Chapter 5
Polycomb Complexes: Chromatin Regulators Required for Cell Diversity and Tissue Homeostasis

Miguel Vidal

Abstract The Polycomb group (PcG) products are a set of evolutionary conserved proteins that form chromatin regulator complexes that control expression of developmentally relevant genes. PcG activity is essential not only to maintain the developmental potential of pluripotent cells from which specialized cell types arise, but also to ensure the directionality of the differentiation process. In the adult, these PcG functions are essential for normal cell homeostasis and their deregulation is often associated with cell transformation events. PcG-dependent transcriptional control involves posttranslational modifications of histones, decreased DNA accessibility, and other mechanisms. While the stability of Polycomb-determined chromatin landscapes is rather stable in differentiated cells, in pluripotent cells it is characteristically dynamic in order to accommodate the execution of developmental genetic programs. Best known as repressors of gene expression, recent evidence points at roles during gene activation. Besides gene expression control, PcG products also participate in other essential functions such as DNA damage response, indicating that these proteins are involved in a wide spectrum of cellular and organismal functions in need of detailed characterization.

Keywords Polycomb • PRC1 • PRC2 • Chromatin regulators • Chromatin compaction • Histone modifiifiers • Developmental potential • CpG islands • Stem cell • Progenitors • Cell homeostasis

M. Vidal (✉)
Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu, Madrid 28040, Spain
e-mail: mvidal@cib.csic.es

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5.1 Introduction

The Polycomb group (PcG) of genes was first discovered during the genetic analysis of development in the fruit fly *Drosophila melanogaster*. A first mutant, named *extra sex combs* (Slifer 1942), referred to the presence of additional bristles in the legs that male flies use during mating. Mutations with similar phenotypes were isolated and the genes grouped under the denomination of one of them, Polycomb (Lewis 1978). These mutants showed homeotic transformations, i.e., a part of the body, for example, an anterior leg with no sex combs acquiring the identity of another part, as that of a posterior leg with sex combs (or, if considering embryos, anterior thoracic segments resembling posterior abdominal segments). The molecular nature of these defects lies on the ectopic expression of homeotic genes which are responsible for segment identity (Hox genes) (Riley et al. 1987). After molecular cloning of *Drosophila* Polycomb genes, mammalian homologs were identified and their inactivation in loss-of-function mouse models was also accompanied by homeotic transformations of the axial skeleton (Akasaka et al. 1996; del Mar Lorente et al. 2000; der Lugt et al. 1994).

For a long time, Polycomb products were considered exclusively as developmental regulators. Subsequent work showed their implication in a wide variety of functions that include parental imprinting (monoallelic expression), adult stem cell self-renewal, pluripotency, and, when deregulated, oncogenic transformation (Bracken and Helin 2009; Mills 2010; Sparmann and van Lohuizen 2006). Polycomb targets include genes associated with transitions within cell lineages on their way to full differentiation. Cell identity genes are Polycomb silenced just before their activation in the subsequent cell state and, at the same time, those genes defining the vanishing cell type are repressed in the new state (Bracken et al. 2006; Mohn et al. 2008; Xie et al. 2013). It is now absolutely clear that ordered differentiation of pluripotent cells cannot occur without the activity of the Polycomb system (Pasini et al. 2007; Shen et al. 2009). In turn, reprogramming from differentiated cells towards pluripotent states also requires Polycomb activity (Onder et al. 2012; Pereira et al. 2010). Importantly, Polycomb regulates self-renewal of pluripotent progenitors and proliferative of their differentiated progeny contributing to tissue homeostasis (Calés et al. 2008; Klauke et al. 2013; Lessard and Sauvageau 2003; Luis et al. 2011). Thus, Polycomb is a malleable regulatory system for selective use of the genome in the generation of cell diversity.

Polycomb functions depend, at least in part, on their activities as catalyzers of chromatin modifications. Polycomb products are a heterogeneous collection of proteins that act in complexes. Their best-known activity in transcriptional control is as negative regulators of gene expression, although reportedly they are also associated with gene activity. Polycomb complexes contain, in addition to PcG products, "non-Polycomb" subunits that were not identified in the original genetic screens. The Polycomb system is evolutionary ancient and conserved, from plants and fungi (not yeast) to mammals (Schuettengruber et al. 2007; Shaver et al. 2010; Whitcomb et al. 2007). Although thought specific for multicellular organisms,

homologs are found in unicellular alga (Shaver et al. 2010) suggesting co-option for cell lineage functions.

Here, I will discuss recent advances in our understanding of the molecular aspects of Polycomb action and their role as chromatin regulators and architectural chromatin proteins. Recruitment to targets and their regulation, with a bias towards mammalian cells, is also examined [see some excellent recent reviews (Lanzuolo and Orlando 2012; Simon and Kingston 2013)]. I first present an overview of gene regulation, from DNA sequence and chromatin states to three-dimensional organization of the genome (Gibcus and Dekker 2012) as a framework to explain Polycomb action.

