic cyclins (cyclin A and B) for degradation, resulting in inactivation of M/Cdks (mitotic cyclin dependent kinase) complexes (Hershko, 1999). Finally, APC-fzr functions during late anaphase in order to target specific sets of proteins for degradation and drive exit from mitosis (Sigrist and Lechner, 1997).

TABLE 4. List of proteins identified in PSC IP from Drosophila nuclear extracts using the LTQ Orbi-Trap MS

Name	Mascot Score (Number of unique peptides)	Domains	Functions
PSC (Posterior sex combs; 170 kDa)	5858 (82)	Zn finger RING-type; coiled coil	PCC; chromatin silencing
Sce/dRING (Sex combs extra; 48 kDa)	2132 (22)	Zn finger RING-type	PCC; chromatin silencing; E3 Ub ligase
PH (Polyhomeotic;168 kDa)	1638 (15)	Sterile alpha motif (SAM); coiled coil	PCC; chromatin silencing
PC (Polycomb; 44 kDa)	1251 (17)	Chromodomain	PCC; chromatin silencing
Cdk1 (Cyclin-dependent-kinase1; 34 kDa)	1079 (14)	Serine/threonine protein kinase	cyclin-dependent protein kinase activity; G2/M transition of mitotic cell cycle
Cyclin B (59 kDa)	923 (13)	Cyclin destruction box	G2/M transition of mitotic cell cycle; anaphase; cytokinesis
Cdc23 (78 kDa)	713 (10)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 8; mitotic anaphase
morula (92kDa)	680 (11)	Cullin	Anaphase promoting complex subunit 2; ubiquitin protein ligase binding; mitotic anaphase
Nurf-38 (38 kDa)	595 (9)	Inorganic pyrophosphatase domain	chromatin remodelling
Cdc27 (102 kDa)	518 (8)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 3; binding; mitotic anaphase
Cdc16 (82 kDa)	477 (8)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 6; binding; mitotic anaphase
PHO (Pleiohomeotic;58 kDa)	413 (5)	Zn finger C2H2-type	DNA binding; chromatin silencing
Scm (Sex comb on midleg; 96 kDa)	368 (5)	MBT repeat; Sterile alpha motif (SAM)	PRC1; Transcriptional repression
Fzr (fizzy-related protein; 54kDa)	367 (4)	WD40 repeat	Anaphase promoting complex activator; exit from mitosis
APC4 (Anaphase promoting complex subunit 4; 88 kDa)	351 (5)	WD40 repeat	Anaphase promoting complex subunit 4

PCC function analysis through expression profiling

To determine the individual functions of each PCC subunit, we utilized RNAi-mediated gene knockdown by treating *Drosophila* S2 cells with dsRNA (Worby et al, 2001) directed against dRING, PSC, PC and PH. As shown in Fig. 2A, Western immunoblotting confirmed that RNAi resulted in almost complete depletion of the targeted factors, without significantly destabilizing associated subunits. However, after dRING knockdown, PSC is upregulated, suggesting a potential feedback loop via dRING-mediated PSC poly-ubiquitylation (our unpublished results).

To investigate the roles of PCC subunits in gene expression control, we extracted RNA form S2 cells treated with dsRNA against individual subunits. Labeled RNA was hybridized on Affymetrix *Drosophila* Genome 2 arrays (http://www.affymetrix.com/support/technical/datasheets/drosophila2_datasheet.pdf), containing 18,500 probe sets representing all known transcripts and variants. Expression indexes were calculated using the RMA algorithm (Irizarry et al., 2003). Examination of RMA expression indexes revealed a bimodal distribution with low values for a large portion of the probe sets. Prior to further analysis, we removed genes that were expressed at very low levels from the data set, using the minimum covariance determinant algorithm (Rousseeuw et al., 1999). To assess the technical variability of microarray experiments, we hybridized each RNA sample from mock- and RNAi- treated cells twice. For each replica, we found a high correlation between expression indexes ($r > 0.9$; $P< 0.001$). Three-independent RNAi knockdown experiments were performed for each subunit using distinct cell batches. For mock-treated cells we performed 12 independent experiments. Then, we applied one-way ANOVA on each probe set to identify genes that changed significantly ($p<0.05$) upon RNAi treatment. For these approximately 5,500 genes, we determined gene expression profiles by taking the ratios between average gene expression indexes obtained from specific RNAi- and mock-treated cells. Next, we performed Principal Component Analysis (PCA), a powerful mathematical procedure that helps to uncover relationships between the transcriptomes of various regulators ((Moshkin et al., 2007). PCA is a linear transformation that finds and projects original variables to the fewest principal components (PCs), accounting for most of the variance in the data set. Expression profiling of subunits of the trxG BAP and PBAP complexes, BRM, MOR, SNR1, Polybromo, BAP170 and OSA, were used for comparison (described Moshkin et al., 2007). About 77 % of the variance in gene expression profiles obtained after individual depletion of the ten proteins analyzed here is explained by PC1-3 (Fig. 2B). PCA revealed that dRING, PC, PSC and PH expression profiles are in close proximity, showing that they are correlated, but they don't overlap completely. As expected, trxG proteins, BRM and its associated proteins, anti-correlated with PCC subunits.

The values for each PC were derived from a linear combination of the original gene expression profiles. To identify and visualize the genes that are co- or differentially regulated by PCC subunits, we selected the top 5% of genes at the right and left tails of the PC1 and PC2 value distributions. Hierarchical cluster analysis of the top scoring PC1 genes revealed that many are coordinately up or down regulated by PCC subunits. As illustrated in Fig. 2C, the most notable feature of hierarchical clustering of top-scoring PC2 genes is the differential behavior of mainly PH, where the majority of genes are down regulated, and then PSC, in comparison to PC and dRING top target genes.

In order to gain insight into the relationship between the PCC transcriptomes, we decided to use an unbiased statistical analysis of the whole data set. Spearman correlation analysis and the derived cluster dendrogram revealed a clear separation between PH, PSC and PC-dRING transcriptomes (Fig. 2D). Venn diagram analysis also revealed that PCC subunits share a large set of target genes, but also regulate a considerable amount of distinctive targets (Fig. 2E). It has to be mentioned that PH and PSC seem to regulate a higher number of unique genes.

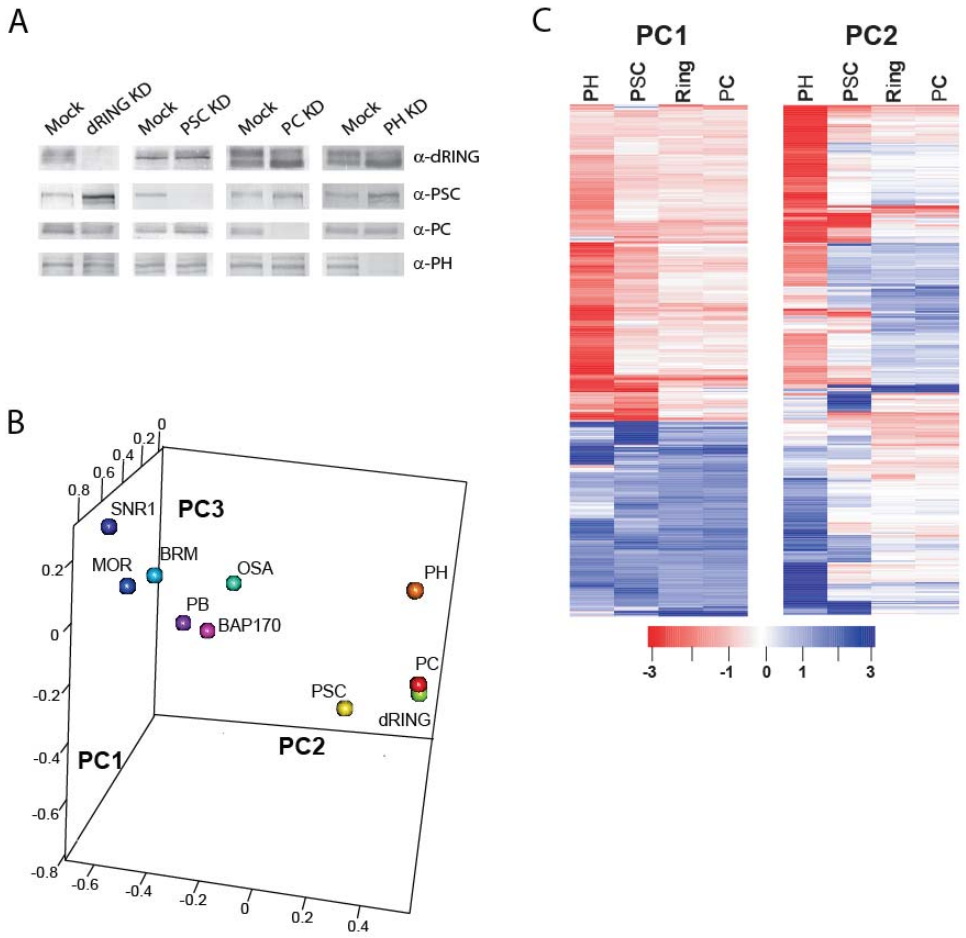
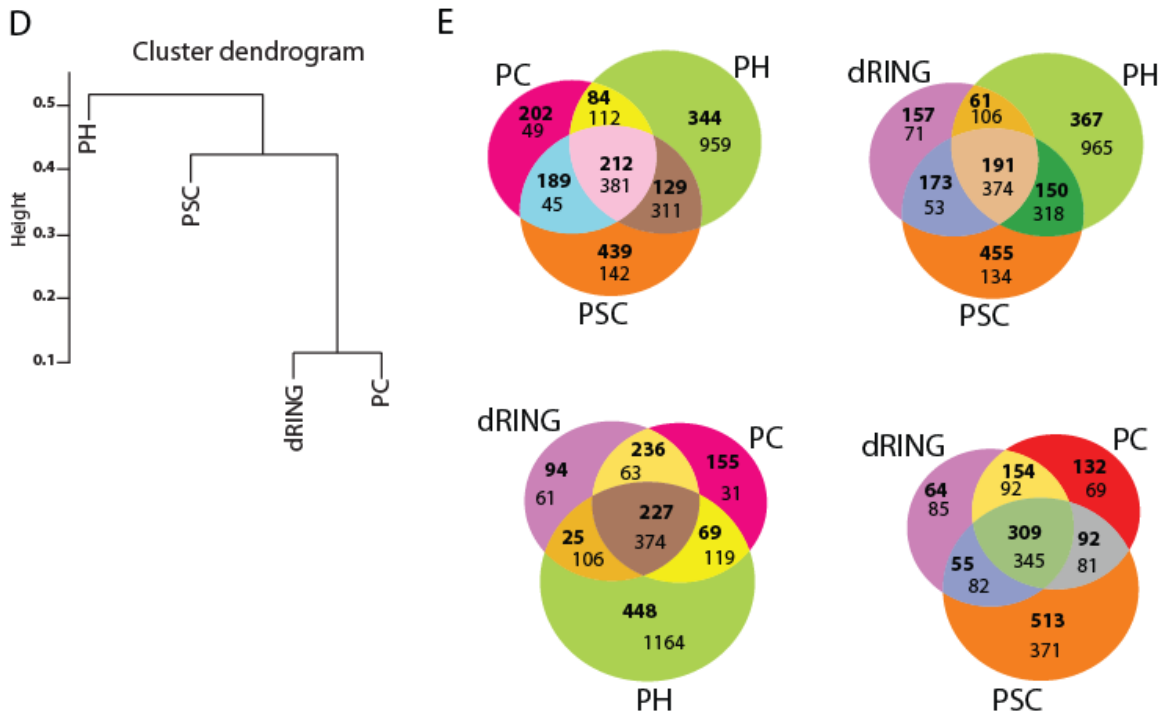


Figure 2. PCC subunits control overlapping, but also distinct transcriptomes
A) S2 cells were either mock-treated or incubated with dsRNAs directed against the PCC subunits, dRING, PSC, PC and PH. Whole-cell extracts were prepared and analyzed by western immunoblotting.
B) Representation of ten expression profiles in a three-dimensional transcriptome space, derived after Principal Component Analysis (PCA). The expression profiles are shown as a projection on the first three PCs after varimax rotation. Each transcriptome represent significant gene targets after three independent biological replicate experiments.
C) Heat map depicting the agglomerative hierarchical clustering of genes, with the highest absolute scores for PC1 and PC2. Changes in gene expression compared to the mock are depicted in blue (up) and red (down) on a log2 scale. White indicates no change.



D) Cluster dendrogram represents the agglomerative hierarchical cluster analysis on microarray expression profiles based on Spearman correlation coefficients. PH and PSC clusters are separated from PC-dRING cluster.
E) Venn diagrams depicting the overlap and differences between the transcriptional targets of dRING, PSC, PC and PH. Numbers indicate significant target genes affected by depletion of the indicated factor(s). The up-regulated genes are indicated in **Bold**.

Thus far, we have analyzed PCC functions in transcription control without considering the biological functions of their target genes. To identify the biological processes regulated by each PCC subunit based on gene expression profiles, we used another unbiased statistical analysis of GO (Gene Ontology) terms. GO terms provide a functional annotation for gene products taken from the corresponding model organism database (Ashburner et al., 2000). GO terms are structured into branched graphs with a common root, describing gene products according to their functional annotations. We focused our analysis on the biological process annotations and GO terms that were significantly up or down regulated as determined by the Student *t* test at a *P* value of < 0.01. Gene ontology analysis suggested the involvement of PcG silencers in a wide range of cellular processes, supporting the notion that they play broad roles in transcription control (Ringrose, 2007). As indicated on Table 5, PCC subunits are implicated on a broad functional network and regulate genes that belong to the same functional groups, signaling, development, cell cycle regulation and proliferation. However, there are differences on the number, identity and regulation level of these target genes in most of the cases. Basically, each PCC subunit shows stronger preference for certain groups of genes. In conclusion, epistatic analysis by expression profiling suggested that dRING, PSC, PC and PH, cooperate during PCC-driven gene regulation, though they appear to have additional functions apart from PCC.

TABLE 5. Top 5% genes regulated by PCC subunits

	PC		PH		PSC		dRING	
GENE GROUP	**UP**	**DOWN**	**UP**	**DOWN**	**UP**	**DOWN**	**UP**	**DOWN**
DNA replication	4	12	2	33	3	12	4	6
DNA repair	8	9	4	26	8	8	7	6
chromatin regulation	20	25	39	86	45	27	35	15
intracellular signaling cascade	117	27	118	91	157	42	100	22
sexual reproduction	53	20	60	100	82	38	53	24
behavior	18	7	30	30	30	10	15	14
neurogenesis	63	12	59	57	87	33	57	10
cell cycle	31	11	30	47	29	24	30	11
cytokinesis	13	1	11	22	16	8	9	1
segregation	8	4	6	32	11	11	5	4
mitosis	14	28	21	23	30	21	23	21
mitotic spindle organization	20	11	24	61	29	26	30	18
cytoskeleton organization	23	6	46	53	49	22	25	5
meiosis	11	7	10	35	12	18	8	7
proliferation	34	1	37	17	34	8	35	1
cell fate determination	40	8	39	12	46	6	37	6
differentiation	36	5	32	16	26	5	29	2
development	186	37	208	148	237	63	190	38
apoptosis	5	5	12	43	12	22	5	6
cell death	14	7	27	20	33	10	9	5
growth	38	2	47	16	62	7	35	3
cell shape	9	1	12	10	14	4	9	3

Numbers of selective top target genes of PC, PH, PSC and dRING involved in important cellular functions. Genes that play more general roles, implicated in biological and metabolic processes, transcription, translation, and intracellular transport are not mentioned.

Discussion

Several lines of genetic and biochemical evidence indicate that the precise molecular mechanisms of PcG functions are quite complicated. The isolation and detailed characterization of differential PcG complexes will give further insight into the understanding of molecular mechanisms involved in PcG-mediated silencing. Thus far, purifications of different PcG complexes suggest that their composition depends on the conditions of purification. In this study, we purified PRC1 related complexes from *Drosophila* embryo nuclear extracts and we identified potential core PRC1-interactors by Mass-spectrometry. Our results suggest a great diversity among PcG complexes. The identity of PCC-associated proteins varies, depending on the individual PCC subunits. Moreover, RNAi experiments in *Drosophila* S2 cells, followed by microarray expression analysis, revealed that each PCC subunit regulates a significant number of unique genes, apart from the common PCC target genes.

As indicated on Fig.1, all four immunopurifications using specific affinity purified antibodies, yielded the PCC subunits, PC, PH, PSC and dRING. Also, there were a few other partially overlapping proteins, namely Scm, PHO, Su(z)2 and Nurf-38. The presence of Scm was expected since it consists a substoichiometric component of PRC1 complex (Saurin et al., 2001). Identification of the sequence specific DNA-binding PcG protein PHO is quite interesting, since it has been implicated previously in targeting PRC1-class complexes (Mohd-Sarip et al., 2002). Detection of Su(z)2 is also intriguing since it is a functional homolog of PSC. As it has been shown recently (Lo et al., 2009), when co-expressed in insect cells with PC, PH and dRING, Su(z)2 can reconstitute a functional complex that binds DNA, compacts chromatin and inhibits chromatin remodeling. In conclusion, Su(z)2 is a potential component of a PRC1-like complex, that exerts its effects together with PCC subunits during gene silencing. Nurf-38 is another potentially interesting associated factor that could facilitate PRC1-driven repression through modulation of chromatin structure. In conclusion, the function of the overlapping proteins in our purifications indicates that they could cooperate with PCC subunits during gene silencing.

In the case of Polycomb-directed immunopurification, apart from PCC subunits and the overlapping proteins mentioned above, another factor was also isolated, called CHIP. CHIP consists an Hsp70-interacting E3-ubiquitin ligase, whose activity seems to depend on its dimerization. Interestingly, PHO is present at highly induced genes on polytene chromosomes and is required for *hsp70* recovery after heat shock (Beisel et al., 2007). The colocalization of PHO with strong signals of active Pol II on polytenes together with the effect of a *pho*-null mutation on the recovery of induced *hsp70*, indicates that PHO may be directly involved in the repression of highly active genes. Since CHIP is an Hsp70-interacting E3-ubiquitin ligase, it is possible that it associates with Polycomb indirectly via PHO, and work together on the repression of highly induced genes.

Regarding the unique Polyhomeotic-associated factors, mass-spectrometry analysis revealed the presence of Connector of kinase to AP1 (Cka) and two proteins of unidentified function, CG17494 and CG2982. Cka is implicated in developmental processes and signaling cascades as JNK cascade, dorsal closure and eye development. It is intriguing that during wound healing, JNK pathway activation leads to downregulation

of PcG gene expression and this mechanism is conserved during cell fate change. The presence of the putative nuclear signaling domain FHA in CG17494 suggests that it could be involved in signaling cascades during cell proliferation, repair or cell cycle regulation. Finally, the JmjC-protein CG2982 is another interesting associated factor, since its ortholog NO66 has potential role in ribosome biogenesis and heterochromatin formation. In conclusion, these factors could associate with PH within a PRC1-related complex and collaborate during signaling and heterochromatin regulation mechanisms.

The purification of dRING-associated factors identified putative interacting proteins, which function in chromatin modification, gene repression and nucleopore complex function. By using Polycomb-depleted extracts, we identified a novel dRING-containing complex (dRAF). Apart from dRING, this complex contained PSC, dKDM2, Megator, CG4877 and Ulp1 (Lagarou et al., 2008). We considered dRING/PSC/KDM2, the core of dRAF complex with potential interactions with the other factors. We uncovered a repressive trans-histone mechanism operating during PcG gene silencing, where the core dRAF removes an active mark from histone H3, and adds a repressive one to H2A (described in detail in Chapter 3).

The proteins that associate exclusively with PSC are implicated in cell cycle regulation. These include cyclin B/ cdk1, and a large part of the Anaphase Promoting Complex (APC). Activation of cyclin B/cdk1 initiates mitotic entry and progression till metaphase, while APC targets the mitotic cyclin B for degradation, resulting in the inactivation of MPF, and finally exit from mitosis. This suggests that PSC could play a pivotal role in mitosis, apart from PCC. Interestingly, RNAi of PSC in *Drosophila* S2 cells results in decreased cell number, impaired proliferation, and FACS analysis shows that PSC-depleted S2 cells exhibit a G2/M arrest phenotype (our unpublished results, Chapter 4). Furthermore, our knockdown studies in flies reveal that PSC is essential during development and might be required for proper anaphase progression (described in detail in Chapter 4).