5.2 Chromatin Landscape, Topological Organization, and Selective Use of the Genome

The diversity of cell types in multicellular eukaryotes is the result of differential use of the coding potential of the genome. This is achieved through regulated access of genomic sites to DNA-binding proteins (transcription factors). Controlled localization determines the nature of contacts between sites in chromatin within a highly, topologically organized structure.

5.2.1 Chromatin States

Polycomb complexes are endowed with catalytic activities that can modify histones and other substrates. DNA access is influenced by nucleosomes, whose mobility, in turn, can be conditioned by posttranslational modifications in canonical histones and by the presence of histone variants (Cosgrove et al. 2004). These modifications also affect binding and activity of chromatin-associated proteins, confirming coevolution of regulated DNA accessibility with packaging mechanisms for large DNA molecules. The close relationship between chromatin regulators, histone modifications, and transcriptional activities is apparent in the predictive power of chromatin states to identify DNA regulatory elements (Zhou et al. 2010). Remarkably, out of the large collection of possible combinations of histone marks, just a small number of functionally meaningful sets, or chromatin states, can be distilled. Thus, thousands of promoters and enhancers can be categorized into three and four discrete chromatin state types, respectively, whereas all genomic regions depending on whether transcriptionally active or repressed fit into three and four states, respectively. For example, nucleosomes with histone H3 di- and tri-methylated at lysine 4 and acetylated at lysines 9 and 27 correlate with active promoters, while mono- and di-methylated K4 in histone H3 is found in weak/poised enhancers (Ernst et al. 2011). Characteristically, one of the silenced states is identified by

nucleosomes enriched in histone H3 tri-methylated at lysine 27 (H3K27me3), a Polycomb-specific modification (Margueron and Reinberg 2011).

Similarly, combinations of chromatin regulators that add or remove covalent modifications, also known as "writers" and "erasers," respectively, as well as proteins that recognize these modifications, i.e., the "readers" (Musselman et al. 2012; Taverna et al. 2007), correlate with distinctive sets of chromatin states (Ram et al. 2011). Six major combinations of chromatin-associated modifiers and "readers," or regulatory modules, have been identified in pluripotent and hematopoietic cells. Four of these correspond to two types each of promoters and enhancers, another to transcribed regions, and a last one to repressed regions binding Polycomb proteins. Generally, these modules include modifiers of opposing activity, but modifiers at Polycomb-silenced promoters are all of repressive nature (Ram et al. 2011). Independently, Drosophila chromatin is partitioned to five states (Filion et al. 2010): two distinct classes of transcriptionally active euchromatic domains, two distinct transcriptionally inactive domains, heterochromatic states, of which one is enriched in heterochromatin protein 1 (HP1) while the other contains Polycomb proteins, and chromatin associated with the nuclear lamina; the latter (Lamin-Associated Domains, LADs) includes a large fraction of the genome and is transcriptionally inert (Filion et al. 2010).

5.2.2 Topological Organization of Chromatin and Gene Control

The definition of chromatin states does not take into account restrictions derived from the three-dimensional configuration resulting from chromatin fiber folding. How this actually occurs is still not known. However, it is clear that it is subjected to limitations imposed by the long polymeric nature of chromatin and the effects of associated proteins (Iyer et al. 2011). Computationally generated models have been tested for their ability to fit experimental observations (Dekker et al. 2013). In one of them, the Multi-Loop-Subcompartment model, chromatin segments of $\simeq$1 megabase (Mb) pairs are proposed to fold in small loops separated by short linkers, in a rosette-like configuration (Jhunjhunwala et al. 2008). Looping, as an organizing principle, is consistent with genome-wide chromatin contacts mapped using chromosome conformation capture techniques (de Wit and de Laat 2012). At high resolution—high DNA sequencing depth and comparisons of contacts between smaller DNA fragment, $<$100 kb—the analysis shows chromatin organized in domains termed Topologically Associating Domains (TADs) (Dixon et al. 2012; Hou et al. 2012; Nora et al. 2012; Sexton et al. 2012). TADs are defined by differences in the probabilities of contacts between sites, whereby sites contained within the domains contact more frequently than with sites outside. TADs across cell types and between mouse and humans are highly similar and independent from transcriptional status (Dixon et al. 2012), indicating a strong architectural