In order to investigate the role of PCC subunits in transcriptional regulation, we performed RNAi-mediated subunit depletion of PCC followed by microarray analysis. Epistatic analysis through whole-genome expression profiling revealed that each subunit is essential for transcriptional control of many genes, shares a large set of target genes within PCC, but also regulates a large number of distinct genes. This observation comes in agreement with the distribution of PCC subunits on salivary gland polytene chromosomes, showing an overlapping but also distinct pattern of binding sites (Gorfinkiel et al., 2004). Although there is variability on the number, identity and level of regulation among target genes, these belong to the same functional groups involved in signaling and developmental pathways, proliferation, and cell fate determination. This indicates that each subunit is equally required for global in vivo functionality of PCC. In comparison to PC and dRING, the number of PH- and PSC-target genes is much higher, suggesting that they might have extra function apart from PCC (Chapter 4). According to this valuable statistical analysis of gene expression profiles, dRING, PSC, PC and PH targets correlate during PCC-regulated gene expression, but also function independently.

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Chapter 3

dKDM2 Couples Histone H2A Ubiquitylation to Histone H3 Demethylation During Polycomb Group Silencing

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dKDM2 couples histone H2A ubiquitylation to histone H3 demethylation during Polycomb group silencing

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Transcription regulation involves enzyme-mediated changes in chromatin structure. Here, we describe a novel mode of histone crosstalk during gene silencing, in which histone H2A monoubiquitylation is coupled to the removal of histone H3 Lys 36 dimethylation (H3K36me2). This pathway was uncovered through the identification of dRING-associated factors (dRAF), a novel Polycomb group (PcG) silencing complex harboring the histone H2A ubiquitin ligase dRING, PSC and the F-box protein, and demethylase dKDM2. In vivo, dKDM2 shares many transcriptional targets with Polycomb and counteracts the histone methyltransferases TRX and ASH1. Importantly, cellular depletion and in vitro reconstitution assays revealed that dKDM2 not only mediates H3K36me2 demethylation but is also required for efficient H2A ubiquitylation by dRING/PSC. Thus, dRAF removes an active mark from histone H3 and adds a repressive one to H2A. These findings reveal coordinate *trans*-histone regulation by a PcG complex to mediate gene repression.

[*Keywords*: Polycomb; epigenetic; chromatin; histone modification; ubiquitin]

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Polycomb group (PcG) and trithorax group (trxG) genes encode for antagonistic transcriptional coregulators that play critical roles in stem cell biology, development, and cancer (Ringrose and Paro 2004; Sparmann and van Lohuizen 2006; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). They were first identified genetically in the fruit fly *Drosophila* as factors required for maintaining the correct expression of homeotic genes throughout development (Maeda and Karch 2006). However, over the years it has become clear that many PcG and trxG proteins play diverse regulatory roles in gene control. Generally speaking, PcG proteins function as transcriptional repressors whereas trxG proteins act as activators.

PcG silencing involves the activities of at least two major types of complexes, PRC1 and PRC2 (Ringrose and Paro 2004; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). *Drosophila* PRC1 contains four core PcG proteins—Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC), and Sex combs extra (SCE), usually referred to as dRING—which constitute the PC core complex (PCC). Mammalian RING1B and fly dRING are ubiquitin E3 ligases responsible for histone H2A monoubiquitylation (H2Aub), a histone modification associated with transcriptional silencing (Wang et al. 2004; Weake and Workman 2008). PRC2-class complexes harbor the histone H3 Lys 27 (H3K27) methyltransferase Enhancer of zeste [E(z)], extra sex combs (ESC), and CAF1 p55 (Ringrose and Paro 2004; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). Recently, a related complex has been described containing the PcG protein PC-like (PCL) that appears to be particularly important for H3K27 trimethylation (Nekrasov et al. 2007; Sarma et al. 2008). One important but still poorly understood issue remains the question of how PcG complexes are recruited to silence specific genes. In *Drosophila*, a key tethering factor is the sequence-specific DNA-binding PcG protein PHO that binds PcG response elements (PREs) and can initiate the recruitment of PcG silencers (Brown et al. 1998; Fritsch et al. 1999; Mohd-Sarip et al. 2002, 2005, 2006). PHO has also been purified in complex with the INO80 ATP-dependent chromatin remodeler and, as PHORC, in complex with the PcG protein SFMBT (Klymenko et al. 2006).

Modulation of chromatin structure has emerged as a key molecular mechanism through which PcG and trxG proteins control gene expression. Histones are subjected to a wide range of reversible post-translational modifica-

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tions including acetylation, phosphorylation, methylation, and ubiquitylation (Berger 2007; Bhaumik et al. 2007; Ruthenburg et al. 2007). These histone modifications can promote recruitment of specific regulatory factors and modulate chromatin accessibility. For many histone modifications, the transcriptional output is critically dependent on what specific residues are modified. For example, lysine methylation can either be associated with gene silencing (H3K9, H3K27, and H4K20) or with gene activation (H3K4, H3K36, and H3K79). Complicating matters further, these lysines can be mono-, di-, or trimethylated, each of which may have distinct functional consequences. Whereas initially believed to be a permanent mark, recent research has identified a range of specific histone lysine demethylases (KDMs) (Shi 2007). An important group of KDMs are characterized by the presence of a JmjC demethylase domain. Like the lysine methyltransferases (KMTs), KDMs display exquisite substrate specificity and distinct KDMs target different methylated lysines. Depending on what mark they target, KDMs act as either transcriptional corepressors or coactivators.

Another important post-translational histone modification is monoubiquitylation of histones H2A and H2B (Osley 2006; Weake and Workman 2008). H2B monoubiquitylation (H2Bub) at Lys 120 by the E3-ligase BRE1 is an active mark, linked to transcriptional elongation. In contrast, H2Aub at Lys 119 has been implicated in PcG transcription silencing, human X-chromosome inactivation, and heterochromatin formation. Thus, H2Aub and H2Bub appear to be antagonistic histone modifications. The E3 responsible for the majority of H2A ubiquitylation is mammalian RING1B or fly dRING (Wang et al. 2004). The ubiquitylation activity of RING1B is strongly stimulated by association through RING–RING formation with BMI1, a PSC homolog (Cao et al. 2005; Ben-Saadon et al. 2006; Buchwald et al. 2006). We note that H2Aub is the target of diverse regulatory pathways. For example, during the DNA-damage response, histones H2A and H2AX are ubiquitylated by RNF8 (Mailand et al. 2007). Selective H2Aub deubiquitylation can be mediated by a number of ubiquitin proteases involved in cell cycle control and transcription (Joo et al. 2007; Nakagawa et al. 2008; Weake and Workman 2008).

To gain more insight in the enzymatic network underpinning PcG silencing, we characterized dRING and its associated factors. Although it is a bona-fide PRC1 subunit, we found that a significant fraction of dRING is part of a separate assemblage we named dRAF (dRING-associated factors). We identified dKDM2 as a key dRAF subunit that plays a pivotal role in a novel *trans*-histone pathway involving the removal of an active histone H3 methyl mark and formation of the repressive H2Aub mark during PcG silencing.

Results

Identification of dRAF

In the course of our biochemical characterization of *Drosophila* PcG protein complexes in partially purified embryo nuclear extracts (Fig. 1A), we came to realize that several PRC1 subunits exist outside this complex. Immunopurification using affinity-purified antibodies directed against PC yielded the PRC1 subunits dRING, PH, PSC, and PC itself, as established by SDS–polyacryl-

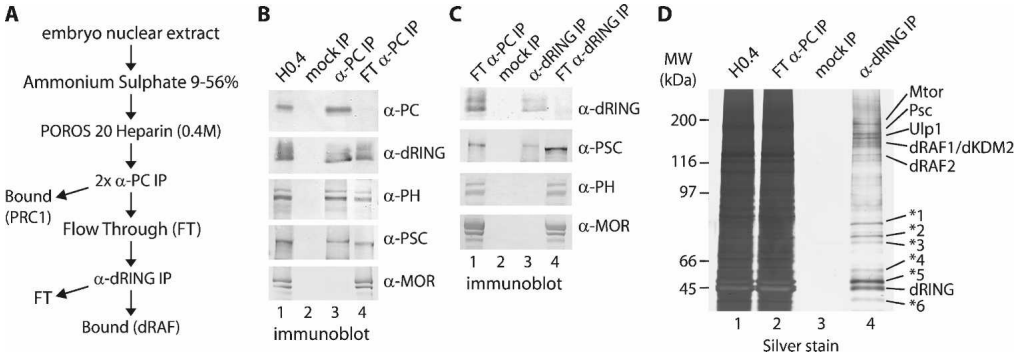

Figure 1. Identification of dRAF. (*A*) Outline of the chromatographic scheme used to purify dRAF. (*B*) dRING, PH, and PSC also exist outside PRC1. Protein A Sepharose beads coated with either control anti-GST antibodies (Mock IP) or affinity-purified antibodies directed against PC (α-PC IP) were used to immunopurify PC and associated factors from partially purified and concentrated embryo nuclear extracts (H0.4). Bound and unbound material (FT α-PC IP) was analyzed by Western immunoblotting using antibodies directed against the indicated proteins. (*C*) dRING and PSC form part of a complex distinct from PRC1 and devoid of PC and PH. The unbound fraction after 2 subsequent PC immunodepletions (FT α-PC IP) was incubated with protein A Sepharose beads coated with affinity-purified antibodies directed against dRING. Bound (α-dRING IP) and unbound (FT α-dRING IP) material was analyzed followed by Western immunobloting. (*D*) Identification of dRAF. H0.4, twice PC-immunodepleted (FT α-PC IP) input of the mock-, or dRING immunopurified (α-dRING IP) fractions were resolved by SDS-PAGE and visualized by silver staining. Proteins present in bands excised from a gel run in parallel were identified by nanoflow LC-MS/MS mass spectrometry. Identified proteins are indicated. Their mascot score and number of unique peptides identified are Mtor: 4741 (Mascot score), 55 (number of unique peptides); PSC: 2226, 31; dKDM2 (dRAF1, CG11033): 1920, 23; Ulp1: 1566, 20; dRAF2 (CG4877): 1312, 17; dRING (SCE): 1160, 12. Asterisks indicate background proteins we routinely observe in our immunopurifications: (*1) Heat shock cognate 4; (*2) γ-tubulin; (*3) RanGap; (*4) β-tubulin; (*5) yolk protein 1 and 2; (*6) RFC.

amide electrophoresis (PAGE) followed by Western immunoblotting (Fig. 1B, lane 3). To our surprise, inspection of the PC-depleted flow-through (FT) revealed the presence of significant amounts of dRING, PH, and PSC (Fig. 1B, lane 4). Thus, these proteins are not solely present as stable PRC1 components. Moira (MOR), a core subunit of the *Drosophila* (P)BAP chromatin remodeling complexes, served as a negative control for the PC-immunopurification. In conclusion, these results indicated that substantial amounts of dRING, PH, and PSC might be present either as free molecules or as components of protein assemblages other than PRC1.

To identify a potentially novel dRING-containing complex, we immunopurified dRING from the PC-depleted extracts using protein A beads coated with affinity-purified α-dRING antibodies. After extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, bound and unbound material were resolved by SDS-PAGE and analyzed by Western blotting (Fig. 1C). PSC copurified with dRING (Fig. 1C, lane 3), although a significant amount remained in the unbound fraction (Fig. 1C, lane 4). In contrast to PSC, PH was not associated with dRING. Thus, it appeared that dRING and PSC are part of a protein complex that is distinct from PRC1 and lacks PC and PH. We visualized the dRAFs by SDS-PAGE followed by silver staining (Fig. 1D).

Mass spectrometric analysis confirmed the presence of dRING and PSC. A number of additional proteins were identified suggesting a number of potential links between PcG silencing and distinct cellular processes. (1) As discussed above, dRING and PSC are classic PcG proteins involved in histone H2A ubiquitylation. (2) Megator (Mtor) is a dynamic subunit of the nuclear pore complex that also exists separately in the nucleoplasm and interacts functionally with the MSL dosage compensation complex and has been implicated in mitotic spindle assembly (Qi et al. 2004; Mendjan et al. 2006). (3) Ulp1 is a SUMO peptidase that, like Mtor, is found associated with the nuclear pore complex (Hay 2007). (4) dRAF1 (CG11033), the fly homolog of KDM2 (Tsukada et al. 2006; Frescas et al. 2007; Shi 2007), is a particularly intriguing protein harboring an F-box, CXXC-type zinc finger, PHD finger, and JmjC demethylase domain. (5) Finally, dRAF2 (CG4877) contains a MYND zinc finger, a protein–protein interaction domain implicated in the recruitment of corepressors. In summary, our analysis suggests a greater complexity among PRC1-related PcG complexes than previously appreciated. Below we will focus on the functional characterization of dRAF.

dRAF and PRC1 are separate PcG complexes

First, we set out to confirm that PRC1 and dRAF are indeed separate complexes. We performed a series of co-immunoprecipitations from crude embryo nuclear extracts using antibodies directed against dRING, PC, PH, and PSC. Western immunoblotting showed that dKDM2 and dRAF2 are stably associated with dRING and PSC in crude embryo nuclear extracts (Fig. 2A). Albeit somewhat less efficiently, Mtor and Ulp1 also clearly interacted with dRAF. In contrast, none of these proteins copurified with the PRC1-specific PC or PH. Immunoprecipitations using antibodies against dRAF2, Mtor, and dKDM2 yielded both dRING and PSC, but not PC or PH (Fig. 2B). To investigate the association of dRING with dKDM2 and other interacting proteins further, we followed them over a series of purification steps (Fig. 2C). The core dRAF subunits dRING, PSC, and dKDM2 coelute with dRAF2, Mtor, and Ulp1 during size-exclusion chromatography, behaving as a large multiprotein assemblage (Fig. 2D). These proteins peaked in fractions corresponding to apparent molecular masses ranging from ~600 to 900 kDa. PRC1 subunits PC and PH also migrate as a large complex, although PC displayed significant trailing. The chromatin remodeler ISWI served as a reference and has a smaller apparent molecular mass. Glycerol gradient sedimentation analysis of a Sephacryl S-300 column peak fraction (#14) revealed clearly overlapping cosedimentation of dRAFs as a large complex (Fig. 2E). Again, not all proteins displayed an identical distribution over the gradient, possibly reflecting complex disassembly or heterogeneity. For example, PC, dRING, and to a lesser extent, PSC showed broad distribution profiles compared with dKDM2, dRAF2, and PH, suggesting dissociation from PRC1 and dRAF.

In conclusion, dRAF and PRC1 form two distinct PcG complexes that share PSC and dRING as common subunits. PC and PH are absent from dRAF and define PRC1, whereas dKDM2 is missing from PRC1 and defines dRAF. We consider dRING/PSC/KDM2 the core of the dRAF complex with potential interactions with other factors, which are not explored further here. The intriguing presence of dKMD2, harboring regulatory motifs such as a JmjC type demethylase domain and an F-box, in a complex with dRING raised a number of interesting possibilities. First, the identification of dRAF as a separate entity sharing subunits with PRC1 suggested that these two complexes might work together during transcriptional silencing. If so, the transcriptional profile of cells lacking dKDM2 and other dRAF or PRC1 subunits would be expected to be similar. Second, dKDM2 would be expected to behave genetically as a transcriptional silencer. Thus, in vivo dKDM2 is predicted to cooperate with PC but to antagonize gene activation by particular trxG methyltransferases. Third and most interestingly, the association between dKDM2 and dRING suggests that dRAF might couple histone demethylation and histone ubiquitylation. In the sections below, we present evidence supporting these three predictions.

dKDM2 and PRC1 control overlapping transcriptomes

To investigate their relative roles in gene expression control, we treated S2 cells with dsRNA directed against dKDM2, dRING, PSC, PC, and PH. As shown in Figure 3A, this caused a significant reduction in the protein level of the targeted factors, without significantly destabilizing associated subunits. However, PSC is up-regulated following a dRING knockdown, suggesting a po-

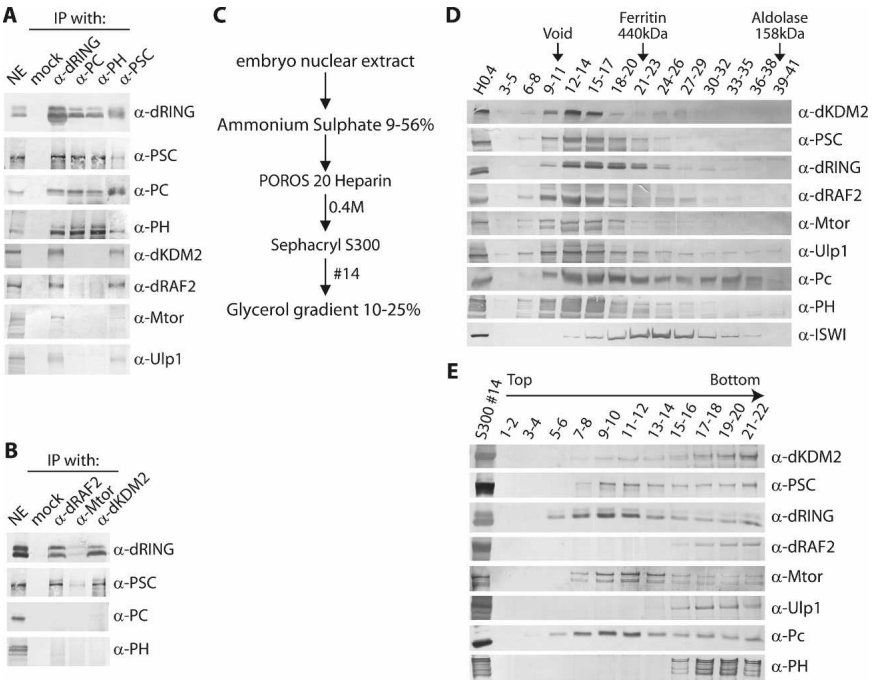

Figure 2. dRAF and PRC1 are separate PcG complexes that share dRING and PSC. (*A*) dRING, PSC, dKDM2, dRAF2, Mtor, and Ulp1 associate in crude *Drosophila* embryo nuclear extracts (NE). Nuclear extract was incubated with Protein A Sepharose beads coated with either control anti-GST antibodies (mock) or affinity-purified α-dRING, α-PC, α-PH, or α-PSC antibodies. After extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting. Lane *1* represents 10% of the input material used in the binding reactions. (*B*) dRING and PSC, but not PC or PH, coimmunoprecipitate with dRAF2, Mtor, and dKDM2. Coimmunoprecipitations were performed and analyzed as described above. (*C*) Outline of the chromatographic scheme used to characterize the dRAF complex further. (*D*) The POROS 20 Heparin 400 mM eluate fractionated by Sephacryl S-300 size-exclusion chromatography. The indicated fractions were combined and resolved by SDS-PAGE, followed by immunoblotting with antibodies directed against dKDM2, PSC, dRING, dRAF2, Mtor, Ulp1, Pc, PH, and ISWI. The core dRAF subunits and interactors peaked in fractions corresponding to apparent molecular masses ranging from ~600 to 900 kDa. The elution of the voided volume (void) and the elution of the markers ferritin (440 kDa) and aldolase (158 kDa) are indicated. (*E*) The dRAF peak fraction #14 from the Sephacryl S-300 column was centrifuged through a 10%–25% glycerol gradient, and collected fractions were examined for the presence of dRAF or PRC1 subunits by immunoblotting.

tential feedback loop via dRING-mediated PSC polyubiquitylation (Buchwald et al. 2006; our unpublished results). For each subunit we performed three fully independent RNAi-mediated knockdown experiments using distinct cell batches. For comparison, we used mock-treated cells and expression analysis of cells depleted for selective subunits of the BAP, PBAP, and ISWI chromatin remodelers (Moshkin et al. 2007).

Previously, we found that principal component analysis (PCA) is an effective mathematical tool to uncover relationships between the transcriptomes of various regulators (Moshkin et al. 2007). PCA is a linear transformation that finds and projects original variables to the fewest principal components (PCs), accounting for most of the variance in the data set. About 77% of the variance in gene expression profiles obtained after individual depletion of the 12 proteins analyzed here is explained by PC1–3. As illustrated by Figure 3B, PCA revealed that the dKDM2, dRING, PC, PSC, and PH expression profiles clearly cluster together, showing that they are highly correlated. As expected of trxG proteins, BRM and its associated proteins anti-correlated with the PcG proteins. Likewise, the ISWI transcription profile was distinct from the PcG proteins. Venn diagram analysis also revealed that dRAF and PRC1 share a large set of target genes (Fig. 3C). Gene ontology analysis suggested the involvement of PcG silencers in a wide range of cellular processes, supporting the notion they play broad roles in transcription control (Ringrose 2007). In conclusion, epistatic analysis by expression profiling suggested a functional cooperation between PRC1 and dRAF.

dkdm2 is an enhancer of Pc

We next wished to test directly whether dKDM2, like PC, might be involved in silencing of the homeotic loci in vivo. We obtained three independent mutant fly lines harboring distinct P-element insertions in the *dkdm2* gene, which is located on the third chromosome. $dkdm2^{KG04325}$ and $dkdm2^{DG12810}$ are homozygous lethal, while $dkdm2^{EY01336}$ is homozygous viable hypomorph. Neither homozygous $dkdm2^{EY01336}$ nor heterozygous $dkdm2^{KG04325}$ and $dkdm2^{DG12810}$ animals displayed obvious homeotic transformations. To test the effects of *dkdm2* mutations on homeotic gene silencing, we crossed each of the three alleles into flies carrying either the Pc^{1} or Pc^{3} allele. We found that in the trans-heterozygous progeny, the *dkdm2* mutant alleles significantly increased the frequency of a range of homeotic transformations (Fig. 4A,B). These include the appearance of sex combs on the second (L2–L1) and third (L3–L1) pairs of legs, transformation of the fourth abdominal segment into the semblance of the fifth (A4-A5), and wing-to-haltere (W–H) transformations. In all cases, the frequency of transformation is much higher in flies trans-heterozygous for one of the *dkdm2* mutants and either Pc^{1} or Pc^{3} compared with animals carrying only a *Pc* mutation. These strong genetic interactions were ob-

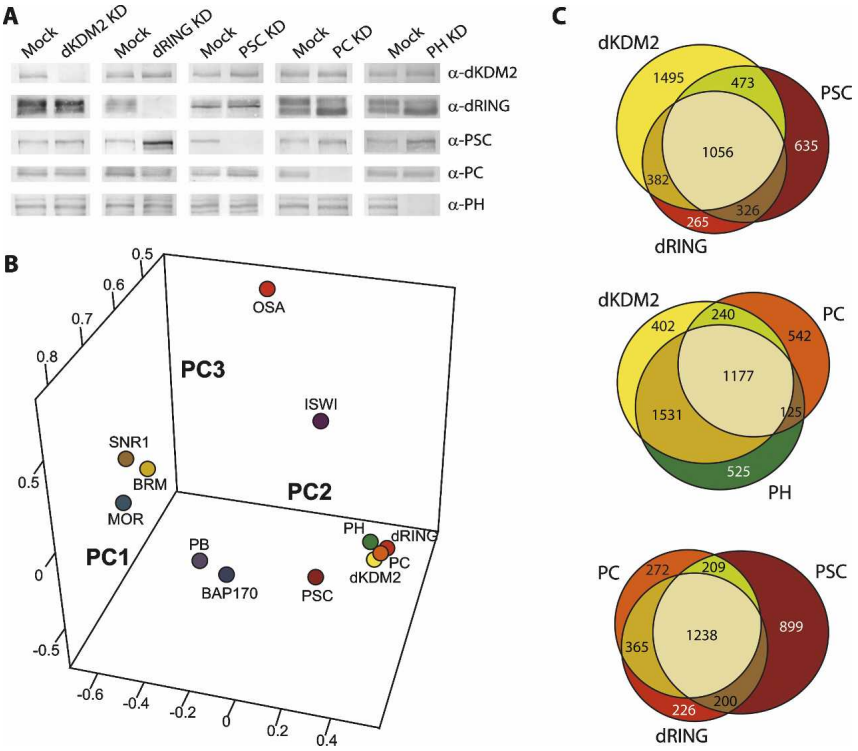

Figure 3. dKDM2 and PRC1 control overlapping transcriptomes. (*A*) S2 cells were either mock-treated or incubated with dsRNAs directed against selective dRAF or PRC1 subunits dKDM2, dRING, PSC, PC, and PH. Whole-cell extracts were prepared and analyzed by Western immunoblotting. (*B*) Representation of 12 expression profiles in a three-dimensional transcriptome space, derived after PCA. RNA was isolated, labeled, and hybridized on Affymetrix *Drosophila* Genome 2 arrays. Expression indexes were calculated using the Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003). The Minimum Covariance Determinant algorithm (Rousseeuw and van Driessen 1999) was used to remove genes that were expressed at very low levels. Next, we applied one-way ANOVA on each probe set to identify genes that changed significantly ($P < 0.05$) upon RNAi treatment. For these ~5500 genes, we determined gene expression profiles by taking the ratios between average gene expression indexes obtained from specific RNAi- and mock-treated cells. Expression profiling of subunits of the trxG BAP and PBAP complexes, BRM, MOR, SNR1, OSA, Polybromo, BAP170, and OSA, as well as ISWI has been described (Moshkin et al. 2007). The expression profiles are shown as a projection on the first three PCs after varimax rotation. Each transcriptome represent significant gene targets after three independent biological replicate experiments. (*C*) Venn diagrams depicting the overlap and differences between the transcriptional targets of the dRAF-signature subunit dKDM2; dRING and PSC, shared by dRAF and PRC1; and the PRC1-selective subunits PC and PH. Numbers indicate significant target genes affected by depletion of the indicated factor(s).

served using different independent alleles, making it highly unlikely that they were due to nonspecific influences of genetic background. In summary, genetic interaction studies established that *dkdm2* acts as an enhancer of *Pc*, supporting the notion that dRAF and PRC1 cooperate in vivo.

dkdm2 is a suppressor of histone methyltransferases trx and ash1

We used a genetic approach to test whether, like the PcG silencers, dKMD2 might counteract trxG protein-mediated transcriptional activation. The trxG genes *trx* and *ash1* encode for histone methyltransferases that are associated with gene activation. TRX is a well-established histone H3K4 methylase (Bhaumik et al. 2007). Although there appears to be less consensus on the ASH1 target, a recent study made a compelling argument that ASH1 mediates H3K36me2 (Tanaka et al. 2007). Flies carrying a mutant trx^1 or $ash1^{10}$ allele display transformations of abdominal segment A5 toward the likelihood of A4, recognizable by a loss of pigmentation in A5 (Fig. 4C). Moreover, these animals frequently show a partial haltere-to-wing (H–W) transformation. In our crosses we analyzed the progeny of homozygous trx^1 or $ash1^{10}$ females crossed with either wild-type or *dkdm2* mutant males. *Trans*-heterozygous animals in which a *dkdm2* allele is combined with either a trx^1 or $ash1^{10}$ mutant allele display a strikingly reduced frequency of homeotic transformations (Fig. 4D). We conclude that *dkdm2* acts both as an enhancer of *Pc* silencing and as a suppressor of trx^1 and $ash1^{10}$ mutants. Because dKDM2 is a putative histone demethylase it is highly likely that its function involves the removal of an activating methyl mark.

dKDM2 is required for H3K36me2 demethylation and H2A ubiquitylation in cells

A prominent feature of dKDM2 is the presence of a JmjC demethylase motif. Yeast has a single KDM2 homolog, Jhd1 (yKDM2), but there are two human homologs: hKDM2A (JHDM1a or FBXL11) and hKDM2B (JHDM1b or FBXL10). Both hKDM2A and yKDM2 have been shown to catalyze H3K36me1/2 demethylation, although removal of the me2 mark appeared more efficient than that of me1 (Tsukada et al. 2006). However, hKDM2B was recently found to be a nucleolar protein that mediates selective demethylation of H3K4me3 (Frescas et al. 2007). To determine the histone target of dKDM2 we used RNAi-mediated depletion of S2 cells. In addition to dKDM2 we also depleted S2 cells for dRING, PSC, PC, and PH (Fig. 3A). Next, we isolated bulk histones from mock- and RNAi-treated cells by acid extrac-

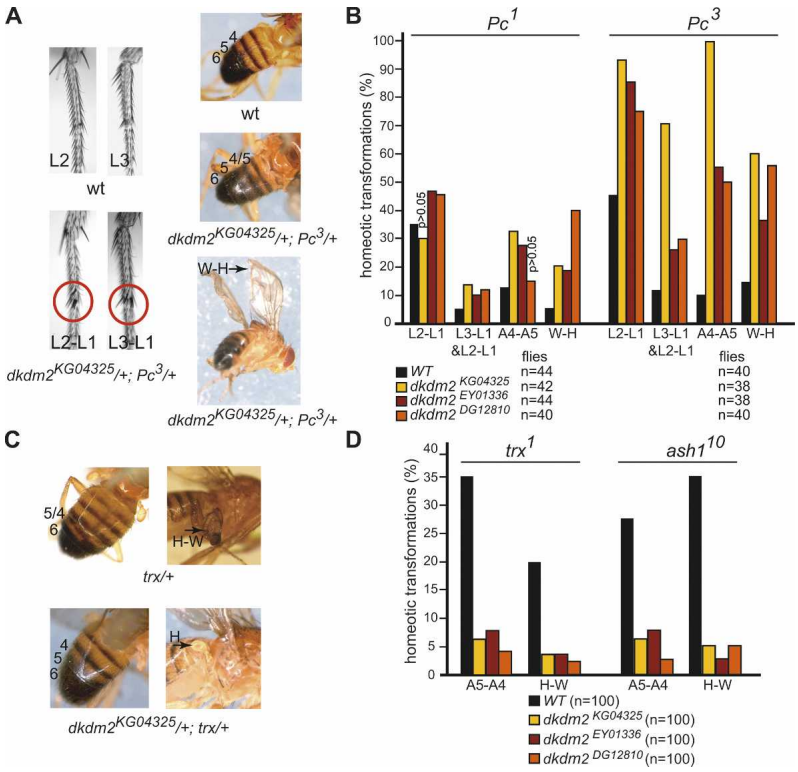

Figure 4. *dkdm2* is an enhancer of *Pc* but a suppressor of *trx* and *ash1*. (*A*) Representative examples of homeotic transformations that were scored in the transheterozygous progeny of a series of crosses in which each of the $dkdm2^{KG04325}$ $dkdm2^{EY01336}$ or $dkdm2^{DG12810}$ mutant alleles or a wild-type (wt) allele were combined with either Pc^1 or Pc^3 mutations. Sex combs are a row of dark, thick bristles, which normally only occur on the first pair of legs of male flies. In flies with defective Pc silencing, sex combs also appear on the second (L2–L1) or third (L3–L1) leg. Transformation of the fourth abdominal into the semblance of the posterior fifth (A4-A5) can be detected by the increased pigmentation of A4. Some flies display a defective wing development indicative of a wing-to-haltere (W–H) transformation. (*B*) Graphical representation of the frequencies of homeotic transformations in flies heterozygous for Pc^1 or Pc^3 mutations but wild type for *dkdm2* (black bars), or transheterozygous animals carrying Pc^1 or Pc^3 combined with either $dkdm2^{KG04325}$ (yellow bars), $dkdm2^{EY01336}$ (red bars), or $dkdm2^{DG12810}$ (orange bars) mutations. The frequency of homeotic transformations is significantly higher in flies transheterozygous for *dkdm2* and *Pc* mutations compared with flies heterozygous for only *Pc* mutations, as determined by Student's *t*-test ($P < 0.05$). Two exceptions are indicated. (*C*) Representative examples of homeotic transformations observed in the transheterozygous progeny of homozygous trx^1 or $ash1^{10}$ females crossed with either wild-type or *dkdm2* mutant males. Abbreviations of the homeotic transformations: (H–W) haltere-to-wing; (A5–A4) transformation of the fifth abdominal segment into the semblance of the fourth. (*D*) Graphical representation of the frequencies of homeotic transformations in flies heterozygous for either Trx^1 or $ash1^{10}$ (black bars) or transheterozygous animals carrying either trx^1 or $ash1^{10}$ in combination with the indicated *dkdm2* mutations. The frequency of homeotic transformations is significantly lower in flies transheterozygous for *dkdm2* and either trx^1 or $ash1^{10}$ compared with trx^1 or $ash1^{10}$ heterozygotes wild type for *dKDM2*, as determined by Student's *t*-test ($P < 0.05$).

tion. The levels of H3K36me1, H3K36me2, H3K36me3, and H3K4me3 were examined by Western immunoblot analysis using modification-specific antibodies (Fig. 5A). dKDM2 depletion caused a strong increase in H3K36me2 levels, whereas H3K36me1, H3K36me3, and H3K4me3 remained unchanged. In contrast, depletion of dRING, PSC, PC, or PH did not affect any of these histone marks. We conclude that dKDM2 selectively demethylates H3K36me2.

The second prominent feature of KDM2 is the presence of an F-box, a protein–protein interaction domain found in a class of proteins involved in ubiquitylation target selection. Previous research established that dRING is a key factor in histone H2A ubiquitylation (Wang et al. 2004). Moreover, it seemed plausible that PSC, like its mammalian homolog BMI1 (Cao et al. 2005; Buchwald et al. 2006), will stimulate the activity of dRING. A confounding factor is, however, that dRING and PSC are subunits of two distinct complexes, dRAF and PRC1. This raises the question of the relative importance of dRAF versus PRC1 for histone H2A ubiquitylation. Related to this question, we were particularly interested in the possibility that dKDM2 might modulate histone H2A ubiquitylation by dRING/PSC. Following cellular depletion of dKDM2, we observed a striking loss of histone H2Aub, as revealed by Western immunoblotting (Fig. 5B). H2Aub was detected using antibodies directed against histone H2A, conjugated ubiquitin, or the E6C5 monoclonal antibody that can recognize H2Aub. The effect of dKDM2 depletion on H2Aub is highly specific, as we did not detect any change in the levels of H2Bub, as determined using antibodies directed against H2B or ubiquitin. The anti-ubiquitin antibody can be used to distinguish between H2Bub and H2Aub because the former migrates slightly slower than the latter on 18% SDS-PAGE. Importantly, the reduction in H2Aub levels due to loss of dKDM2 was comparable with that observed in cells lacking the H2A E3 ligase, dRING, or PSC. In contrast to loss of dKDM2, depletion of the PRC1 subunits PC or PH had no effect on H2Aub levels. These findings suggest that, at least in these cells, dRAF is more critical for bulk cellular histone H2A ubiquitylation than PRC1. We conclude that dKDM2 has a dual function in chromatin regulation: First, dKDM2 mediates histone H3K36me2 demethylation, removing an active chromatin mark. Second, dKDM2, together with dRING/PSC, is responsible for the majority of histone H2A ubiquitylation in cells, a repressive chromatin mark.

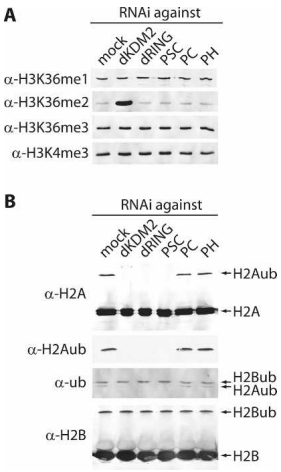

Figure 5. Depletion of endogenous dKDM2 causes increased histone H3K36me2 and loss of H2Aub. (*A*) Reduction of dKDM2 levels leads to a selective increase in H3K36me2. S2 cells were either mock-treated or incubated with dsRNAs directed against selective dRAF or PRC1 subunits dKDM2, dRING, PSC, PC, and PH (see Fig. 3A). Histones were purified by acid extraction and resolved by SDS-PAGE followed by Western blotting using the indicated antibodies directed against selective methyl marks. (*B*) dKDM2, dRING, and PSC are required for histone H2A ubiquitylation but do not affect H2Bub. The ubiquitylation status of histones purified from RNAi-treated S2 cells were analyzed by Western blotting using antibodies directed against either H2A, H2Aub, ubiquitin (ub), or H2B. The nonubiquitylated histones as well as H2Aub and H2Bub are indicated.

dRAF mediates H3K36me2 demethylation

Our depletion studies in cells indicated that dKDM2 is a bifunctional enzyme required for H3K36me2 demethylation as well as H2A ubiquitylation. To obtain direct evidence for this notion and to gain insight into the molecular mechanism of action of dKDM2, we decided to reconstitute both reactions in vitro. We used the baculovirus expression system to coexpress Flag-tagged dKDM2 (F-dKDM2), dRING, and PSC in Sf9 cells. Immunopurification on an anti-Flag column followed by peptide elution yielded a dRAF core assemblage comprising F-dKDM2 and apparently stoichiometric amounts of PSC and dRING (Fig. 6A). These results showed that dKDM2, dRING, and PSC form a stable trimeric complex. Likewise, we assembled and purified PCC, composed of F-PH, PC, PSC, and dRING, F-dKDM2 alone, a heterodimeric F-dRING/PSC complex, F-PSC alone, F-dRING alone, and heterodimeric F-PH/PC. As a substrate for our reconstituted reactions, we used purified endogenous oligonucleosomes harboring a representative full range of histone modifications. We first tested the ability of the recombinant dKDM2/dRING/PSC complex to demethylate H3K36me2 in vitro. Incubation of oligonucleosomes with dKDM2/dRING/PSC resulted in the efficient removal of H3K36me2, but not of H3K36me1, H3K36me3, or H3K4me3 (Fig. 6B). As expected, PCC did not display any demethylase activity. dKDM2 alone also efficiently demethylate H3K36me2, establishing that dRING and PSC were dispensable for this enzymatic activity (Fig. 6C). In conclusion, as suggested by our RNAi-mediated depletion experiments, these in vitro reconstitution assays establish that dRAF mediates the selective removal of the H3K36me2 mark.

dKDM2 stimulates H2A ubiquitylation by dRING/PSC

We next compared the ability of the dRAF core complex and PCC to ubiquitylate histone H2A. Approximately equimolar amounts of either dKDM2/dRING/PSC or PCC were incubated with nucleosomes, followed by SDS-PAGE and Western blotting using antibodies directed against H2A, ubiquitin, or H2Aub. We only observed efficient ubiquitylation in the presence of dKDM2/dRING/PSC, but not in reactions containing PCC (Fig. 6D). The reaction was specific for histone H2A because there was no detectable ubiquitylation of H2B. We note that the endogenous H2Aub or H2Bub is not detectable with the histone amounts loaded on these gels. In conclusion, the results from our in vitro reconstitution dovetail perfectly with the results of dKDM2 depletion, which suggested that dRAF, rather than PRC1, is responsible for bulk H2A ubiquitylation.

To investigate the role of dKDM2 in more detail, we incubated nucleosomes with various combinations of dRAF subunits (Fig. 6E). dRING alone mediates only a low level of H2A ubiquitylation (Fig. 6E, lanes 2–4). In agreement with the observations of Buchwald et al. (2006) for BMI1, we detected low amounts of H2Aub in reactions containing PSC (Fig. 6E, lanes 5–7) and a modestly increased efficiency when both PSC and dRING were present (Fig. 6E, lanes 15–17). In contrast, dKDM2 robustly stimulated H2A ubiquitylation by dRING (Fig. 6E, lanes 18–20) as well as by dRING/PSC (Fig. 6E, lanes 21–23). By itself dKDM2 did not affect H2Aub (Fig. 6E, lanes 8–10) nor stimulated PSC (Fig. 6E, lanes 12–14). Again, the reactions were highly selective, as in the same assay histone H2B was not ubiquitylated (data not shown). The PRC1 selective subunits PC/PH did not stimulate H2A ubiquitylation by dRING/PSC (Fig. 6F, lanes 4–11), whereas dKDM2 strongly activated an amount of dRING/PSC that by itself did not yield detectable H2Aub (Fig. 6F, lanes 12–15).