underlying principle. TADs are separated by short genomic segments or domain boundaries, enriched in CCCTC-binding factor CTCF (Shen et al. 2012), one of the proteins bound to insulators. These are DNA segments defined in transgenic assays by their ability to "shelter" regulatory elements from each other. TAD boundaries are important for spatial partitioning in domains (Nora et al. 2012). Cell type-specific contacts imply promoters and regulatory elements within the domains (Dixon et al. 2012; Nora et al. 2012) at loop-attachment points (Lin et al. 2012). At a lower resolution, chromosome conformation capture studies partition spatially the genome in interspersed compartments A and B. Compartment A correlates with gene-rich, highly expressed, DNAse I-sensitive genomic regions and contains accessible "open" chromatin, in opposition to closed chromatin in compartment B. Regions in compartment A, when analyzed as 1 Mb segments, also correlate with histone H3K36me3 and H3K27me3 marks. However, considered as shorter 100 kb segments, all above correlations hold except that for H3K27me3 (Lieberman-Aiden et al. 2009). Smaller, independently defined TADs are contained within A or B compartments. Three-dimensional chromatin architecture studied at yet higher resolution in pluripotent ES cells and neural progenitors showed that invariant TADs contain cell type-specific subdomains determined by looping interactions between regulatory sequences (Philips-Cremins et al. 2013). Major determinants of these spatial arrangements are, in addition to CTCF, the Mediator complex and cohesins, whose previously known roles as transcriptional regulators possibly derive from their activities as architectural proteins. Smaller chromatin loops linking enhancers and promoters involve Mediator and cohesins while interactions between more distant regions involve CTCF and cohesins. Cell lineage commitment and further differentiation would thus be characterized by specific sub-TAD level of chromatin organization (Philips-Cremins et al. 2013). In summary, eukaryotic chromosomes are folded in a highly ordered fashion within the 3D space of the nucleus.

Examples of how transcriptional activity is reflected in three-dimensional domain structure are the α-globin gene and the HoxD cluster (Baù et al. 2010; Noordermeer et al. 2011). At a larger scale, differentiation events correlate with spatial reorganization of chromatin; examples are the variations in LADs during neural differentiation of embryonic stem (ES) cells (Peric-Hupkes et al. 2010) or the changes in chromatin contacts that accompany B-cell development (Lin et al. 2012). By segregating genes encoding regulators of developmental competence (Kohwi et al. 2013) or cell lineage commitment (Lin et al. 2010) to transcriptionally inert regions (as in compartments B), the stability and direction of developmental processes are insured. Then, upon differentiation signals, activating transcription factors confer transcriptional competency to a previously silent compartment. Contacts between enhancer–promoter and promoter–promoter (Li et al. 2012; Lin et al. 2012) within TADs as well as with those in adjacent TADs coalesce into spatially discrete RNA pol II-enriched sites, possibly coinciding with transcription factories (Chakalova et al. 2005; Cook 2010). Inactive genes in these TADS, however, would locate away from the factories, in a configuration

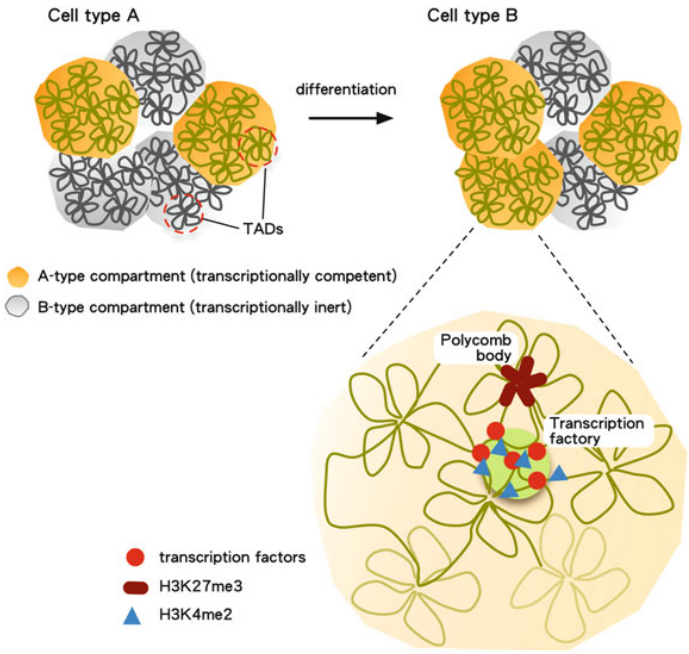

Fig. 5.1 Simplified overview of Polycomb repression and chromatin topology. Chromatin is segregated in large compartments depending on transcriptional activity. Within these compartments chromatin is folded in much smaller architectural units (Topological Associating Domains, or TADs) regardless of transcriptional status. CTCF and cohesins (not shown) delineate and sustain contacts at TADs boundaries. Differentiation cues resulting in differentiation of cell A into cell B concur with acquisition of transcriptional competence that allows coordinated activation of loci (organized in tissue-specific chromatin interactions) within a given TAD. Association of repressed genes is (reversibly) stabilized by Polycomb proteins, whereas transcription factor-dependent association between promoter/enhancer within TADs and with those in other TADs stabilizes association into regions of localized transcription (transcription factories). Only two of the associated histone marks, characteristic of repressed and active genes, are indicated. By stabilizing contacts between not activated loci, Polycomb contributes to decrease undesired fluctuations in gene expression. While robust, the silent state of Polycomb targets is responsive to developmental programs