Because dRAF links H3K36me2 demethylation to H2A ubiquitylation, we wondered whether these distinct biochemical reactions might be interdependent. H3K36me2 demethylation and H2A ubiquitylation can occur concomitantly in the same reaction (Fig. 7A). Because dKDM2 alone suffices to efficiently catalyze H3K36me2 demethylation (Fig. 6C), this reaction clearly does not depend on H2A ubiquitylation. However, it remained possible that H2A ubiquitylation might be dependent on H3K36me2 demethylation. To address this issue, we first purified endogenous mononucleosomes to test whether H2Aub and H3K36me2 could coexist within the same nucleosome. We used antibodies directed against H3K36me2 to immunodeplete the mononucleosome pool for this histone mark (Fig. 7B). All

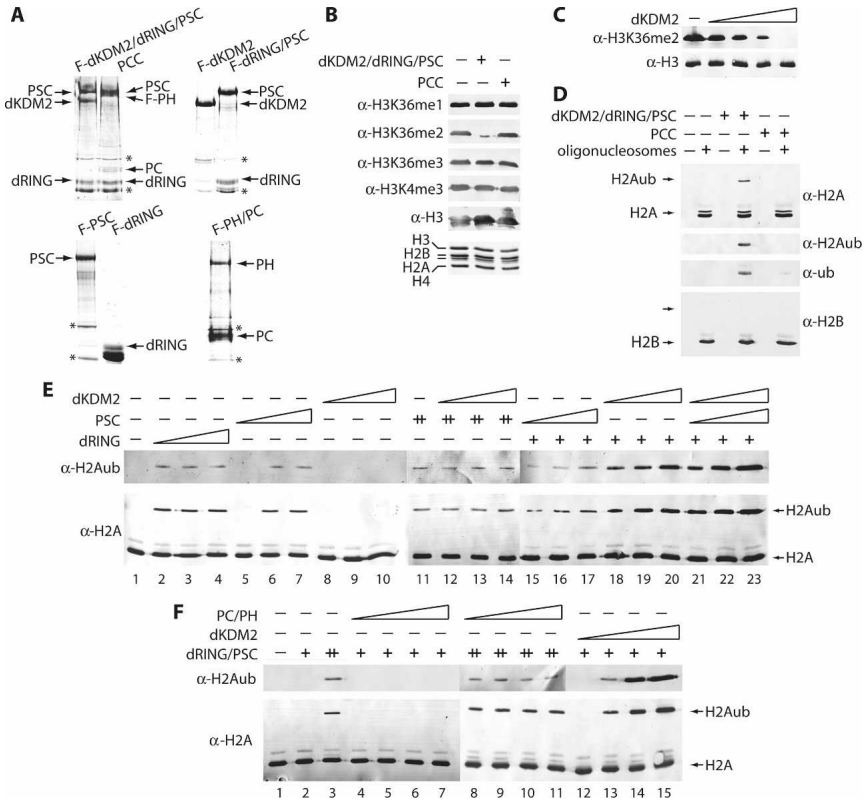

Figure 6. dKDM2 stimulates selective H2A ubiquitylation by dRING-PSC. (*A*) We (co)expressed and purified dRAF and PRC1 core subunits as various multiprotein assemblies or by themselves using the baculovirus system. Purified factors and complexes include Flag-tagged dKDM2 (F-dKDM2)/dRING/PSC, PCC comprising F-PH/PSC/PC/dRING, F-dKDM2, F-dRING/PSC, F-PSC, F-dRING, and F-PH/PC. Following extract preparation, immunopurification, and elution under native conditions using Flag-peptides, proteins were resolved by SDS-PAGE and visualized by Coomassie staining. Asterisks indicate nonspecific background proteins. (*B*) dRAF core complex demethylates H3K36me2. Oligonucleosomes were incubated with either buffer control, dKDM2/dRING/PSC, or PCC. Approximately equimolar amounts (~30 nM) of each protein complex were added, as judged by Coomassie staining (shown in *A*). Reaction mixtures were resolved by SDS-PAGE followed by Western blotting using antibodies directed against the indicated methyl marks or the core domain of histone H3. The *bottom* panel shows the core histones present in the reaction visualized by Coomassie staining. (*C*) dKDM2 alone is sufficient to demethylate H3K36me2. Oligonucleosomes were incubated with either a buffer control or increasing amounts of dKDM2 (~10, 20, 40, or 80 nM). Analysis was as described above. (*D*) dRAF core complex (dKDM2/dRING/PSC) ubiquitylates histone H2A, but not H2B. Oligonucleosomes or a buffer control were incubated in the presence of approximately equimolar amounts (~30 nM) of either dKDM2/dRING/PSC or PCC. Reaction mixtures were resolved by SDS-PAGE followed by Western blotting using antibodies directed against H2A, H2Aub, ubiquitin (ub), and H2B. Note that the amount of histones loaded did not allow detection of endogenous H2Aub or H2Bub. (*E*) dKDM2 stimulates histone H2A ubiquitylation by dRING and dRING/PSC. Oligonucleosomes were incubated with increasing amounts of dRING, PSC, or dKDM2 (~20, 40, or 80 nM), dKDM2 was also titrated in the presence of ~40 nM (++) PSC, and dKDM2 and/or PSC were titrated in reactions containing ~20 nM (+) dRING. Analysis was as described above. (*F*) PC/PH does not stimulate H2A ubiquitylation by dRING/PSC. Oligonucleosomes were incubated in the presence of either 20 (+) or 40 nM (++) dRING/PSC and increasing amounts of PC-PH (~10, 20, 40, or 80 nM). As a control, dKDM2 (~10, 20, 40, or 80 nM) was added to reactions containing 20 nM (+) dRING/PSC. Analysis was as described above.

H3K36me2 was recovered in the immunoprecipitate (Fig. 7B, lane 2) and absent from the unbound FT fraction (Fig. 7B, lane 3). Conversely, only nonubiquitylated H2A was present in H3K36me2-selected nucleosomes (Fig. 7B, lane 2) and all H2Aub was detected in the H3K36me2-depleted fraction (Fig. 7B, lane 3). We conclude that the active H3K36me2 mark and the repressive H2Aub mark do not coexist within a single nucleosome. To test directly whether H2A ubiquitylation was dependent on H3K36me2 demethylation, we compared H3K36me2-containing and H3K36me2-depleted mononucleosomes as substrates in this reaction. As shown in Figure 7C, the presence or absence of H3K36me2 did not influence the efficiency of H2A ubiquitylation by dRING/PSC/dKDM2. As a final test, we mutated two key residues of the dKDM2 JmjC demethylase domain and expressed and purified wild-type and mutant versions of dKDM2 (Fig. 7D). Whereas dKDM2(T241A) and dKDM2(H244A) were unable to demethylate H3K36me2, their ability to ubiquitylate H2A remained unaffected (Fig. 7E). From these results we conclude that stimulation of H2A ubiquitylation by dKDM2 is independent of demethylation of H3K36me2.

Taken together, these results confirmed that dKDM2 is involved in two completely different biochemical reactions. Firstly, dKDM2 is a histone demethylase that specifically removes the active H3K36me2 mark. Secondly, dKDM2 strongly stimulates histone H2A ubiquitylation by dRING/PSC, thus promoting a mark associated with silent chromatin. Collectively, our knockdown studies in cells and our in vitro reconstitution reactions suggested that dRAF rather than PRC1 is the major H2A ubiquitylating enzyme in cells.

Discussion

In this study, we investigated the molecular mechanisms involved in PcG-mediated gene silencing. The major findings of this work are the following. First, we identified a novel PcG silencing complex we named dRAF,

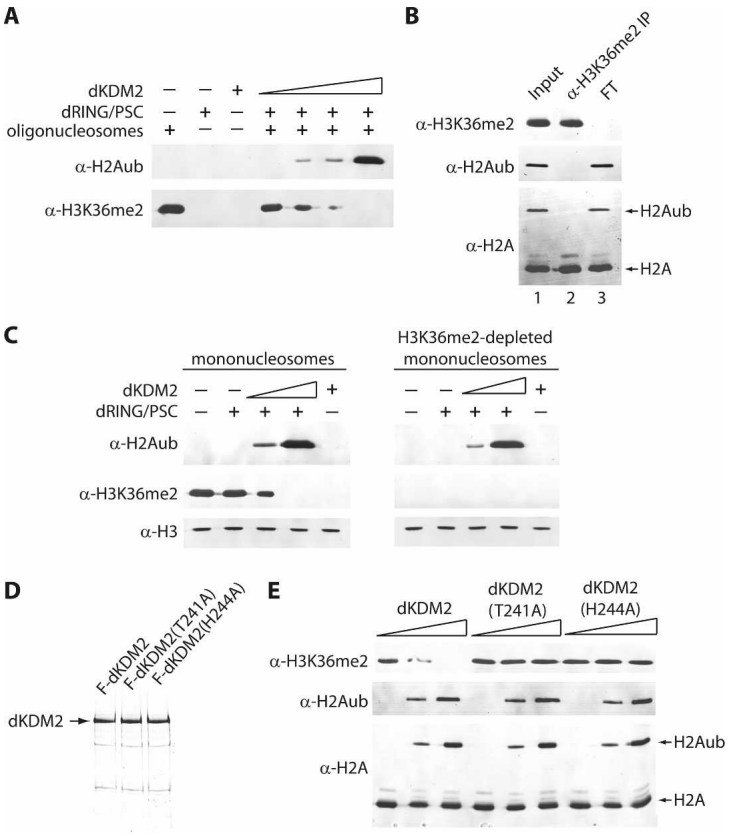

Figure 7. H2A ubiquitylation and H3K36me2 demethylation can occur concomitantly but are not interdependent. (*A*) H2A ubiquitylation and H3K36me2 demethylation can occur concomitantly in the same reaction. Oligonucleosomes or a buffer control were incubated in the presence of dRING/PSC (~30 nM), dKDM2 (~30 nM), or with increasing amounts of dKDM2 (~10, 20, 40, or 80 nM) in the presence of ~30 nM dRING/PSC. Reaction products were resolved by SDS-PAGE followed by Western blotting using the indicated antibodies. (*B*) H3K36me2 and H2Aub are mutually exclusive nucleosomal marks in bulk chromatin. Approximately 2 mg of purified endogenous mononucleosomes were immunopurified using Protein A Sepharose beads coated with antibodies directed against H3K36me2. Input, bound, and unbound FT fractions were collected, resolved by 18% SDS-PAGE, and analyzed by Western blotting using antibodies directed against H3K36me2, H2Aub, and H2A. (*C*) Stimulation of H2A ubiquitylation by dKDM2 is independent of H3K36me2. Mononucleosomes (*left* panel) or H3K36me2-depleted mononucleosomes (*right* panel) were incubated in the presence of either ~30 nM dRING/PSC alone or together with ~40 or ~80 nM dKDM2 or ~80 nM dKDM2. Analysis was as described above. (*D*) Wild-type Flag-tagged dKDM2 (F-dKDM2) or mutants F-dKDM2(T241A) and F-dKDM2(H244A) were expressed in Sf9 cells using the baculovirus system. Following extract preparation, immunopurification, and elution under native conditions using Flag-peptides, proteins were resolved by SDS-PAGE and visualized by Coomassie staining. (*E*) H3K36me2 demethylation defective dKDM2 mutants remain fully able to stimulate H2A ubiquitylation by dRING/PSC. Oligonucleosomes were incubated with ~20, 40, or 80 nM of dKDM2, dKDM2 (T241A), or dKDM2 (H244A), respectively. Reaction products were resolved by SDS-PAGE followed by Western blotting.

harboring core subunits dKDM2, dRING, and PSC. Whereas dRING and PSC are also part of PRC1, the other two PRC1 core subunits, PC and PH, are absent from dRAF. In addition, we found that significant amounts of PSC and PH are not associated with either PRC1 or dRAF, suggesting they might form part of other assemblages. In short, our work suggests a greater diversity among PcG complexes than previously anticipated. Second, genome-wide expression analysis revealed that dKDM2 and PRC1 share a significant number of target genes. Third, we found that *Pc* and *dkdm2* interact genetically and cooperate in repression of homeotic genes in vivo. Fourth, dKDM2 counteracts homeotic gene activation by the trxG histone methyltransferases TRX and ASH1. Fifth, we uncovered a novel *trans*-histone pathway acting during PcG silencing. dKDM2 plays a central role by removal of the active H3K36me2 mark and promoting the establishment of the repressive H2Aub mark by dRING/PSC. Finally, our observation that dKDM2 is required for bulk histone H2A ubiquitylation by dRING/PSC, suggests that dRAF rather than PRC1 is the major histone H2A ubiquitylating complex in cells.

The term *trans*-histone pathway was first coined to describe that H2B ubiquitylation is required for H3K4 and H3K79 methylation, whereas the reverse is not the case (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002; Sun and Allis 2002; Bhaumik et al. 2007; Laribee et al. 2007; Tanny et al. 2007; Weake and Workman 2008). Recently, it was found that H2Bub determines the binding of Cps35, a key component of the yeast H3K4 methylase COMPASS complex (J.S. Lee et al. 2007), providing insight in the molecular mechanism by which two positive marks are coupled. Here, we described a different type of *trans*-histone regulation where the removal of the active H3K36me2 mark is directly linked to repressive monoubiquitylation of H2A. A recent study strongly argued that ASH1 mediates H3K36me2 (Tanaka et al. 2007). Significantly, our genetic and biochemical analysis revealed an in vivo antagonism between dKDM2 and ASH1. Thus, dKDM2 appears to reverse the enzymatic activity of trxG protein ASH1 through H3K36 demethylation, whereas it does not affect H3K4 methylation. The observation that chromatin binding of TRX is ASH1 dependent (Rozovskaia et al. 1999) is likely to be part of the explanation of the strong genetic interaction between *dkdm2* and *trx*. The association of the H3K27me2/3 demethylase UTX with the MLL2/3 H3K4 methylase complexes is an example of coupling removal of a repressive mark to the establishment of an active mark (M.G. Lee et al. 2007).

Our work revealed that the key H2A E3 ubiquitin ligase dRING is part of two distinct complexes, PRC1 and dRAF. A previous study identified the mammalian

BCOR corepressor complex, which harbors RING1, NSPC1, and FBXL10 and other proteins, absent from dRAF (Gearhart et al. 2006). These findings suggest that BCOR and dRAF represent a variety of related but distinct silencing complexes. Reduction of dKDM2 caused a dramatic loss of H2Aub levels, which was comparable with that observed after depletion of dRING or PSC. However, knockdown of PRC1 subunits PC or PH had no effect on H2Aub. These observations suggest that dRAF rather than PRC1 is responsible for the majority of H2A ubiquitylation in cells. This notion was reinforced by in vitro reconstitution experiments, suggesting that dRAF is a more potent H2A ubiquitin ligase than PRC1. An unresolved issue remains the molecular mechanisms that underpin the opposing consequences of either H2A or H2B ubiquitylation. It is intriguing that H2Aub appears to be absent in yeast, present but less prominent than H2Bub in *Drosophila* (see Fig. 5; our unpublished results), and abundant in mammalian cells. An attractive speculation is that H2Aub becomes more important when genome size increases and noncoding regions and transposons need to be silenced.

In summary, we identified the PcG complex dRAF, which employs a novel *trans*-histone pathway to mediate gene silencing. dKDM2 plays a pivotal role by coupling two distinct enzymatic activities, H3K36me2 demethylation and stimulation of H2A ubiquitylation by dRING/PSC. Our results indicate that dRAF is required for the majority of H2Aub in the cell. dKDM2 cooperates with PRC1 but counteracts trxG histone methylase ASH1. These findings uncovered a repressive *trans*-histone mechanism operating during PcG gene silencing.

Materials and methods

Plasmids, recombinant proteins, immunological procedures, and antibodies

Details of cloning procedures are available upon request. For baculovirus expression, the coding sequence of full-length dKDM2, dKDM2(T241A), and dKDM2(H244A) were cloned into a modified version of the shuttle vector pVL1392 (Pharmingen) expressing an in-frame N-terminal Flag tag. For antibody production, dRING (full length), PSC (amino acids 1107–1602), dKDM2 (amino acids 1–353), dKDM2 (amino acids 647–923), dRAF2 (amino acids 1–440), Mtor (amino acids 367–716 and 1097–1419), and Ulp1 (amino acids 1–493) were cloned in pGEX-2TKN, a derivative of pGEX-2TK (Pharmacia) and expressed as glutathione S-transferase (GST) fusion proteins. Protein purification, immunization, and affinity purifications were as described (Harlow and Lane 1998; Chalkley and Verrijzer 2004). The following antibodies were used: guinea pig: α-dRing: SN11 and SN12; α-dKDM2: GR368; α-PSC: GR254; α-Ulp1: SNC045; rabbit: α-dKDM2: GR207; α-dRAF2: SN1915; α-Mtor: SN1912; α-PC: SN965; α-PH: SN964 (Mohd-Sarip et al. 2002); α-MOR: SN670 and SN671 (Mohrmann et al. 2004); α-ISWI (Kal et al. 2000); α-PSC monoclonal hybridoma supernatant clone 6E8 (Martin and Adler 1993); H3K36me1 (Abcam Ab9048); H3K36me2 (Upstate Biotechnologies 07-369); H3K36me3 (Abcam Ab9050); H3K4me3 (Abcam Ab8580); H3 (Abcam Ab1791); H2A (Upstate Biotechnologies 07-146); H2Aub (E6C5) (Upstate Biotechnologies 05-678); H2B (Upstate Biotechnologies 07-371); ubiquitin (Affiniti Research Products Ltd. PW8810). Immunoblotting experiments were performed using standard procedures. Coimmunoprecipitation experiments were performed as described (Mohd-Sarip et al. 2002). All critical immunoprecipitations and Western blotting experiments were repeated with different antisera. *Drosophila* embryo nuclear extract fractionation by $(NH4)_2SO_4$ precipitation, POROS Heparin and Sephacryl S-300 size-exclusion chromatography, and glycerol gradient sedimentation were performed as described (Mohrmann et al. 2004).

Drosophila *genetics*

Flies were maintained under standard conditions. $dkdm2^{KG04325}$, $dkdm2^{EY01336}$, and $dkdm2^{DG12810}$ strains were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu). Pc^1, Pc^3, trx^1, $ash1^{10}$ mutant alleles were provided by F. Karch (Geneva). Information on these stocks can be found at FlyBase (http://www.flybase.org). Five females heterozygous for either Pc^1 or Pc^3 mutations were crossed with five wild-type or *dkdm2* heterozygous mutant males. For the crosses involving *trx* or *ash1*, we analyzed the progeny of five homozygous trx^1 or $ash1^{10}$ females crossed with five wild-type or various *dkdm2* heterozygous mutant males. All crosses were performed in parallel at 25°C, and the frequencies of homeotic transformations were counted. Significance analysis was performed by Student's *t*-test for proportions.

RNAi-mediated knockdowns and genome-wide expression analysis

RNAi-mediated depletion and expression profiling and statistical analysis were performed as described (Moshkin et al. 2007). Briefly, dsRNA targeting the various subunits was synthesized using an Ambion Megascript T7 kit according to the manufacturer's protocol. Oligonucleotide sequences used to generate dsRNA will be provided upon request. Cells were incubated in the presence of dsRNA for 4 d. RNA samples from three independent biological replicates were isolated, prepared, and hybridized with Affymetrix microarrays. PCA was performed as described (Moshkin et al. 2007). Venn Diagrams were constructed using *t*-test, assigning 1 for significantly up-regulated genes ($P < 0.05$) and −1 for significantly down-regulated genes ($P < 0.05$). Co-occurrence of up- and down-regulated genes is shown as overlap on the Venn diagrams. All statistical analysis was performed using R and Bioconductor packages. Details will be provided upon request.

In vitro demethylation and ubiquitylation assays

Reconstituted PCC, dKDM2/dRING/PSC, dKDM2, dRING/PSC, PSC, dRING, and PH/PC were expressed using the baculovirus system and purified as described previously (Francis et al. 2001; Mohd-Sarip et al. 2002). Mono- and oligonucleosomes were prepared essentially as described with minor modifications (Bulger and Kadonaga 1994). Chromatin was treated with sarkosyl (0.05% final concentration) for 5 min and immediately loaded onto 5%–30% sucrose gradients. After ultracentrifugation in a SW28 rotor for 16 h at 26,000 rpm at 4°C, 3-mL fractions were collected and those containing either mononucleosomes or oligonucleosomes with a repeat length of 10–15 were used. Histone demethylase assays were carried out as described (Tsukada et al. 2006) using ~200 µg of oligonucleosomes per 400-µL reaction, incubated for 1 h at 30°C. Ubiquitylation reactions contained 200 µg of oligonucleosomes in 2-mL reactions containing 25 mM Hepes-KOH (pH 7.6), 2 mM $MgCl_2$, 70 mM

KCl, 0.6 mM DTT, 5 mM ATP, 2 mM NaF, 10 mM Okada acid, 0.484 µg of ubiquitin activating enzyme E1 (Boston Biochem, catalog no. E-305), 2.58 µg of ubiquitin-conjugating enzyme UbcH5c (Boston Biochem, catalog no. E2-627), and 6.64 µg of His_6-ubiquitin (Alexis Corporation, catalog no. BSTU-530-M002) and were incubated for 1 h at 30°C. For coupled ubiquitylation/demethylation reactions, the respective buffers were simply combined 1:1. Reaction mixtures were concentrated by standard TCA precipitation and resolved in SDS-loading buffer. Ten percent of each reaction was resolved by 18% SDS-PAGE followed by Western blotting.