characterized by H3K27me3 enrichment (Lin et al. 2012). Figure 5.1 depicts a simplified view of chromatin organization linking changes in transcription status and nuclear location during differentiation. Clustering of silent loci is often visualized as speckled areas enriched in Polycomb products known as Polycomb bodies (Mao et al. 2011). Contacts between Polycomb-repressed genes (Bantignies et al. 2011) in Polycomb bodies and their contribution to functional spatial segregation within the topological organization of chromatin are well documented in flies (Delest et al. 2012).

5.2.3 Control of Gene Expression by Regulation of RNA sPolymerase II Activity

Some correlative evidence links the presence of Polycomb products on promoters to an essential step in the regulation of RNA pol II activity: pausing transcriptionally engaged polymerase to prevent productive elongation (Core et al. 2012; Rahl et al. 2010). On a majority of promoters, RNA Pol II is stalled by the activity of the negative elongation factor (NELF) and DRB sensitivity inducing factor (DSIF) or pausing factors (Adelman and Lis 2012; Levine 2011; Zhou et al. 2012). Following binding in an initially hypophosphorylated state, cyclin-dependent kinase 7, a subunit of general transcription factor complex TFIIH, phosphorylates serine 5 (S5P) in the multicopy (52 times) heptapeptide YSTSPS located at RNA pol II C-terminal region. Along with this modification, a short nascent transcript is synthesized, 7-methyl-guanosine added to its 5′ end, and then pausing factors halt elongation. Release from the paused state into full elongation occurs when cyclin-dependent kinase 9 (Cdk9), a subunit of P-TEFb complex, phosphorylates (and inactivates) DSIF, NELF, and also serine 2 of RNA pol II (S2). In vivo imaging shows Cdk9 co-localization in transcription factories, with the paused (S5P) form of RNA pol II, but no so much with the form engaged in processive polymerization (S2P) (Ghamari et al. 2013). In mammalian pluripotent cells, developmental loci repressed by Polycomb bind the nonproductive form of RNA pol II phosphorylated at S5, but not at S2 (Brookes et al. 2012).

5.3 Polycomb-Mediated Posttranslational Modifications

5.3.1 Polycomb-Specific Histone Modifications

Catalytic activities in Polycomb subunits are essential for gene repression and other functions. Substrates of Polycomb-dependent posttranslational modifications include principally histones, but also a variety of other proteins.

In addition to histone H3 methylation (H3K27me3), Polycomb complexes mono-ubiquitylate the C-terminal region of histone H2A (H2AUb1), at lysine 119. The enzymes responsible for these modifications reside in separate biochemical entities or Polycomb-Repressive Complexes (PRCs). Histone ubiquitylation activity resides in PRC1 complexes, whereas histone methyltransferase (HMTase) belongs to PRC2 complexes (the number reflects that the complex was isolated after PRC1). The precise function of these and other histone modifications is intensely debated. A "histone code," as determined by specific combinations of histone modifications, would reflect instructions for transcription changes (Strahl and Allis 2000). Thus, some histone marks are considered as "activating" and other "repressing." The enrichment in both marks, H3K4me3 (activating) and

H3K27me3 (repressing), at Polycomb-silenced promoters underlies, in part, their naming as bivalent regions (Bernstein et al. 2006a). Beyond the semantic part of the argument, other authors propose that histone modifications are primarily determined by transcription and chromatin remodeling (Henikoff and Shilatifard 2011). Certainly, specificity can be appreciated in the binding of chromatin complexes to regions with particular combinations of histone marks (Musselman et al. 2012). However, the complexity of these combinations is rather limited, as stated by the small number of chromatin states observed. Therefore, more important than directing binding, histones modified in one or another way probably allosterically influence the activity of chromatin regulatory proteins (Rando 2012). Indeed, Polycomb HMTase is just one example (see below).