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Chapter 4

Posterior sex combs is involved in cell cycle regulation

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Summary

Polycomb group (PcG) and trithorax group (trxG) proteins are evolutionary conserved epigenetic regulators required for maintaining appropriate repression and activation of homeotic genes throughout development. Several mammalian PcG members have been implicated in the control of cellular proliferation and neoplastic development. Genome-wide mapping studies of PcG binding in *Drosophila* and mammals identified several key cell cycle regulators as common target genes. Here, we address the role of PSC in cell cycle regulation. *Drosophila* S2 PSC-depleted cells show impaired proliferation and a G2/M arrest phenotype. Knockdown studies in flies indicate that PSC is essential during development and might be required for normal anaphase progression. Mass-spectrometry analysis revealed that PSC associates with cyclin B/cdk1 and many subunits of the Anaphase Promoting Complex (APC/C) in *Drosophila* embryo nuclear extracts. Our results suggest a mechanistic model where PSC could be required for complete anaphase progression and exit from mitosis.

Introduction

Polycomb group (PcG) and trithorax group (trxG) proteins were first identified in *Drosophila* as a set of transcription factors that specify cell identity along the anteroposterior axis of segmented animals by maintaining their correct repression and activation, respectively, throughout development (Kennison, 1995). Early expressed gap and pair rule proteins have made the initial decision, whether the homeotic gene expression state is “off” or “on”. PcG proteins are epigenetic regulators responsible for the stable propagation of homeotic gene repression through chromatin modification (Simon, 1995). Recent studies provide evidence that the PcG maintenance system regulates many other target genes in addition to homeotic genes, involved in development, cell proliferation, stem cell identity and cancer (Martinez and Cavalli, 2006; Ringrose and Paro, 2004; Schwartz and Pirrotta, 2007; Sparmann and van Lohuizen, 2006).

Cellular transformation and malignant outgrowth is usually the outcome of inappropriate gene expression and genome instability caused by perturbations in chromatin structure. PcG proteins were potential key candidates for cancer pathogenesis, since they exert their function through modulation of chromatin structure. The first association between PcG and cancer development came from the characterization of *Bmi-1*, a mouse homologue of the *Drosophila Psc* (*posterior sex combs*), which encodes a core PRC1 (PCC) subunit. *Bmi-1* was identified as a proto-oncogene that cooperates strongly with MYC to promote the generation of B- and T-cell lymphomas (Haupt et al., 1991; van Lohuizen et al., 1991), but the basis for this cooperation was not understood. Several studies suggested a possible mechanism for this cooperation, where they show

that BMI1 inhibits MYC-induced apoptosis through negative regulation of the *ink4a-ARF* locus (Jacobs et al., 1999a; 1999b). This locus encodes the two tumor suppressors p16 and p19arf, both of which restrict cellular proliferation in response to aberrant mitogenic signaling (Lowe et al, 2003). Although the PcG protein most strongly associated with neoplastic development is BMI1, a clear correlation has been established between the aberrant expression of other mammalian PcG members and tumorigenesis (Kirmizis et al., 2003; Tokimasa et al., 2001; van Kemenade et al., 2001; Varambally et al., 2002; Visser et al., 2001; Wang et al., 2004). In addition to being essential regulators of embryonic development, certain mammalian PcG proteins have also emerged as key players in the maintenance of adult stem cell populations (Valk-Lingbeek et al., 2004). Several PcG proteins have been implicated in the regulation of the self-renewal capacity of specific stem cell types, but the most compelling evidence exists for BMI1. Mice lacking *Bmi-1* exhibit homeotic posterior transformations coupled with strong proliferative defects during lymphocyte development and neurological disorders (van der Lugt et al., 1994). Moreover, BMI1 has been shown to play an essential role during the self-renewal of hematopoietic and neural stem cells (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003).

Genetic interaction studies in flies provided the first suggestive evidence that *Drosophila* PcG genes might have a role in mitosis. For instance, *E(z)* (*Enhancer of zeste*) was identified in a screen for essential cell cycle genes (Gatti and Baker, 1989). *E(z)* mutants exhibited reduced mitotic frequencies and proliferation defects (Phillips and Shearn, 1990). Early embryos derived from mothers heterozygous mutant for *polyhomeotic* (*ph*) exhibited chromatin bridges at anaphase resulting from sister chromatids failing to properly segregate (Lupo et al., 2001). In a later study, the PcG members *ph*, *Pc* (*Polycomb*), *Psc* (*Posterior sex combs*), *E(z)* and *Asx* (*additional sex combs*) were analyzed for mitotic defects (O' Dor et al., 2006). All mutants except *E(z)* exhibited segregation defects and a variety of mitotic defects, not previously described, indicating that *Drosophila* PcG genes are required for proper cell cycle progression. Analysis of clones of *Psc-Su(z)2* or *ph* mutant cells in larvae imaginal discs has shown tumor-like phenotypes that are characterized by unrestricted cell proliferation and a failure to exit the cell cycle at the end of larval development (Beuchle et al., 2001), supporting the link between *Drosophila* PcG genes and cell proliferation. Furthermore, in ChIP experiments, a PRE was identified in a region spanning the promoter and the first intron of the *Drosophila CycA* (*Cyclin A*) gene, which shares some but not all properties with homeotic PREs (Martinez et al., 2006). A series of results in this work suggested that PcG proteins directly regulate *CycA*, linking them to cell cycle control *in vivo*.

Several research groups have taken a global approach through genome-wide mapping of PcG protein binding in order to identify PcG target genes. Studies in human and mouse embryonic stem (ES) cells suggested that PcG proteins repress directly a large group of genes involved in neurogenesis, hematopoiesis, axial patterning and other developmental processes, the activation of which would otherwise promote differentiation (Boyer et al., 2006; Lee et al., 2006). Moreover, some PcG targets encode for components of signal transduction pathways like TGFb (Transforming growth factor beta), BMP (Bone Morphogenetic Protein) and Wnt, required for lineage differentiation and stem cell maintenance and proliferation during embryonic development. Deregulation of these signaling pathways has been associated with cancer development

(Bierie et al., 2006; Reya et al., 2005). Comparable results were obtained from a similar approach in human embryonic fibroblasts (Bracken et al., 2006). In the *Drosophila* system, genome-wide PcG profiling in tissue-cultured cells (Schwartz et al., 2006; Tolhuis et al., 2006) and in different developmental stages *in vivo* (Negre et al., 2006; Oktaba et al., 2008), further confirmed that PcG target genes represent an assembly of key regulators involved in cell cycle, development, differentiation and cell fate specification. Taken together, the global identification of PcG target genes implied a dynamic regulation of PcG function during differentiation.

In this work, we aimed to characterize the function of PCC subunits, namely PC, PH, PSC and dRING, during cell cycle progression and development. Knockdown studies in *Drosophila* showed that each PCC subunit is essential during development. Interestingly, RNAi of each PCC subunit in *Drosophila* S2 cells revealed that only PSC-depleted cells exhibit impaired proliferation and a G2/M arrest phenotype. Mass-spectrometry analysis identified many subunits of the Anaphase Promoting Complex (APC/C) and cyclin B/cdk1 as PSC-associated factors in *Drosophila* embryo nuclear extracts. Our results so far suggest a mechanistic model where PSC might work together with APC/C in *Drosophila* S2 cells in order to provide proper anaphase progression and exit from mitosis.

Materials and methods

Cell culture, RNAi, antibodies, and immunodetection

Drosophila S2 cells were cultured and treated with double-stranded RNA (dsRNA) for four days as described (Worby et al., 2001). Double-stranded RNA was synthesized using an Ambion Megascript T7 kit according to the manufacturer's protocol. All immunological procedures were performed by standard methods (Mohrmann et al., 2004). Antibodies against dRING, PC, PH used for immunobloting have been described previously (Lagarou et al., 2008). For PSC antibody production, PSC (aa 1107-1602) was cloned in pGEX-2TKN, a derivative of pGEX-2TK (Pharmacia) and expressed as glutathione S-transferase (GST) fusion protein. Protein purification, immunization and affinity purifications were as described (Chalkley and Verrijzer, 2004). Two different PSC antibodies were used here: guinea pig anti-PSC, GR463 and GR464. Furthermore, we used the antibodies: rabbit anti-phospho-histone H3 (Upstate, # 06-570), rabbit anti-H3 (Abcam Ab1791), mouse anti-tubulin (Sigma, monoclonal T5168), monoclonal hybridoma anti-cyclin B and anti-cyclin A (Developmental studies Hybridoma Cell Bank, concentrated clones F2-F4c and A12, respectively) and rabbit anti-cdk1 (Santa Cruz Biotechnology Inc., sc-53).

Flow cytometric analysis

Cells treated with dsRNA for four days, were collected, washed with PBS 1X (phosphate-buffered saline) and then fixed with 70% ethanol as described previously (Budzowska et al., 2004). After overnight fixation on ice, the cells were washed with PBS and resuspended in 400 µl PBS containing 0.1% Triton X-100, 0.1 mg/ml propidium iodide, and 0.1 mg/ml RNase. The cells were incubated for 30 min at 37 ° C and analyzed on a FACScan (Becton Dickinson).

Biochemical purification procedures

Protein A Sepharose beads coated with affinity-purified antibodies directed against PSC (guinea pig GR463/464) were used to identify PSC associated factors from concentrated embryo nuclear extracts. After extensive washes with a buffer containing 600mM KCl and 0.1% NP-40, bound proteins were resolved by SDS-PAGE and visualized by coomassie staining (data not shown). Proteins present in bands excised from a gel run in parallel were identified by nanoflow LC-MS/MS (see Materials and Methods Chapter 2). For the co-immunoprecipitation experiments, *Drosophila* embryo nuclear extracts were incubated with Protein A Sepharose beads coated with either control anti-GST antibodies (mock) or affinity-purified anti-PSC (GR463/464), anti-dRING (SN12), anti-PC (SN965) and anti-PH (SN964) antibodies. After extensive washes with a buffer containing 250 mM NaCl and 0.1% NP-40, bound proteins were resolved by SDS-PAGE and analyzed by western immunoblotting.

***Drosophila* genetics**

Flies were maintained under standard conditions. All crosses were performed in parallel at 25°C. Strains were obtained from the DRSC (*Drosophila* RNAi Screening Center, at Harvard Medical School, http://www.flyrnai.org/) and from the Bloomington *Drosophila* stock centre (http://flystocks.bio.indiana.edu/).

SEM (Scanning Electron Microscopy)

Specimens for Scanning Electron Microscopy were prepared as described in Braet et al. (1997). Briefly adult flies were fixed for a few hours in a fixative containing 4% formaldehyde and 1% glutaraldehyde in sodium phosphate buffer (pH 7.2). Subsequently, the flies were dehydrated in graded ethanol and incubated in hexamethyldisilazane. After drying, the specimens were mounted onto aluminum stubs with adhesive carbon tabs and sputter coated for 1 min using an Agar automatic sputter coater. The specimen was then ready to view on a HITACHI TM1000 scanning electron microscope.

Drosophila embryo fixation and immunostaining

Drosophila wild type and PSC-depleted embryos were collected at 25°C on laying medium supplemented with live yeast paste. 0-3 h embryos were washed, dechorionated in 50% bleach solution, and immediately transferred into 5 ml of 3.7% formaldehyde in 1X PBS plus 5 ml heptane. Embryos were shaken gently for 20 s and then fixed with rotation at room temperature for 20 min. After fixation, the formaldehyde layer was removed and methanol was added to devitellinize the embryos. Embryos were either directly processed for staining or stored in methanol at -20°C. For immunostaining, fixed embryos were rehydrated in 5 ml of freshly prepared PBST (1X PBS, 1% bovine serum albumin, 0.05% Triton X-100) solution for 20 min at room temperature on a rotator, followed by several rinses in fresh PBST solution. After primary antibody incubation, embryos were washed 3 times 20 min each in 1 ml PBST, and then incubated with the appropriate secondary antibodies (Alexafluor; Molecular Probes) in PBST in the dark. Embryos were washed 3 times 20 min each in 1 ml PBST and rinsed in PBS, before treatment in mounting medium with 4', 6'-diamidino-2-phenylindole (DAPI) counterstain (Vectashield with DAPI; H-1200; Vector Laboratories). Using a Leica DM6000 microscope with Texas red, DAPI filters and fluorescein isothiocyanate, captured images were processed by using Photoshop.

Results

Each PCC subunit is essential during *Drosophila* development

Previously, we investigated the role of PCC subunits, namely PC, PH, PSC and dRING, in transcriptional regulation, by performing RNAi-mediated subunit depletion of PCC followed by microarray analysis (described in Chapter 2). Epistatic analysis through whole-genome expression profiling revealed that each subunit's target genes within the development, cell cycle and proliferation functional groups overlap significantly, showing functional cooperation during PCC-driven gene expression in a variety of developmental processes. However, there are differences concerning the number, identity and the regulation level of these target genes. Also, each subunit regulates a significant number of distinct genes. Consistent with this, we observed that in comparison to PC and dRING, the number of PSC- and PH-target genes is much higher. This suggested that PSC and PH might have additional functions apart from PCC.

In order to gain more insight into the *in vivo* role of PCC subunits (PCCs) in development, we depleted each subunit separately in flies by using the UAS-GAL4 system. This system gives the ability to knockdown genes in a tissue-specific manner, providing a powerful way to investigate the role of these genes in defining cellular

identity. Specifically, we crossed PCC-RNAi flies with different GAL4-drivers, so that the progeny produce double stranded RNA *in vivo*, so called "inducible RNAi" in specific tissues. As it is illustrated on Figure 1, we used four different GAL4-drivers, namely GAL4-sd (scalloped), GAL4-ey (eyeless), GAL4-GMR (Glass Multiple Reporter) and GAL4-actin, which when crossed with PCC-RNAi flies drive the depletion of PCCs in specific tissues.

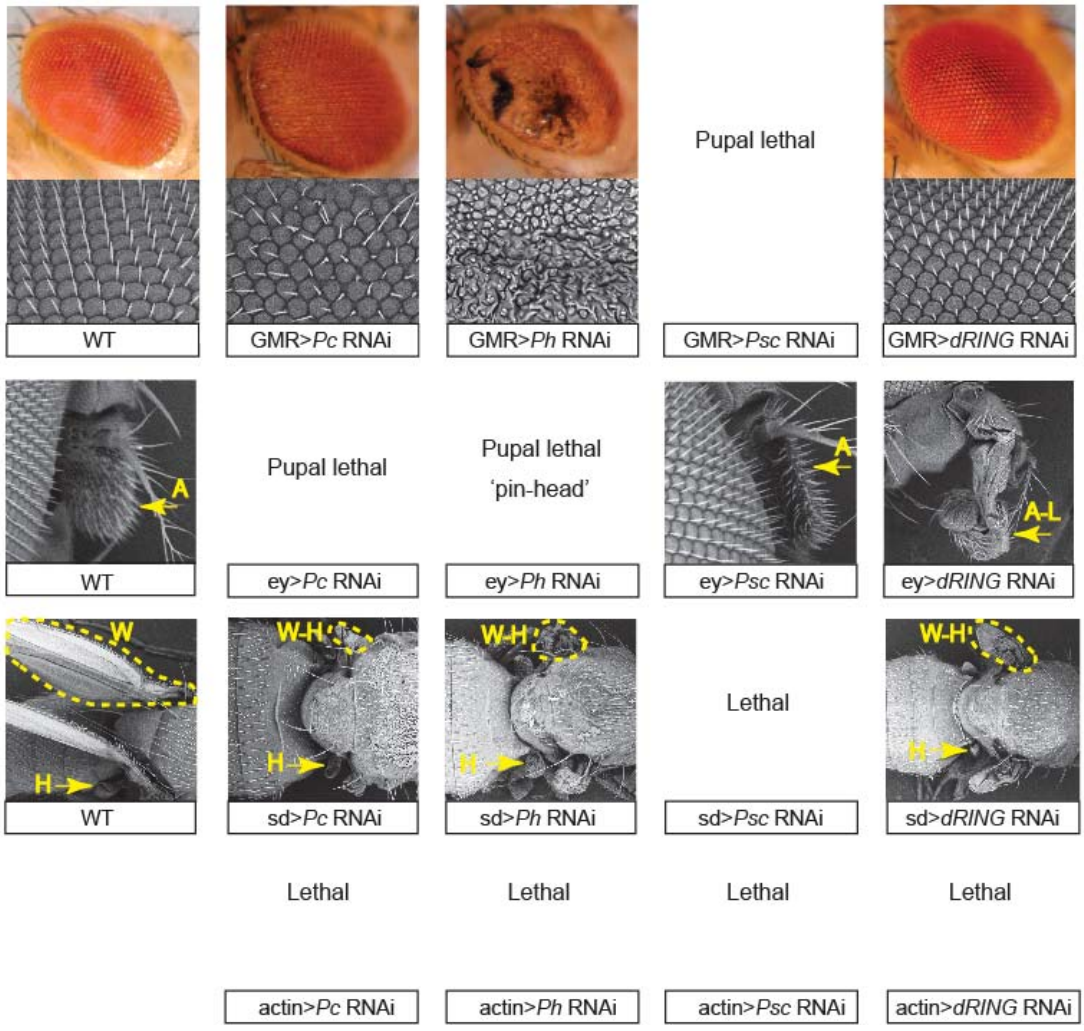


Figure 1. Illustration of the phenotypes of tissue-specific knockdown of PCC subunits in *Drosophila*. Crosses of PCC-RNAi flies with four different GAL4-drivers, GAL4-sd, -ey, -GMR and -actin, which drive the depletion of PCCs in specific tissues.

In particular, *scalloped* is a neuronal wing driver that drives depletion of each PCC in the wing. *GMR* and *eyeless* drive the depletion of each PCC in the eye, and

finally, *actin* drives widespread constitutive depletion of each subunit in the whole organism. The results of our knockdown studies in flies are illustrated in Figure 1. Essentially knock down of PC, PH and dRING using GAL4-sd produced strong phenotypes in the wing wherein in most of the cases the wings were highly reduced, resulting to a wing to haltere transformation, whereas in the case of PSC, its knockdown in the wing is lethal. This indicates that each PCC subunit is required very early in the development of wing tissue. Knock down using GAL4-GMR disrupted the facets in the eye giving the exterior of the eyes a rough appearance, resulting in eye developmental defects or in lethality. The different subunits give similar but variable phenotypes in terms of how strong the phenotype is, depending on the efficiency of the knockdown. For example, knockdown of PH, and not of PC or dRING, with GMR results in the formation of necrotic spots. Furthermore, knock down using GAL4-ey gives stronger phenotypes that GAL4-GMR, probably because ey has broad range of expression in the head region. In addition, the presence of each PCC subunit is essential during development, since their ubiquitous depletion via the constitutive GAL4-actin driver is lethal. Taken all together, the phenotypes derived from all the crosses suggest that each PCC subunit plays a role in tissue development. However, in comparison with the other PCC subunits, PSC knockdown results in lethal phenotypes in most of the cases, indicating that PSC might play a more significant role in developmental pathways in *Drosophila*.

PSC is involved in cell cycle regulation

Our knockdown studies in flies and our genome-wide expression profile analysis revealed that all PCC subunits (PCCs), and mainly PSC, each play essential roles during developmental pathways. To determine the effects of PCCs on cell proliferation, we performed RNAi-mediated subunit depletion in tissue-cultured *Drosophila* S2 cells, followed by accurate counting of the cell number from days 1 to 4, after RNAi treatment. According to the resulting proliferation curve, depletion of PC and dRING does not affect cell proliferation, since in their absence S2 cells appear to proliferate progressively similar to control untreated S2 cells. In contrast, depletion of PSC, and less so of PH, causes a dramatic reduction in cell proliferation (Figure 2A). Moreover, observation of the cell phenotype revealed that PC- and dRING- knocked down cells exhibit a normal phenotype as control cells. On the contrary, after knockdown of PSC, but also of PH to a lesser extent, there is a large number of accumulating dead cells (data not shown).

We next addressed the role of PCCs in cell cycle progression. Knockdown of PCCs in S2 cells was followed by Fluorescence Activated Cell Sorter (FACS) analysis. While the control (untreated) cells and cells depleted for the other PCC subunits have no effect on the cell cycle profile in flow cytometry, PSC-depleted populations have a reproducibly decreased proportion of cells in G1 and S phase, with an accompanying increase in G2/M phase (Figure 2B). Moreover, depletion of PSC led to an increased number of polyploid and aneuploid cells (Figure 2B).

Next, we wanted to characterize the effect of PSC on the cell cycle and define at which exact stage, namely G2 phase or mitosis, PSC is implicated. We tested the levels

of the mitosis marker Histone 3 Phosphorylated at Serine-10 (H3PS10) on whole cell extracts derived from control and RNAi-treated S2 cells, using a highly specific antibody.

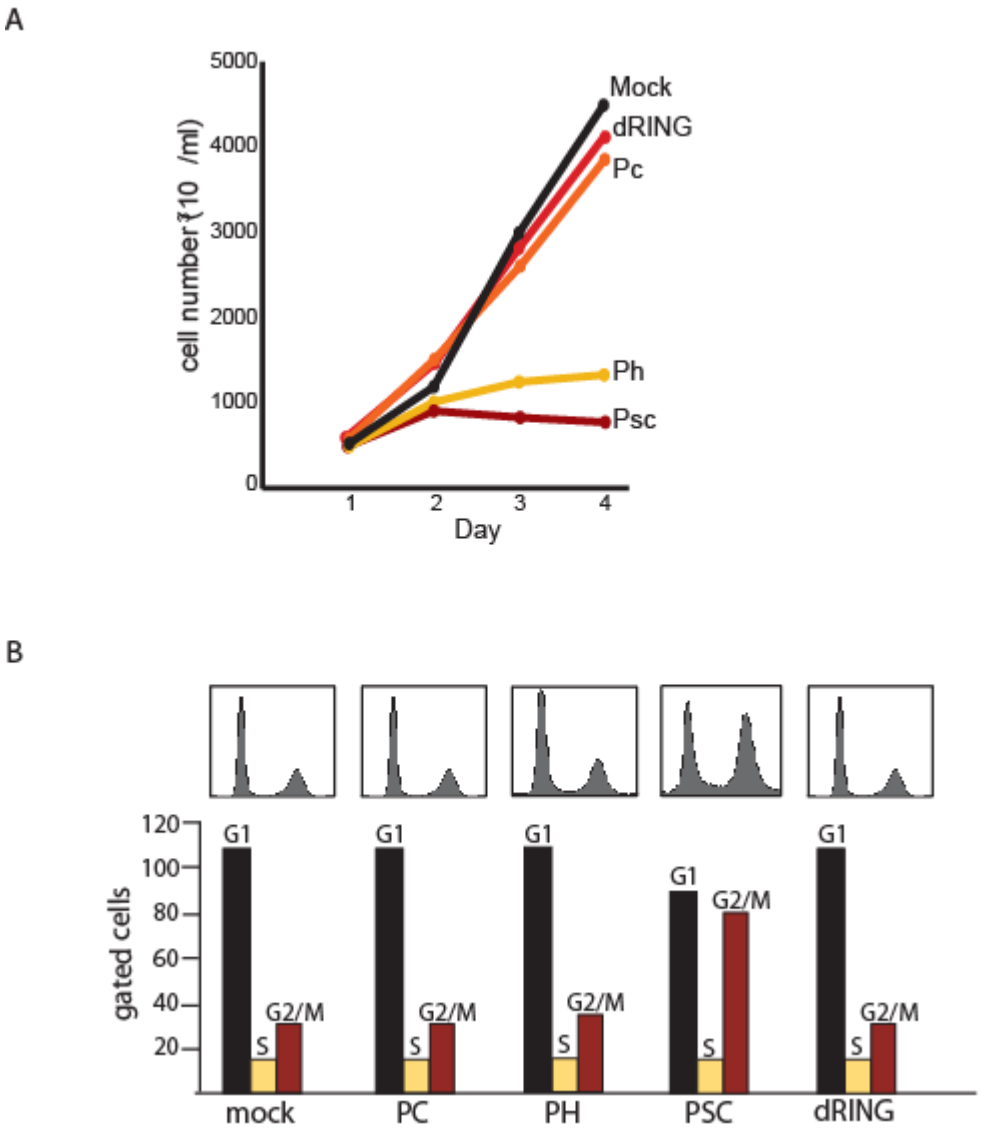
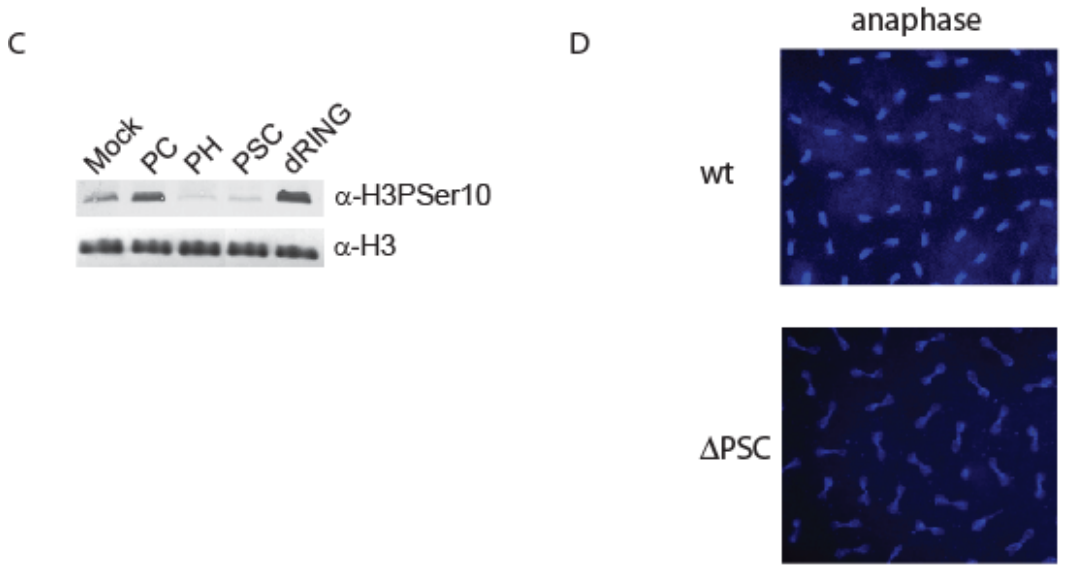


Figure 2. PSC affects cell cycle.
A) Proliferation rate of control S2 and S2 cells treated with dsRNA directed against each PCC subunit.
B) Cell cycle distribution of control S2 and S2 cells depleted for each PCC subunit, as determined by FACS analysis. Quantification is based on gated cells. The ungated FACS profiles are not shown.



C) Western immunoblot analysis of whole-cell extracts from control and RNAi treated cells with antibodies against either histone H3 phosphorylated on Ser 10 (H3PS10) or bulk H3.
D) Wild type (wt) and PSC-depleted early embryos (ΔPSC) (0-3 h) were stained with DAPI. In contrast to wild type, PSC-depleted embryos exhibit chromatin bridges.

Phosphorylation of H3Ser10 occurs during late G2 phase. As mitosis proceeds, this histone modification spreads along the chromosomes till prophase, reaching its highest levels at metaphase. According to the "ready production label" hypothesis, the presence of H3 phosphorylated Ser10 signals to the cell that it has reached the metaphase stage (Prigent and Dimitrov, 2003). Upon metaphase/anaphase transition the phosphorylation mark has to be removed. As it is shown in Figure 2C, in comparison to control and PC-, dRING-depleted cells, depletion of PSC and PH results in significant reduction of H3PS10 levels. However, FACS analysis of PH-depleted cells does not indicate any defect in cell cycle profile. We suppose that PH affects cell cycle in an indirect way, which will not be discussed further here. Concerning PSC there are two possibilities according to the H3PS10 status: either PSC-depleted cells display a reduced level of histone H3 phosphorylation consistent with a blocked transition from G2 phase to mitosis, or PSC could be implicated in a later stage during mitosis e.g. anaphase, preventing cells from exiting mitosis.

In order to characterize further and determine the exact cell cycle phase in which PSC is involved, we used a convenient system to study mitotic progression. Specifically, we studied the phenotype of wild type and PSC-depleted early embryos from 0-3 hours after egg laying. Wild type (wt) embryos undergo normal anaphase, with all segregating nuclei being separated from their former sister chromatids with no chromatin bridges visible in the space between them (Figure 2D). In contrast, in the absence of PSC (ΔPSC), embryos exhibit segregation defects at anaphase caused by formation of

chromatin bridges (Figure 2D). Chromatin bridges consist of chromatin that has failed to segregate with the rest of the chromosomes and therefore appear to bridge a pair of daughter nuclei. In conclusion, we suggest that PSC is required for proper anaphase progression.

Identification of PSC Associated Factors

We sought to elucidate the molecular mechanisms of PSC in cell cycle regulation by isolating the endogenous PSC and its associated partners. We performed stringent immunopurification by using highly specific affinity-purified antibodies against PSC (see Chapter 2). Mass spectrometric analysis revealed the presence of many key cell cycle regulators as PSC-associated factors (Table 1). These are cdk1 (cyclin-dependent kinase 1), cyclin B and many subunits of the Anaphase Promoting Complex (APC/C) namely APC2 (morula), Cdc23 (APC8), Cdc27 (APC3), Cdc16 (APC6), APC5 (imaginal discs arrested), APC4 and the late activator of APC/C, fizzy-related protein (fzr). Also, APC1 (shattered) subunit was detected but in low mascot score (not indicated on Table 1).

TABLE 1. List of proteins identified in PSC IP from Drosophila nuclear extracts using the LTQ Orbi-Trap MS

Name	Mascot Score (Number of unique peptides)	Domains	Functions
PSC (Posterior sex combs; 170 kDa)	5858 (82)	Zn finger RING-type; coiled coil	PCC; chromatin silencing
Sce/dRING (Sex combs extra; 48 kDa)	2132 (22)	Zn finger RING-type	PCC; chromatin silencing; E3 Ub ligase
PH (Polyhomeotic;168 kDa)	1638 (15)	Sterile alpha motif (SAM); coiled coil	PCC; chromatin silencing
PC (Polycomb; 44 kDa)	1251 (17)	Chromodomain	PCC; chromatin silencing
Cdk1 (Cyclin-dependent-kinase1; 34 kDa)	1079 (14)	Serine/threonine protein kinase	cyclin-dependent protein kinase activity; G2/M transition of mitotic cell cycle
Cyclin B (59 kDa)	923 (13)	Cyclin destruction box	G2/M transition of mitotic cell cycle; anaphase; cytokinesis
Cdc23 (78 kDa)	713 (10)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 8; mitotic anaphase
morula (92kDa)	680 (11)	Cullin	Anaphase promoting complex subunit 2; ubiquitin protein ligase binding; mitotic anaphase
Nurf-38 (38 kDa)	595 (9)	Inorganic pyrophosphatase domain	chromatin remodelling
Cdc27 (102 kDa)	518 (8)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 3; binding; mitotic anaphase
Cdc16 (82 kDa)	477 (8)	Tetratricopeptide repeat (TPR)	Anaphase promoting complex subunit 6; binding; mitotic anaphase
PHO (Pleiohomeotic;58 kDa)	413 (5)	Zn finger C2H2-type	DNA binding; chromatin silencing
Scm (Sex comb on midleg; 96 kDa)	368 (5)	MBT repeat; Sterile alpha motif (SAM)	PRC1; Transcriptional repression
Fzr (fizzy-related protein; 54kDa)	367 (4)	WD40 repeat	Anaphase promoting complex activator; exit from mitosis
APC4 (Anaphase promoting complex subunit 4; 88 kDa)	351 (5)	WD40 repeat	Anaphase promoting complex subunit 4

The complex of cdk1 (also called cdc2) and cyclin B is the main regulator of the entry into mitosis, and multiple factors control its activity (Harper and Elledge, 1996; King et al., 1996; Lew and Kornbluth, 1996). Association of cyclin B with its partner cdk1 leads to activation of its kinase function. Degradation of cyclin B is necessary to downregulate the kinase activity of cdk1 and to permit sister chromatid separation, disassembly of the mitotic spindle, chromosome decondensation and cytokinesis (Murray, 1995).

The Anaphase Promoting Complex or Cyclosome (APC/C) is the major ubiquitin ligase required for mitosis (Aquaviva and Pines, 2006; Kerscher et al., 2006; Pickart and Eddins, 2004; van Leuken et al., 2008). APC/C is a multisubunit E3 ubiquitin-protein ligase of the RING-domain family, which targets mitotic regulators such as cyclins and securin for degradation by the 26S proteasome at very precise times in mitosis (Sullivan and Morgan, 2007, Figure 3). The subunits of the APC/C identified in PSC-immunopurification include the cullin of the enzymatic core of the complex, TPR-containing subunits, scaffolding subunits, and the late activator of the APC/C, fzr (fizzy-

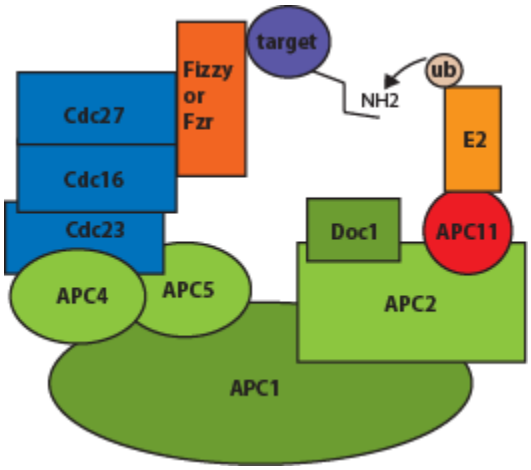


Figure 3. The Anaphase Promoting Complex/ Cyclosome (APC/C). The APC contains 12 or 13 subunits, 11 of which are evolutionary conserved. In yeast, nine of them are essential for normal activity and are thought to interact. APC2 and APC11, which harbor a cullin and a RING-finger domain, respectively, form the enzymatic core of the complex. Cdc27, Cdc16 and Cdc23 subunits contain several TPRs (tetratricopeptide repeat) involved in protein-protein interactions, that contribute differently to the function of APC/C. APC4 and APC5 could have a scaffolding role, connecting the enzymatic core to the regulatory TPR subunits. APC/C associates with activators of the Cdc20/Fizzy (Fzy) and Cdh1/Fizzy-related (Fzr) families in a cell-cycle dependent manner. Fzy and Fzr recognize the APC/C substrates by specific motifs, D-box (destruction box) and 'KEN-box', respectively, and drive their ubiquitylation by APC/C/fzy and APC/C/fzr. APC/C/fzy indirectly triggers the degradation of cohesin, the protein complex that binds sister chromatids together, allowing anaphase onset. APC/C/fzy also targets the mitotic cyclins A and B for degradation in prometaphase and metaphase, respectively, resulting in inactivation of M/Cdks (mitotic cyclin-dependent-kinases) complexes (Hershko, 1999). APC/C/fzr functions during late anaphase till G1 targeting cyclins and other proteins for degradation in order to prevent their premature accumulation and premature entry into S phase (Sigrist and Lechner, 1997).

related protein) (see Table 1 and Figure 3 for details). The function of APC/C is regulated by phosphorylation, as well as by various activators and inhibitors that alter its substrate specificity at different phases of the cell cycle. Although the central role of the APC/C in mitosis is well established, it is still under intense investigation how it works and is regulated.

PSC interacts with cyclin B/cdk1

Mass-spectrometry analysis identified several key cell cycle regulators as PSC-associated factors that could explain the role of PSC in cell cycle regulation. The sequential activation and inactivation of cyclin dependent protein kinases (cdks) ensures the proper timing and order of cell cycle events. An appealing model is that each class of cyclin is responsible for driving the phosphorylation of a subset of Cdk substrates, and that each subset is dephosphorylated when its respective cyclin is destroyed. Here, we isolated cyclin B/cdk1, which is also called Maturation Promoting Factor (MPF) and controls the cell cycle at the G2/M transition stage (Ohi and Gould, 1999). Cyclin B, the levels of which rise in G2, promotes the completion of chromosome condensation and spindle assembly, thereby driving cell-cycle progression into metaphase. Interestingly, we also detected many subunits of the Anaphase Promoting Complex or Cyclosome (APC/C), which ubiquitylates cyclin B at the metaphase-anaphase transition (Hershko et al., 1994), initiating the destruction of cyclin B. One of the identified subunits is fzr, which activates APC/C during late anaphase forming the APC/C/fzr. This complex targets cyclin B and other proteins during late anaphase, playing essential role on the timely exit from mitosis (Pimentel and Venkatesh, 2005). These findings imply that PSC might be involved in cell cycle regulation through functioning with APC/C/fzr during late anaphase.

We set out to confirm that PSC interacts with its associated factors identified by mass-spectrometry. Because of *Drosophila* antibody limitations, so far we could only examine the interaction of PSC with cdk1 and cyclin B. We performed a series of co-immunoprecipitations from crude *Drosophila* embryo nuclear extracts using antibodies directed against dRING, PC, PH and PSC. Western immunoblotting showed that PSC associates stably with cdk1 and cyclin B in crude embryo nuclear extracts (Fig. 4A). In contrast, cdk1/cyclin B did not co-purified with the other PCC subunits. In addition, we tested if PSC associates with cyclin A, the other mitotic cyclin which forms a complex with cdk1 earlier than cyclin B. PSC does not associate with cyclin A, showing that the interaction is specific for the cyclin that appears later during mitosis, cyclin B. Immunoprecipitations using antibodies against cdk1 and cyclin B, yielded low amounts of PSC, but clearly no interaction with PC, PH or dRING (Fig. 4B). This finding indicates that the association of cdk1/cyclin B with PSC is substoichiometric. PSC is a RING-containing putative E3 ligase, shown to enhance H2A ubiquitylation by dRING

during PcG silencing (Lagarou et al., 2008). Thus, it could be possible that PSC ubiquitylates cyclin B and targets it for degradation by APC/C/fzr.

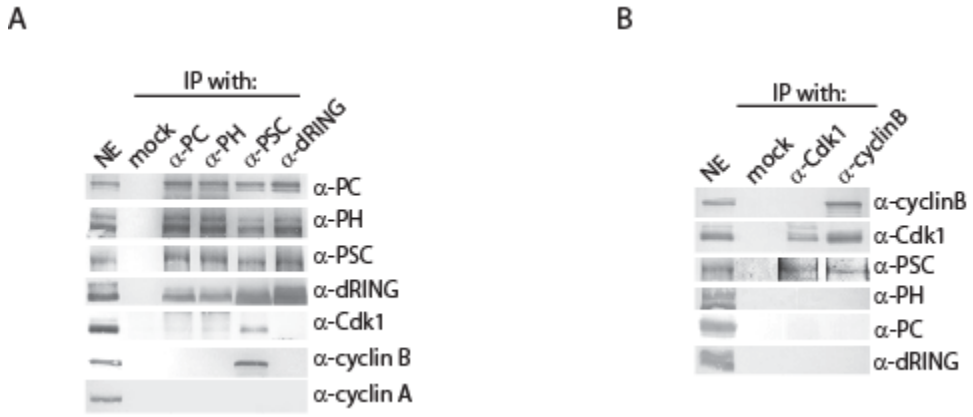


Figure 4. PSC interacts with cyclinB/cdk1 in *Drosophila* embryo nuclear extracts
A) PSC, but not dRING, PH, PC, associates with cyclinB/cdk1 in crude *Drosophila* embryo nuclear extracts (NE). First lane (NE) represents 10% of the input material used in the binding reactions.
B) PSC, but not dRING, PH, PC, co-immunoprecipitate with cyclinB/cdk1 in crude *Drosophila* embryo nuclear extracts (NE). First lane (NE) represents 10% of the input material used in the binding reactions.