5.3.2 Polycomb Methyltransferases

In mammalian cells, a PRC2 complex containing Enhancer of Zeste homolog 2 (EZH2), Suppressor of Zeste 12 homolog (SUZ12), and Embryonic Ectoderm Development (EED) marks in vitro nucleosomes with H3K27me3 (Cao et al. 2002; Kuzmichev et al. 2002). Of all subunits in the complex, which also contained AE-binding protein 2 (AEBP2) and the retinoblastoma binding protein 4 (RBBP4/RbAP48), only EZH2 contains a SET domain, characteristic of most lysine methyltransferases. A similar complex, containing the ortholog E(Z), was identified in Drosophila (Czermin et al. 2002; Müller et al. 2002). Complexes in mammalian cells containing the paralog EZH1 also show H3K27-specific HMTase activity (Margueron et al. 2008; Shen et al. 2008) and, in some contexts, as in ES cells, EZH1 and EZH2 are functionally redundant (Shen et al. 2008). Additionally, mammalian EZH2 in a PRC2 variant has been shown to methylate in vitro lysine 26 of linker histone H1 (Kuzmichev et al. 2004).

H3K27me3 is the hallmark of Polycomb activity, although how mechanistically it is linked to transcriptional silencing actually is still unclear. SET domain deletion in EZH2 drastically decreases H3K27me3 levels (Shen et al. 2008). Important new evidence strongly supports that unmodified histone H3K27 is the in vivo substrate of Polycomb methyltransferase and that gene repression is linked to methylation: using Drosophila as a model, the deletion of the gene encoding histone H3 and subsequent complementation with unmethylatable K27R variant were found to phenocopy the E(Z) mutation (Pengelly et al. 2013). This demonstrated that Polycomb-dependent repression is inexorably linked to H3K27 methylation. For some targets at least, this function may be linked to PRC1 recruiting (Cao et al. 2002) (see below).

EZH2 HMTase activity depends on its association with subunits EED, SUZ12, RBBP4, and AEBP2. Some of these subunits sense chromatin structure through specific histone contacts so that H3K27me3 nucleosomes stimulate and H3K4me3 or H3K36me2,3 nucleosomes inhibit EZH2 activity (Ciferri et al. 2012; Margueron

et al. 2009; Schmitges et al. 2011). HMTase substrate specificity is determined by the SET domain, as indicated by mutations Y641F or A677G, which make H3K27me2 a preferred substrate rather than H3K27me0 and H3k27me1 used by wild type EZH2 (McCabe et al. 2012b; Sneeringer et al. 2010). Interestingly, these mutations were identified in patients with B-cell lymphoma and correlate with augmented H3K27me3 levels (McCabe et al. 2012a; Sneeringer et al. 2010).

The recent modeling of the three-dimensional structure of PRC2 has helped to explain the contrasting effects of interactions with the chromatin landscape. Critical contacts between the SET motif and the SANT domains of EZH2 are thought to respond to conformational changes in EED and SUZ12, the samplers of histone H3 methylated at K27 or K4/K36, respectively (Ciferri et al. 2012). The model also explains why EZH2 catalytic activity is prevented only on the K27 that resides in the same histone tail with methylated K4 or K36 (Voigt et al. 2012). AEBP2 contacts all other PRC2 subunits assisting in its integrated responses. Thus, PRC2 appears to be a catalytic device with intrinsic ability for spreading repression-compatible histone modifications towards adjacent nucleosomes until it is confronted with inhibitory signals from transcriptionally active regions.

H3K27 methylation is reversed by the action of specific members of the family of Jumonji C (JMJC) demethylases [for more details, see a recent review (Kooistra and Helin 2012)]. KDM1 lysine (K)-specific demethylase 6B (KDM6b/JMJD3) and lysine (K)-specific demethylase 6A (KDM6a/UTX) remove methyl groups from H3K27me3 and H3K27me2 up to the mono-methylated form. Only the Jumonji C domain-containing histone demethylase 1 homolog D (JHDM1D/KDM7A) demethylates H3K27me1 (and other methylated histones too). H3K27 demethylases are recruited to Polycomb targets in pluripotent cells for differentiation-required gene activation (Agger et al. 2007; Lan et al. 2007; Lee et al. 2007). However, often they are associated with active sites, counteracting any EZH2 activity that could interfere with gene expression (Dahle et al. 2010; De Santa et al. 2009). These JMJC proteins, however, can also act independently of their activity as demethylases, for instance, localizing elongation factors to active genes (Chen et al. 2012).