To test whether cyclin B is a substrate of PSC, we examined the endogenous cyclin B protein levels in tissue-cultured *Drosophila* S2 cells depleted for each PCC subunit by dsRNA treatment. Cells were collected from day 1 to 3 after RNAi treatment. We tested the levels of cyclin B and cdk1 on the cell extracts by western immunoblotting. We also tested the levels of tubulin, as a loading control (Figure 5A). As expected cdk1 levels remain stable, since only its activation status is oscillating during the cell cycle. In mock and PC- and dRING-depleted cells, cyclin B protein expression is absent. This observation indicates that in these cells cyclin B is totally degraded, so that the cells are cycling normally. In contrast, in PSC- and PH-depleted cells we observe detectable levels of cyclin B on day 2, which increase on day 3 after RNAi treatment (Figure 5A). This suggests that in PSC- and PH-depleted cells there is no degradation and concomitant stabilization of cyclin B. We suppose that the effect of PH-depletion on cyclin B levels is indirect. In the case of PSC-depleted cells, there are two options. The first is that these cells are arrested in the G2 phase in which cyclin B levels rise and accumulate. The second option is that these cells are arrested in anaphase, consistent with previous studies in *Drosophila*, where stabilization of cyclin B during metaphase caused arrest in anaphase accompanied by abnormal chromosome movements (Sigrist et al., 1995).

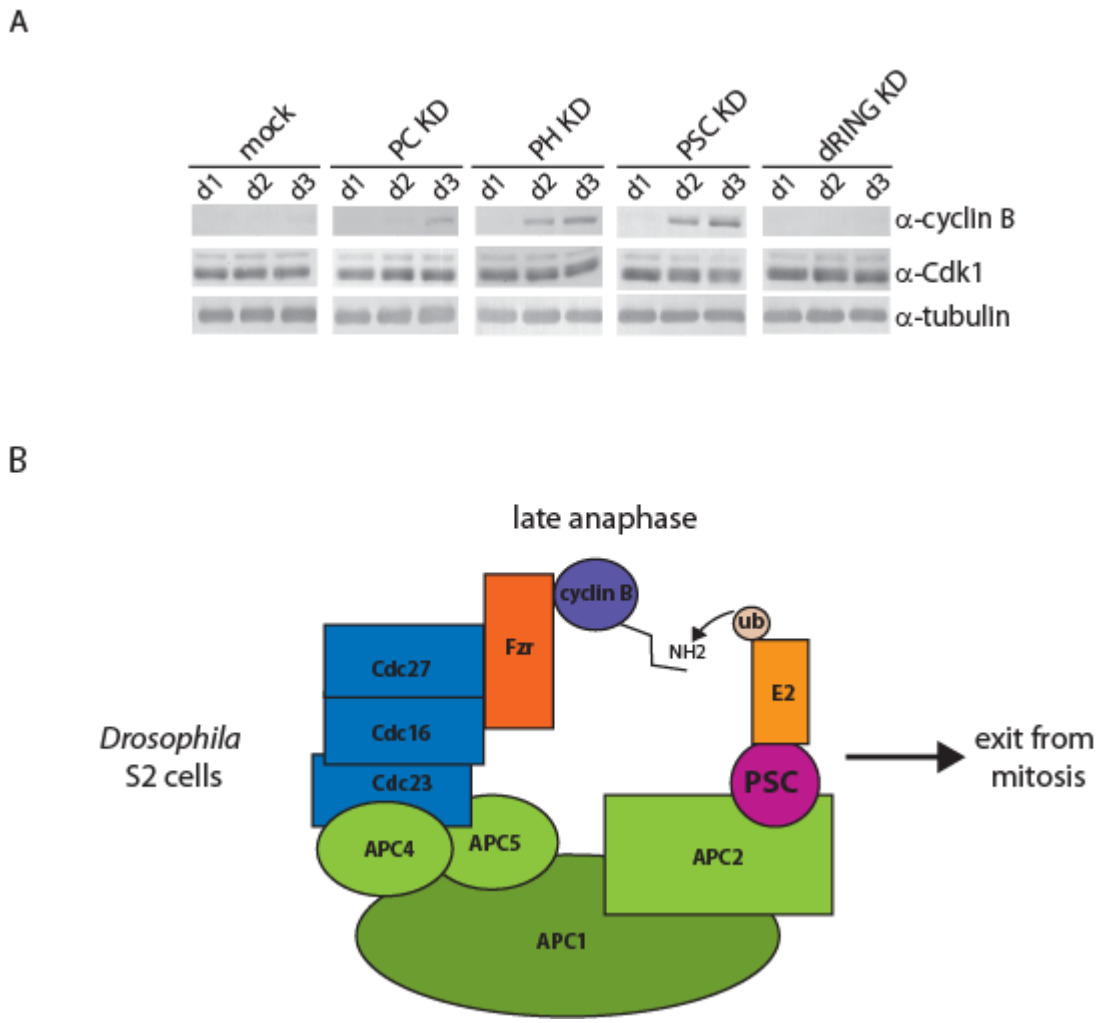


Figure 5. PSC regulates the levels of cyclin B
A) Tissue-cultured *Drosophila* S2 cells were treated with dsRNA directed against PC, PH, PSC and dRING. Cells were collected from day 1 till day 3 after RNAi. Western immunoblot analysis of RIPA extracts was performed from control S2 and RNAi treated cells with antibodies against cyclin B, cdk1, and tubulin (as loading control).
B) Proposed model. PSC functions as the E3 ligase of a differential APC/C/fzr complex, responsible for selective recognition and degradation of cyclin B during late anaphase in *Drosophila* S2 cells.

In order to explain the phenotype of PSC-depleted cells, we would like to favor the second option, that PSC depletion causes arrest of cells in anaphase. This hypothesis dovetails perfectly with our results on early embryos derived from PSC-knockdown flies, which do not undergo normal anaphase progression, but exhibit segregation defects at anaphase caused by formation of chromatin bridges (Figure 2D). Our mass-spectrometry results revealed that PSC associates with many subunits of the Anaphase Promoting Complex (APC/C). These include the late APC/C activator fzr (fizzy-related protein), the cullin of the complex APC2 (morula), several structurally related TPR-containing and

scaffolding subunits, but not the E3 ligase of the complex, namely the APC11 (Table 1). Our hypothesis suggests that at least in *Drosophila* S2 cells PSC could replace functionally the APC11 subunit, in a differential APC/C/fzr complex, responsible for selective recognition and degradation of cyclin B during late anaphase (Figure 5B). In the absence of PSC, there is no degradation and hence stabilization of cyclin B, so that cells arrest in late anaphase and do not exit from mitosis.

Discussion

PcG proteins were initially identified by their role in maintaining Hox gene expression patterns throughout development. Accumulating data suggest that PcG proteins have many other targets apart from Hox genes. Genome-wide profiling studies of PcG proteins in mammals and flies revealed a significant enrichment for several key developmental and cell cycle regulators as PcG targets. These findings added further evidence to the link between mammalian PcG members and tumorigenesis and stem cell maintenance, and to the connection of *Drosophila* PcG genes with proliferation and cell fate determination.

In this work, we investigated the role of PCC subunits, namely PC, PH, PSC and dRING, during *Drosophila* development. Knockdown studies in flies revealed that each subunit is essential during development, and plays a role in tissue development (Figure 1). However, PSC appears to play a more significant role during development than the other PCCs. Knockdown of PCC subunits in tissue-cultured *Drosophila* S2 cells showed that only depletion of PSC, and to a lesser extent of PH affects cell proliferation and survival (Figure 2A). In addition, FACS analysis revealed that only PSC-depleted cells exhibit a G2/M arrest phenotype (Figure 2B), accompanied by a reduction of H3PS10 levels (Figure 2C). Furthermore, PSC-depleted embryos exhibit segregation defects at anaphase caused by formation of chromatin bridges (Figure 2D), suggesting that PSC might be involved in anaphase progression. Mass-spectrometry analysis of PSC-associated factors from *Drosophila* embryo nuclear extracts revealed the presence of cyclin B/cdk1 and many subunits of the Anaphase Promoting Complex (APC/C) (Table 1). Co-immunoprecipitation experiments confirmed that PSC, but not the other PCC subunits, specifically interacts with cyclin B/cdk1 (Figure 4A, 4B). During mitosis, cyclin B is destructed by the RING-type E3 ligase complex APC/C. PSC is a RING-containing putative E3 ligase, that has been shown to enhance H2A ubiquitylation by dRING during PcG silencing (Lagarou et al., 2008). Intrigued by the possibility of cyclin B being a substrate of PSC, we tested the protein levels of cyclin B in PCCs RNAi-treated cells. Our results suggest that in PSC-depleted cells, there is no degradation and therefore stabilization of cyclin B, which prevents these cells from exiting mitosis (Figure 5A). Taken together, we suggest a mechanistic model according to which in *Drosophila* S2 cells, PSC could consist the E3 ligase of a differential APC/C/fzr complex, responsible for selective recognition and degradation of cyclin B during late anaphase (Figure 5B).

Our results would be in agreement with the significant role of *Bmi-1*, a mouse homologue of *Psc*, in proliferation, development and stem cell maintenance (Jacobs et al., 1999a; 1999b; Lessard and Sauvageau, 2003; Park et al., 2003; van der Lugt et al., 1994). Interestingly, expression profile analysis of purified *Drosophila* germline stem cells (GSCs) showed that *Psc* is the most highly enriched PcG transcript in these cells, supporting the view that *Psc/Bmi-1* in particular are used to repress differentiation in a wide variety of stem cells (Kai et al., 2005). Although the precise function of *Psc* in GSCs has yet to be tested, it remains possible that PSC acts in other more specific complexes than PRC1 in stem cells.

Recently, it has been described that loss-of-function *Psc-Su(z)2* or *ph* mutant clones from third-instar larvae exhibit a drastic change in cell cycle phasing, with a substantial increase of the fraction of G2/M cells accompanied by increased nuclear and cellular volume (Oktaba et al., 2008). These findings demonstrate that *ph, Psc* and its functional homologue *Su(z)2* are required for restricting growth and proliferation of imaginal disc cells. These results are in contradiction with our observations concerning the requirement of PSC and PH for progressive cell proliferation. This contrary results are possibly due to the use of different tissues, specifically tissue-cultured *Drosophila* S2 cells derived from late embryonic stages versus larvae imaginal disc cells. In addition, this finding may be another paradigm of the highly dynamic role of PcG proteins during development. Consistent with this, in a study of PcG protein chromosomal binding profiles during different developmental stages (Negre et al., 2006), it has been observed that there are target genes in embryos that are lost at later stages, while other targets are absent in embryos and appear during later development. Moreover, Oktaba et al. (2008) performed ChIP experiments where they showed PcG binding at the *CycB* (*Cyclin B*) PRE. In addition, cyclin B protein levels are elevated in *Psc-Su(z)2* or *ph* mutant clones from third-instar larvae wing imaginal discs, suggesting that PcG proteins directly regulate the levels of *CycB* transcription. Although this is consistent with our data linking the role of PSC in cell cycle with cyclin B regulation, our hypothesis supports that PSC exerts its function post-transcriptionally.

Our data so far suggest a mechanistic model in which PSC might function as the E3 ligase of a differential APC/C/fzr complex, responsible for selective recognition and degradation of cyclin B during late anaphase in *Drosophila* S2 cells. A previous study from Huang and Raff (2002) has raised the possibility that APC/C may not exist as a single complex, but as several related complexes that perform partially non-overlapping functions. Their work suggests the existence of subpopulations of the APC/C that are independently activated to degrade cyclin B in a temporally and spatially co-ordinated fashion. This observation favors our model concerning PSC being part of a differential APC/C/fzr complex. Further work will be required to prove our hypothesis. Specifically, we will reconstitute this complex for in vitro assays using recombinant proteins, in order to show that PSC specifically ubiquitylates cyclin B and targets it for degradation.

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Chapter 5

Discussion

Discussion

Polycomb group (PcG) and trithorax group (trxG) proteins are antagonistic transcriptional co-regulators that maintain the repressed or activated transcriptional states of their target genes through modulation of chromatin structure. Although primarily known for their role in maintaining cell identity during the establishment of the body plan (Pirrotta, 1998), recent studies provide evidence that PcG proteins regulate many other target genes in addition to homeotic genes, involved in development, cell proliferation, stem cell identity and cancer (Martinez and Cavalli, 2006; Sparmann and van Lohuizen, 2006; Valk-Lingbeek et al., 2004). At the molecular level, PcG proteins function as Polycomb Repressive Complexes (PRCs). The best characterized *Drosophila* PcG protein complexes which are working together during PcG silencing via targeting to PREs (Polycomb Response Elements), are termed PRC1, PRC2 and PhoRC (Ringrose and Paro, 2004; Schwartz and Pirrotta, 2007). *Drosophila* PRC1 contains 4 core PcG proteins Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC) and dRING, which constitute the PC core complex (PCC) (Wang et al., 2004). PRC2-class complexes harbor Enhancer of zeste (E(z)), the histone H3 lysine 27 (H3K27) methyltransferase which generates the broad repressive H3K27me3 mark that characterizes the chromatin of PcG target genes (Papp and Muller, 2006). Finally, PHORC consists a core PRE-binding complex that might be needed for PRC1 and PRC2 anchoring at many PcG targets (Klymenko et al., 2006).

In chapter 2, we undertook an unbiased proteomics approach in order to identify PRC1 related complexes and gain more insight into the mechanisms of PcG-mediated repression. Mass-spectrometry analysis revealed that apart from the core PRC1 (PCC), there is variability in the proteins associated with the individual PCC subunits (PCCs). The functional characterization of dRING- and PSC-associated factors is discussed in detail in Chapters 3 and 4, respectively. We also sought to study the individual role of PCCs in gene expression control. Epistatic analysis through whole-genome expression profiling revealed that the target genes of each PCC subunit within the development, cell cycle and proliferation functional groups overlap significantly, showing functional cooperation during PCC-regulated gene expression. However, each subunit regulates also a significant number of distinct genes. In comparison to PC and dRING, the number of PSC- and PH-target genes is much higher, suggesting that PSC and PH might have additional functions apart from PCC. Consistently, in Chapter 3 we found that significant amounts of PSC and PH are not associated with either PRC1 or dRAF, suggesting they might form part of other assemblages. The additional function of PSC apart from PCC is discussed in Chapter 4. In future work, we will aim to unravel potential functions of PH as part of other multiprotein complex(es) apart from PCC. Several studies have provided evidence for the essential role of PH in proliferation, differentiation and signaling pathways. Genetic approaches and clonal analysis revealed that *ph* plays an essential role

in identity specification and patterning of the developing imaginal discs through *engrailed* expression control and the hedgehog signaling pathway during *Drosophila* limb morphogenesis (Randsholt et al., 2000). In addition, *ph* was recovered in a course of a gain-of-function screen for identification of genes with a role during ovarian follicle formation in *Drosophila*, and was shown to be required for somatic cell proliferation and differentiation during this process (Narbonne et al., 2004). Genetic mosaic screens in adult fly brains have reported that in *ph* mutant clones there is loss of subtype identity within otherwise phenotypically wild type brains (Wang et al., 2006). In this study they demonstrated that *Drosophila ph* plays an essential role in maintaining neuronal diversity through metamorphosis, suggesting a link between steroid hormone signaling and the epigenetic function of PH. Moreover, larvae polytene chromosomes mapping studies have indicated that PH shares many binding sites with CycG (Cyclin G), the *Drosophila* homologue of the mammalian Cyclin G1 and G2 (Salvaing et al., 2008). This finding suggests a connection of PH with proliferation and cell cycle regulation, since the vertebrate CycG1 is a tumor suppressor of p53 (Okamoto and Beach, 1994), and CycG2 acts as a negative regulator of cell cycle (Bennin et al., 2002), causing G1/S arrest when overexpressed.

PH-directed immunopurification resulted to the identification of several proteins involved in signaling and heterochromatin regulation, but also of proteins involved in PcG silencing and that overlap between all the PCC-immunopurifications (Chapter 2). To gain further insight into potential functions of PH apart from PCC, we performed few extra purification steps and purified the unique PH-associated factors (PHAFs) from *Drosophila* embryo nuclear extracts depleted for the other PCC subunits, namely PC, dRING and PSC. Mass-spectrometry analysis revealed only the presence of the two proteins identified previously namely CG17494 and CG2982 as PHAFs. CG17494 contains a FHA (Forkhead-associated) domain, a putative nuclear signaling domain (Durocher and Jackson, 2002), found in proteins that participate in establishing or maintaining cell cycle checkpoints, cell proliferation and DNA repair (Mahajan et al., 2008). CG2982 belongs to the MINA53/NO66 group of the JmjC-domain-only family, and its orthologue NO66 is localized to the nucleolus playing a role in ribosome biogenesis and participates in the replication or silencing of certain heterochromatic regions (Eilbracht et al., 2004). Ribosome biogenesis is a highly coordinated process that ensures proper cell growth and proliferation by supporting the synthesis of proteins. Moreover, it has been observed that NO66 co-localizes to specific heterochromatic regions with the nucleolar antigen Ki-67, an FHA-domain protein that provides a specific marker for proliferative cells (Bridger et al., 1998; Endl and Gerdes, 2000). In conclusion, the presence of CG2982 and CG17494 is quite intriguing and could provide further insights into the mechanisms that link PH function to cell cycle, proliferation and signaling. Currently we are working on the functional characterization of these PH-associated factors (PHAFs). So far, our knockdown studies in flies have shown that CG17494 and CG2982 are essential during *Drosophila* development (Doyen et al., our unpublished data).

In Chapter 3, we describe a novel PcG silencing complex, that we named dRAF (dRING-Associated Factors), harboring the core subunits dKDM2, dRING and PSC. Interestingly, dRAF employs a novel trans-histone pathway acting during PcG silencing, where dKDM2 plays a central role by removing the active H3K36me2 mark and promoting the establishment of the repressive H2Aub mark by dRING/PSC (Lagarou et al., 2008). In addition to the JmjC domain and the F-box, involved in histone demethylation and ubiquitylation, respectively, dKDM2 harbors a CXXC-type zinc finger and a PHD finger (Shi, 2007; Tsukada et al., 2006). CXXC-type zinc finger domains specifically recognize and bind unmethylated CpG-rich regions (Voo et al., 2000). It has been shown that the human homologue of dKDM2, JHDM1B/FBXL10/KDM2B is localized to the nucleolus and binds to ribosomal DNA (rDNA) through its CXXC-zinc finger leading to transcriptional repression of ribosomal RNA (rRNA) genes and concomitant inhibition of cell growth and proliferation (Frescas et al., 2007). JHDM1B also has been described to function as a repressor of *c-jun* transcription by binding to the *c-jun* promoter through its CXXC-zinc finger and tethering co-repressors such as Sin3A and HDACs (Koyama-Nasu et al., 2007). The molecular function of JHDM1B (KDM2B) uniquely parallels that of the human paralogue JHDM1A (KDM2A). JHDM1A is enriched in pericentromeric heterochromatin and binds to CpG islands through its CXXC-domain mediating silencing of satellite RNAs and maintenance of heterochromatin (Frescas et al., 2008). It will be very interesting to identify potential binding targets of the CXXC-zinc finger domain of the *Drosophila* homologue that engage dKDM2 in other functions controling gene expression by different mechanisms.

The plant homeodomain (PHD) finger is a highly specialized methyl-lysine binding domain found in a variety of chromatin-associated proteins that are involved in epigenetic regulation. Several studies have reported interaction of PHD finger with trimethylated lysine 4 on histone 3 (H3K4me3), a universal modification at the beginning of active genes that can promote both gene expression and repression. For example, the PHD domain of ING2 (inhibitor of growth 2) tumor suppressor, a native subunit of a repressive mSin3a-HDAC1 histone deacetylase complex, binds with high affinity to H3K4me3. In response to DNA damage, recognition of H3K4me3 by the ING2 PHD domain stabilizes the mSin3a-HDAC1 complex at the promoters of genes that stimulate proliferation, providing a new mechanism by which H3K4me3 functions in active gene repression (Pena et al., 2006; Shi et al., 2006). Also, the PHD of NURF (Nucleosome Remodelling Factor) has been shown to associate preferentially with H3K4me3 tails, coupling H3K4 trimethylation to NURF-mediated ATP-dependent chromatin remodelling during maintenance of Hox gene expression patterns throughout development (Li et al., 2006; Wysocka et al., 2006). Identification of the putative methyl-lysine marks that are recognized by the PHD finger of dKDM2 will provide further insight into the function of this protein in gene regulation.

KDM2B has been implicated in cell cycle regulation and tumorigenesis. One of the earliest reports of *Jhdm1b* function came out of a genetic screen for tumor suppressor genes in mouse lymphomas (Suzuki et al., 2006). Indirect evidence suggested that *Jhdm1b* might act as a tumor suppressor, including a link to the negative regulation of *c-Jun* 14 (Koyama-Nasu et al., 2007), as well as a description of its role in the negative

regulation of rRNA genes (Frescas et al., 2007). However, a recent study provides several lines of evidence that support *Jhdm1b* may indeed be a proto-oncogene. He et al. (2008) have demonstrated that Jhdm1b contributes to the regulation of cell proliferation and senescence by directly repressing the expression of the *p15 Ink4b* tumor suppressor through removal of K3K36me2. Moreover, a *Caenorhabditis elegans* homolog of KDM2B, T26A5.5, has been identified in an RNA interference (RNAi) screen designed to detect mutator genes that contribute to genome stability in *C. elegans* somatic cells (Pothof et al., 2003). It will be of great importance to investigate the role of the *Drosophila* homologue, dKDM2, in cell cycle regulation and development.

Apart from the core dRAF subunits, we also identified two nuclear pore complex associated proteins, namely Mtor (Megator) and Ulp1 (Ubiquitin like protease 1), as potential dRING-interactors. Mtor is a dynamic subunit of the nuclear pore complex that also exists separately in the nucleoplasm and has been implicated in mitotic spindle assembly (Qi et al., 2004). Mtor has also been found to interact functionally with the MSL dosage compensation complex, revealing an unexpected link between dosage compensation and the NPC (Nuclear Pore Complex) (Mendjan et al., 2006). Ulp1 is a nuclear pore-associated SUMO protease that employs C-terminal hydrolase activity to process SUMO (small ubiquitin-like modifier) precursors to the mature forms (Hay, 2007). In *Drosophila* cells Ulp1 is required to maintain the normal spectrum of SUMO conjugates by preventing the escape of SUMO from the nucleus (Smith et al., 2004). SUMO is distantly related to ubiquitin and has many biological functions, including control of gene expression, maintenance of genome integrity, intracellular transport and protein stability (Verger et al., 2003). Recently, several ways have been discovered in which the SUMO and ubiquitin pathways can intersect and communicate (Sun et al., 2007). It would be quite interesting to unravel a possible crosstalk between SUMOylation and ubiquitylation through Ulp1 and dRING functional interaction. We have observed that dRING is localized to the nuclear periphery in *Drosophila* S2 cells (Lagarou et al., our unpublished data) and it will be noteworthy to examine if Mtor and Ulp1 are required for the recruitment of dRING to the nuclear periphery and if so, to determine their importance in PcG silencing and/or other distinct cellular processes.

Polycomb group (PcG)-dependent epigenetic regulation has emerged within the past few years as an important player in the control of proliferation during the acquisition of cell identity. In chapter 4, we address the role of PSC in cell cycle regulation. Knockdown studies in flies and in tissue-cultured *Drosophila* S2 cells showed that PSC plays a significant role in development and proliferation, respectively. Cell cycle analysis revealed that PSC-depleted cells exhibit a G2/M arrest phenotype. Furthermore, PSC-depleted embryos exhibit segregation defects at anaphase suggesting that PSC might be involved in anaphase progression. Identification of PSC-associated factors from *Drosophila* embryo nuclear extracts by mass-spectrometry analysis revealed the presence of cyclin B/cdk1 and many subunits of the Anaphase Promoting Complex (APC/C). We confirmed by co-immunoprecipitation experiments that PSC, but not the other PCC subunits, specifically interacts with cyclin B/cdk1. We also observed that in PSC-depleted cells, cyclin B protein levels are stabilized. Taken together, we hypothesize a model where PSC is the E3 ligase of a differential APC/C/fzr complex, responsible for

selective degradation of cyclin B during late anaphase in *Drosophila* S2 cells. In the absence of PSC, cyclin B levels remain high and inhibit mitotic exit. In order to prove this hypothesis we are currently working on the reconstitution of this complex for in vitro assays using recombinant proteins (Mohd-Sarip et al., our unpublished data). Our main goal is to show that PSC specifically ubiquitylates cyclin B and targets it for degradation.

It is clear from the analysis of the PcG binding-sites on polytene chromosomes that PSC targets additional loci during interphase. Although PC and PH overlap to more than 90% to their binding on chromosomes, Rastelli et al. (1993) have shown that PSC shares more than half of its binding sites with PC. Further evidence for additional function(s) of PSC apart from PCC comes from a study where the distribution of PC, PH and PSC was determined in whole mount embryos throughout embryogenesis and across the cell cycle using high-resolution confocal laser scanning microscopy (Buchenau et al., 1998). PSC was shown to form specific clusters in different parts of the nucleus that are partially coincident with the centromeric heterochromatin and do not colocalize with PC and PH. It was also observed that the behavior of PSC during various cell cycle phases is different than that of PC and PH. Specifically, PSC dissociates from chromatin in prophase, as PC and PH, but in contrast to the other two proteins becomes strongly reassociated with the chromatin during anaphase B (late anaphase), rebinding already being complete by telophase when the other two PcG group members are in the process of reassociation. It is suggested that the reduced dispersion of the PSC protein during mitosis after dissociation from the chromatin may indicate that PSC remains in association with other proteins in a very stable complex. This is consistent with our hypothesis that PSC is a part of a differential APC/C/fzr complex during anaphase.

It has to be mentioned that *Bmi-1*, a mouse homologue of *Psc*, encodes the PcG protein, which is most strongly associated with neoplastic development (Jacobs et al., 1999a; 1999b). Although several mammalian PcG proteins are implicated in the regulation of the self-renewal capacity of specific stem cell types, the most compelling evidence exists for BMI1 (Lessard and Sauvageau, 2003; Park et al., 2003; van der Lugt et al., 1994). Our results suggest that PSC plays a significant role in development and proliferation, indicating that the functions of PcG proteins are evolutionary conserved. Moreover, it has been demonstrated that BMI1 is phosphorylated in a cell cycle-dependent manner and that phosphorylated BMI1 is physically dislodged from the chromatin at the G2/M stage in both primary and tumor cell lines (Voncken et al., 1999). In a latter study it was found that the MAPKAP kinase 3pk interacts with BMI1 and acts as a BMI1 kinase in vitro and in vivo (Voncken et al., 2005). Interestingly, chromatin release of BMI1 by 3pk overexpression leads to re-expression of *p14arf*, one of the gene products of the *Cdkn2a/INK4A* locus involved in the control of proliferation and stem cell renewal (Lessard and Sauvageau, 2003; Voncken et al., 2003). In addition, it is suggested that besides chromatin association, protein/protein interactions and the catalytic activity of BMI1 might be altered upon phosphorylation. In our case, absence of PSC staining in mitotic *Drosophila* S2 cells implies that PSC disassociates from chromatin and is probably phosphorylated (Lagarou et al., our unpublished results). It will be of great interest to identify potential phosphorylation sites of PSC by mass-spectrometry. It is reasonable to suggest that cdk1 might be a kinase of PSC since we have shown that cdk1

stably associates with PSC in *Drosophila* embryo nuclear extracts (Chapter 4). It is possible that phosphorylation of PSC by cdk1 regulates its activity during different stages of the cell cycle. Unraveling the nature of the interaction of PSC with cdk1 and its chromatin association and the exact biological significance of phosphorylation for PSC function will provide further insights into the role of PSC in cell cycle regulation.

In conclusion our work on the identification and functional characterization of differential PRC1-related complexes, has revealed a greater complexity among *Drosophila* PcG complexes and their functions than previously anticipated. Many interesting questions arise, which present a relevant challenge for future research. Future work will improve our understanding on the mechanisms by which PcG proteins are involved in different cellular processes through control of gene expression.

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Summary

Summary

The PcG/trxG system is evolutionary conserved from *Drosophila* to mammals. Although primarily known for their role in maintaining cell identity during the establishment of the body plan, several mammalian PcG members have now been implicated in the control of cellular proliferation and neoplastic development. To date, three distinct *Drosophila* PcG protein complexes have been characterized, which are working together during PcG silencing via targeting to PREs referred as PRC1, PRC2 and PhoRC.

Understanding the mechanisms by which PcG proteins repress gene expression requires the isolation of functional PcG complexes. In Chapter 2 we undertook an unbiased proteomics approach in order to identify PRC1-related complexes from *Drosophila* embryo nuclear extracts. The complexes identified by mass spectrometry analysis consist of the core PRC1 (PCC) subunits, namely PC, PH, PSC and dRING, and have differential associated factors depending on the individual PCC subunit. Our results suggest a great diversity among PRC1-related PcG complexes. We investigated the relative roles of the individual PCC subunits in gene expression control by performing knockdown experiments in *Drosophila* S2 cells, followed by microarray expression analysis. Epistatic analysis through whole-genome expression profiling revealed the essential role of each subunit during transcriptional regulation of many target genes. We observed that all subunits share a large set of target genes within PCC, involved in cell proliferation, signaling cascades and developmental pathways, but they also regulate a significant number of unique genes. These results indicate that although there is cooperation between PC, PH, PSC and dRING during PCC-driven gene expression, the individual PCC subunits appear to have additional functions apart from PCC.

In Chapter 3 we characterized dRING and its associated factors (dRAF). Specifically we immunopurified dRING from Polycomb-depleted extracts using highly specific antibodies against dRING. Mass spectrometric analysis revealed that dRAF lacks the PRC1 subunits PC and PH, but contains additional proteins involved in distinct cellular processes. One of the key dRAF signature subunits is dKDM2, an intriguing protein, harboring regulatory motifs such as a JmjC type demethylase domain and an F-box involved in ubiquitylation. Following a series of purification steps, we proved that dRAF and PRC1 form two distinct PcG complexes that share PSC and dRING as common subunits. dRING/PSC/dKDM2 was considered the core of the dRAF complex with potential interactions with the other factors. Genome-wide expression analysis revealed a functional cooperation between PRC1 and dRAF during gene regulation. Interestingly, dKDM2 greatly enhances the H2A ubiquitylase activity of dRING/PSC on nucleosomes in vitro. Depletion studies in *Drosophila* S2 cells revealed that dKDM2, dRING and PSC, but not PC or PH, are required for bulk histone H2A ubiquitylation (H2Aub). These results suggested that at least in *Drosophila*, dRAF is mainly responsible for H2A ubiquitylation. Genetic interaction assays indicated that dKDM2 participates in

PcG-mediated repression. Moreover, we found that another histone modification is mediated by dRAF, since dKDM2 selectively demethylates histone 3 lysine 36 dimethyl (H3K36me2) in vivo and in vitro. In conclusion, dRAF uncovers a novel trans-histone pathway during PcG silencing, where dKDM2 plays a central role by removing the active H3K36me2 mark and promoting the establishment of the repressive H2Aub mark by dRING/PSC.

In Chapter 4, we worked on the characterization of the function of PCC subunits in development. Knockdown studies in flies indicated that each PCC subunit is essential during tissue development. Knockdown experiments in *Drosophila* tissue-cultured S2 cells revealed that PSC is involved in cell cycle regulation, since PSC-depleted cells are characterized by decreased cell number, impaired proliferation and a G2/M arrest phenotype. In addition, PSC-depleted embryos exhibit segregation defects at anaphase caused by formation of chromatin bridges, suggesting that PSC might be involved in anaphase progression. Using mass spectrometric analysis, we came to realize that PSC associates with several key cell cycle regulators, namely cyclin B/cdk1 and many subunits of the Anaphase Promoting Complex (APC/C), that might explain the role of PSC in cell cycle regulation. Our hypothesis suggests that PSC could function within a differential APC/C complex in order to achieve proper anaphase progression and exit from mitosis in *Drosophila* S2 cells.

Samenvatting

Het PcG/trxG systeem is evolutionair geconserveerd van *Drosophila* tot zoogdieren. Hoewel ze vooral bekend zijn om hun rol in het behoud van celidentiteit tijdens het opzetten van het bouwplan van het lichaam, worden verschillende PcG eiwitten in zoogdieren nu ook betrokken in de regulatie van celproliferatie en ontwikkeling van neoplasie. Drie verschillende *Drosophila* PcG eiwitcomplexen zijn tot dusver gekarakteriseerd, welke samenwerken in PcG repressie via gerichte binding aan PREs, genoemd PRC1, PRC2 en PhoRC.

Een beter begrip van de mechanismen waarmee PcG eiwitten gen expressie onderdrukken vereist de isolatie van functionele PcG complexen. In hoofdstuk 2 hebben we voor een onbevooroordeelde proteoom analyse gekozen om PRC1-gerelateerde complexen te identificeren in *Drosophila* embryo nucleaire extracten. De complexen geïdentificeerd door middel van massa spectrometrie bestaan uit de PRC1 (PCC) kern subeenheden PC, PH, PSC en dRING en verschillende geassocieerde factoren, afhankelijk van de PCC subeenheid. Onze resultaten suggereren een grote diversiteit onder de PRC1-gerelateerde PcG complexen. We hebben de relatieve rol van de individuele PCC subeenheden in regulatie van genexpressie onderzocht door middel van knockdown experimenten in *Drosophila* S2 cellen, gevolgd door microarray expressie analyse. Epistatische analyse van expressie profielen van het complete genoom onthulde de essentiële rol van iedere subeenheid tijdens transcriptie regulatie van vele target genen. We zagen dat alle subeenheden een grote set gemeenschappelijke target genen hebben binnen PCC, die betrokken zijn bij celproliferatie, signaal transductie cascades en ontwikkelingsroutes, maar dat ze ook een significant aantal unieke genen reguleren. Deze resultaten wijzen erop dat, hoewel er samenwerking is tussen PC, PH, PSC en dRING tijdens PCC-gedreven genexpressie, individuele subeenheden ook additionele functies buiten PCC hebben.

In hoofdstuk 3 karakteriseerden wij dRING en zijn geassocieerde factoren (dRAF). We zuiverden dRING op uit Polycomb-gedepleteerde extracten door middel van zeer specifieke antilichamen gericht tegen dRING. Massa spectrometrische analyse liet zien dat de PRC1 subeenheden PC en PH ontbreken in dRAF, maar dat het additionele eiwitten bevat die betrokken zijn in afzonderlijke cellulaire processen. Eén van de meest kenmerkende dRAF subeenheden is dKDM2, een intrigerend eiwit dat motieven bevat zoals een JmjC type demethylase domein en een F-box domein betrokken bij ubiquitinering. Na een aantal zuiveringstappen konden we aantonen dat dRAF en PRC1 twee afzonderlijke PcG complexen vormen, welke PSC en dRING als gemeenschappelijke subeenheden hebben. dRING/PSC/dKDM2 werd gezien als de kern van het dRAF complex, met mogelijke interacties met andere factoren. Genoom-wijde expressie analyse onthulde een functionele samenwerking tussen PRC1 en dRAF tijdens genregulatie. Interessant genoeg vergrootte dKDM2 de H2A ubiquitylase activiteit van dRING/PSC in vitro. Depletie studies in *Drosophila* S2 cellen lieten zien dat dKDM2,

dRING en PSC, maar niet PC of PH, nodig zijn voor het gros van de histon H2A ubiquitinering (H2Aub). Deze resultaten suggereren dat dRAF, in ieder geval in *Drosophila*, hoofdverantwoordelijk is voor H2A ubiquitinering. Genetische interactie studies indiceren dat dKDM2 betrokken is bij PcG-gemedieerde repressie. Ook hebben we gevonden dat een andere histon modificatie gemedieerd wordt door dRAF, aangezien dKDM2 activiteit selectief histon 3 lysine 36 dimethyl (H3K36me2) demethyleert in vivo en in vitro. Samenvattend onthult dRAF een nieuwe trans-histon route tijdens PcG repressie, waarbij dKDM2 een centrale rol speelt door het verwijderen van het actieve H3K36me2 merk en het bevorderen van de totstandkoming van het repressieve H2Aub merk door dRING/PSC.

In hoofdstuk 4 werkten we aan de karakterisering van de functie van PCC subeenheden in de ontwikkeling. Knockdown studies in vliegen wezen aan dat elke PCC subeenheid essentieel is tijdens de ontwikkeling van weefsels. Knockdown experimenten in *Drosophila* S2 cellen in weefselkweek toonden aan dat PSC betrokken is bij cel cyclus regulatie, aangezien PSC-gedepleteerde cellen gekarakteriseerd worden door een afgenomen aantal cellen, verminderde proliferatie en een G2/M arrest fenotype. Bovendien vertonen PSC-gedepleteerde embryos segregatiedefecten tijdens de anafase, die veroorzaakt worden door de vorming van chromatine bruggen. Dit doet vermoeden dat PSC betrokken is bij anafase-progressie. Met behulp van massa spectrometrie analyse vonden we dat PSC verschillende cel cyclus regulatoren bindt, namelijk cycline B/cdk1 en vele subeenheden van het Anaphase Promoting Complex (APC/C), hetgeen de rol van PSC in cel cyclus regulatie zou kunnen verklaren. Onze hypothese suggereert dat PSC zou kunnen functioneren binnen een bepaald APC/C complex om zo een goede voortgang door de anafase en het verlaten van mitose te bereiken in *Drosophila* S2 cellen.

Curriculum Vitae

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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Anna Lagarou Erasmus MC Department: Biochemistry Research School: Postgraduate school Molecular Medicine	PhD period: June 2004-May2009 Promotor: Prof.Dr. C.P. Verrijzer

PhD training	
	Year
General academic skills - Master's Molecular Medicine-Experimental Approach to Molecular and Cell Biology	2004-2005
In-depth courses - Molecular Medicine course-Basic and Translational Oncology, Rotterdam - MGC AIO Course-From Development to Disease, Rotterdam	2005 2005
Poster Presentations - 'Schedule EuTRACC 1st Annual Review Meeting', Prague, Czech Republic - 'Dynamic Organization of Nuclear Function' Meeting, Cold Spring Harbor Laboratory, NY, USA - 2nd 'Transregio5' Symposium 2007 'Chromatin Assembly and Inheritance of Functional States' Munich, Germany	2009 2008 2007
International conferences - 2nd Annual Meeting, EU 6th Framework Programme, 'The epigenome' Network of Excellence. Naples, Italy - 6th Joint Medisch Genetisch Centrum-Cancer Research UK Graduate Student Conference, Liege, Belgium	2006 2005
Conferences - Stem Cells Meeting, 'Development and Regulation', Amsterdam, the Netherlands - Dutch Chromatin Meeting, Rotterdam, the Netherlands - Annual MGC-Symposium (MGC- Medisch-Genetisch Centrum Zuid-West Nederland)	2008 2006 2004-2008

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Few months back, I wouldn't expect to say that, but now I am already missing Holland…

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Anna

List of Abbreviations

APC/C: Anaphase Promoting Complex/Cyclosome
ASH1: absent, small, or homeotic discs 1
Cdk1: Cyclin-dependent-kinase 1
dKDM2: *Drosophila* Histone Lysine Demethylase 2
dRAF: dRING Associated Factors
EED: Embryonic Ectoderm Development
E(Z): Enhancer of Zeste
ESC: Extra sex combs
FHA: Forkhead-associated domain
Fzr: Fizzy-related protein
H2AubK119: Histone H2A ubiquitylated at lysine 119
H3K27me3: Histone H3 trimethylated at lysine 27
JmjC: Jumonji C
Mtor: Megator
NURF38: Nucleosome remodeling factor 38
NURF55: Nucleosome remodeling factor 55
PC: Polycomb
PcG: Polycomb group
PCL: Polycomb-like
PCL-PRC2: Polycomb Repressive Complex 2 with PCL
PH: Polyhomeotic
PHO: Pleiohomeotic
PHOL: Pleiohomeotic-like
PHORC: Pleiohomeotic Repressive Complex
PRC1: Polycomb Repressive Complex 1
PRC2: Polycomb Repressive Complex 2
PREs: Polyconb Response Elements
PSC: Posterior sex combs
SCE (dRING): Sex combs extra
SCM: Sex comb on midleg
SFMBT: Scm-related four MBT domains
SU(Z)2: Suppressor of zeste 2
SU(Z)12: Suppressor of zeste 12
TRX: Trithorax
trxG: trithorax group
Ulp1: Ubiquitin-like protease 1
YY1: Ying-Yang 1