5.3.3 Polycomb H2A Mono-ubiquitin Ligases

Polycomb-dependent histone mono-ubiquitylation of histone H2A, a modification found on 5–15 % of total H2A in mammalian cells (Goldknopf et al. 1975), was identified through biochemical fractionation and following the catalytic activity responsible for the modification (Wang et al. 2004). The addition of the 76 amino acid Ubiquitin (Ub) polypeptide is mediated by an activating enzyme (E1) that transfers Ub to one of several conjugating enzymes (E2); subsequently, E2-Ub associate with a third component, the so-called E3 ligase, that brings in proximity the substrate for ubiquitylation [recently reviewed (Komander and Rape 2012)]. H2A ubiquitylation copurified with a PRC1 complex and functional testing of

individual PRC1 subunits found most activity on the RING-finger protein RING1B/RNF2. This was consistent with the known role of RING-finger proteins as E3 ligases. Other Polycomb RING-finger proteins were present in the complex, but only the RING1 paralogs (RING1A/RING1 and RING1B/RNF2; SCE in Drosophila) act as E3 mono-ubiquitin ligases. The other RING-finger subunits (members of the family of Polycomb group ring finger (PCGF) proteins) function as positive cofactors in the ubiquitylation reaction (Cao et al. 2005; Wang et al. 2004). Thus, Polycomb E3 ligases, as other RING-finger E3 ligases, act as dimers of RING-finger proteins. In vitro studies show that UBCH5C/UBE2D3 is the preferred E2 element in H2A mono-ubiquitylation (Buchwald et al. 2006). Structural studies show that UBCH5C/UBE2D3 associates with RING1B through an interface resulting from the folding of the RING finger, away from the region that binds PCGF subunits (Bentley et al. 2011; Buchwald et al. 2006; Li et al. 2006). Binding to the nucleosome substrate involves DNA and an acidic patch on histone H4 that contact a basic interface demarcated by a RING1B-BMI1/PCGF4 dimer (Bentley et al. 2011). Pairs of RING1-PCGF proteins are the defining unit PRC1 complexes (see below). It is generally assumed that the E3 ligase activity lies mostly with RING1B/RNF2; however, both in vitro (Buchwald et al. 2006) and in vivo evidence (de Napoles et al. 2004) demonstrates that RING1A/RING1 also acts as an E3 ligase.

Polycomb RING1 proteins are the major histone H2A ubiquitin ligases, as shown by the undetectable levels in cells depleted from these proteins (de Napoles et al. 2004). Likewise, SCE is the major H2A ubiquitin ligase in Drosophila (Gutierrez et al. 2011). However, in some contexts additional E3 ubiquitin ligases mono-ubiquitylate histone H2A. For instance, RNA-binding RING-dependent ubiquitin protein ligase (hRUL138/DZIP3) acts as part of a NCoR-HDAC complex that represses chemokine genes (Zhou et al. 2008) or ubiquitin protein ligase E3 component n-recognin 2 (UBR2) that modifies histone H2A during spermatogenesis (An et al. 2010). Also, the Cullin4B-Ring E3 ligase complex (CRL4B), a member of the family of cullin-RING E3 ligases (Jackson and Xiong 2009), has been shown to mono-ubiquitylate histone H2A in cancer cells (Hu et al. 2012), an unexpected observation given its inability to modify nucleosomal H2A in vitro (Wang et al. 2006). The histone variant H2A.Z (H2Av in Drosophila) is found at the silent X-chromosome but also in transcriptionally active regions and in Polycomb-regulated bivalent domains [not in stably Polycomb-silenced sites, though (Creyghton et al. 2008; Ku et al. 2012)]. It can also be mono-ubiquitylated in a RING1-dependent manner (Ku et al. 2012; Sarcinella et al. 2007). Interestingly, H2A.Z ubiquitylation occurs not only at lysine 120 (equivalent to H2A K119) but also at lysines 121 and, to a less extent, 125 (Ku et al. 2012).

What are the consequences of H2A mono-ubiquitylation on transcription? Correlative evidence shows a link between histone Polycomb-dependent H2AUb1 and gene repression in ES cells. Thus, upregulation of gene expression concurrent with H2AUb1 loss in RING1-deficient cells is rescued by wild type RING1B but not by catalytically inert forms (RING1B mutants I53S or I53A) (Endoh et al. 2012).

H2AUb1 dependent and independent Polycomb repression is also seen in Drosophila (Gutierrez et al. 2011). Mechanistically, the question remains to this day without clear answer. In vitro, H2AUb1 nucleosomes are not efficiently tri-methylated at histone H3K4, and this results in transcription initiation failure (Nakagawa et al. 2008).

Regardless of the silencing mechanism, the correlation between gene repression and histone H2AUb1 modification is generally consistent with activation associated with ubiquitin proteases that remove the Ub moiety from histone H2A (Joo et al. 2007; Zhu et al. 2007). Histone H2A deubiquitinating enzymes are a large and structurally diverse set, some acting on several substrates, in addition to H2A. They are members of the family of Ub-specific proteases [USP10 (Draker et al. 2011), USP12 (Joo et al. 2011), USP16 (Joo et al. 2007), USP21 (Nakagawa et al. 2008), USP22 (Zhao et al. 2008b), and USP46 (Joo et al. 2011)], of the Ub C-terminal hydrolases [Brca1-associated protein 1(BAP1) (Scheuermann et al. 2010)], and of the JAB1/MPN/Mov34 metalloenzyme (JAMM) metalloproteases [myb-like, SWIRM and MPN domains 1 (MYSM1) (Zhu et al. 2007)]. Of these, at least USP10 also deubiquitinates H2A.Z (Draker et al. 2011). Another protease, USP16/UBP-M, is responsible for the deubiquitination wave that accompanies mitosis (Joo et al. 2007). It appears that these proteases function in a local context. For instance, in prostate cancer cells, MYSM1, as part of a histone acetyltransferase (HAT)-containing complex, activates androgen receptor (AR)-regulated genes, in a process coupled to removal of linker histone H1 (Zhu et al. 2007). In hematopoietic cells, MYSM1 associates with BRAHMA/SMARCA2, an ATPase of the SWI/SNF type of chromatin remodelers, to activate the B-cell lineage transcription factor EBF1 (Jiang et al. 2011b). These results indicate that MYSM1 and perhaps other H2A deubiquitinases act as part of varied complexes involved in transcriptional activation. However, not every H2A deubiquitinase participates in gene activation. In Drosophila, inactivation of H2A ubiquitin protease Calypso (the homolog in mammals is BRCA1-associated protein 1, BAP1) results in loss of repression at a subset of Polycomb targets (Gutierrez et al. 2011; Scheuermann et al. 2010). Calypso, together with the Polycomb member Additional sex combx (ASX), is part of a Polycomb-repressive deubiquitinase complex (PR-DUB) complex that associates with Polycomb response elements [PREs, DNA sequences that recruit Polycomb complexes (see below)] (Scheuermann et al. 2010). In the absence of Calypso, ubiquitylation and deubiquitylation cycles, a process that has been proposed as necessary for repression, cannot take place. In mammalian cells, BAP1 may function independently of its in vitro H2A-deubiquitylating activity (Scheuermann et al. 2010). Its major impact may result from its ability to stabilize other regulators such as host cell factor-1 (HCF-1) and O-linked *N*-acetylglucosamine transferase (OGT) (Dey et al. 2012) (see below). In agreement with this, Polycomb-dependent repression of Hox genes is not affected by BAP1 inactivation (Abdel-Wahab et al. 2012).

5.3.4 Other Histone Modifying Activities

Some of the subunits in Polycomb complexes not identified genetically as Polycomb products are also histone modifiers. Among them is FBXL10/KDM2B, a DNA-binding protein involved in PRC1 recruiting (see below). FBXL10/KDM2B has a JMJC domain that can demethylate histone H3K36 (He et al. 2008) and H3K4 (Frescas et al. 2007), although how influential this activity is in gene control is not established.

5.3.5 Non-histone Substrates of Polycomb Enzymes

The catalytic activities of Polycomb complexes are not restricted to histones. Even the well-known histone modifiers EZH2 and RING1B/RNF2 have been shown to act on non-histone substrates. An example is the EZH2-dependent methylation of transcription factor GATA4, a modification that weakens its binding to HAT p300 and thus reduces its activating ability (He et al. 2012). Another substrate is transformation-related protein 53 (TRP53) poly-ubiquitylation by RING1B/RNF2 in some tumor cells (Su et al. 2013).

5.3.6 SUMO Modification

Small ubiquitin-like modifier (SUMO) family proteins alter the function of covalently bound substrates analogously to ubiquitylation. SUMO modifications also occur in a stepwise manner: an E1 activating enzyme transfers SUMO polypeptide to the E2 ligase (ubiquitin-conjugating enzyme E2I/UBC9) which upon binding to a substrate-bound E3 adaptor links the SUMO moiety to the substrate [reviewed in Geiss-Friedlander and Melchior (2007)]. The activity of PRC1 subunit chromobox 4 (CBX4/PC2) as a SUMO adaptor was found serendipitously in cotransfection assays with C-terminal-binding protein 2 (CTBP2), an interacting partner known to be SUMOylated (Kagey et al. 2003). Besides CTBP2, CBX4/PC2 SUMOylates a variety of substrates, including de novo DNA methyltransferase 3a (Dnmt3a) (Li et al. 2007), CTCF (MacPherson et al. 2009), or homeodomain interacting protein kinase 2 (HIPK2) (Roscic et al. 2006). CBX4/PC2 itself can be SUMOylated and together with UBC9 and other modified substrates localizes at nuclear bodies enriched in Polycomb products, or Polycomb bodies (Kagey et al. 2003). CBX4/Pc2 SUMOylation regulates PRC1 assembly on chromatin, as deduced from the increased association of complexes containing hyperSUMOylated CBX4/PC2 in tissues deficient in the SUMO-specific protease 2 (Senp2) (Kang et al. 2010). A similar positive effect on Polycomb association is seen upon SUMOylation of *C. elegans* Polycomb protein SOP-2 (Zhang

et al. 2004). In contrast, as a puzzling observation, sumoylation of SOP-2 homolog in Drosophila, Sex Comb on midleg (SCM), is linked to decreased binding to PREs and repressing activity (Smith et al. 2011). These are examples of profound impact on Polycomb complexes mediated by reversible posttranslational modification of their subunits.

5.3.7 Protein Glycosylation

The addition of a single O-linked *N*-acetylgucosamine to serine or threonine residues is a posttranslational modification of functionally diverse proteins, including many important transcriptional regulators [reviewed in Hanover et al. (2012)], among them Drosophila Polyhomeotic (PH) (Gambetta et al. 2009). In fly embryonic tissues, the maintenance of Polycomb-dependent repression is lost in mutants lacking O-linked GlcNAcylation, explaining that the gene encoding the O-linked *N*-acetylglucosamine transferase (OGT), Super sex combs (SXC), is categorized as a Polycomb gene (Gambetta et al. 2009; Sinclair et al. 2009). O-GlcNacetylated proteins are found at Polycomb Regulatory Elements (PRE) DNA sequences. However, while global PH binding decreases in SXC mutant cells, neither H3K27me3 marks of E(Z) occupancy are affected (Gambetta et al. 2009). The full elucidation of OGT impact on Polycomb function needs further studies.

5.4 Polycomb Biochemical Entities

Polycomb complexes are conveniently categorized into PRC1 and PRC2 classes, that not only contain non-overlapping sets of subunits but are enzymatically characterized by their abilities to modify histones H2A (PRC1) or H3 (PRC2). Although biochemically heterogeneous, a minimum set of subunits or complex core is strictly required for their enzymatic activities and is shared among complexes within the same class. Other subunits add regulatory functionality to PRC1 and PRC2, although for many of them their roles have not been determined. A detailed description of known complexes is included in this book in Chap. 6. Here, I present a brief overview of PRC-specific complex cores and additional subunits, focusing on protein motifs related to their activities.

5.4.1 PRC2 Complexes

The organization and regulation of PRC2 has recently been reviewed (O'Meara and Simon 2012). A functional Polycomb HMTase consists of: the catalytic subunit (paralogs, EZH1 and EZH2), histone binding modules (RBBP4/RAbp48, EED),

and regulator (SUZ12) and scaffold (AEBP2) components. EED and RBBP4 are proteins with propeller-like folded WD40 repeats, a structure found in other histone binding proteins. SUZ12 has a VEFS domain (an acidic cluster and a tryptophan/methionine-rich sequence named after its presence at the C-terminal region of proteins VRN2-EMF2-FIS2-Su(z)12) which is essential for HMTase inhibition. EZH paralogs contain, in addition to a lysine methyltransferase SET domain, two SANT domains. From the above described model for the core PRC2 complex between two nucleosomes (Ciferri et al. 2012) it appears that EED binding to histone H3K27me3 contacts a SANT domain to allosterically activate EZH2 (Margueron et al. 2009); conversely, RBBP4-bound histone H3K4me3 or H3K46me3 inhibits EZH2 (Schmitges et al. 2011) through contacts mediated by SUZ12. AEBP2 contacts all other subunits and its three zinc fingers hold potential for DNA binding (Kim et al. 2009). The model suggest that the presence of EED isoforms, differing at their N-terminal region (Kuzmichev et al. 2005), could be functionally relevant given its contact with EZH2 SANT domain. The PRC2 core is organized as a regulatory unit whose stability is crippled in the absence of some subunits, as seen after depletion of EED or SUZ12 (Montgomery et al. 2005; Pasini et al. 2004).

Non-core PRC2 subunits are mostly involved in PRC2 interaction with histones. These include the Plant homeodomain (PHD) proteins of the Polycomb-like (PCL) family: PHD finger protein 1 (PHF1/PCL1), metal response element binding transcription factor 2 (MTF2/PCL2) and PHD finger protein 19 (PHF19/PCL3) and jumonji, AT-rich interactive domain 2 (JARID2). One or another PCL subunit facilitates association with H3K36me3 regions through their PH domains and JARID2 plays important roles in PRC2 binding and modulation of its activity.

5.4.2 PRC1 Complexes

The core element of PRC1 complexes is a heterodimer of RING-finger proteins: a E3 ligase for histone H2A mono-ubiquitylation (either RING1A or its paralog RING1B) and a member of the Polycomb group of Ring-Finger (PCGF) family, which act as a positive cofactor. A variable number of additional subunits, in distinct sets, associate with core elements defined by each of the six PCGF proteins (Gao et al. 2012; Gearhart et al. 2006; Levine et al. 2002; Ogawa et al. 2002; Sánchez et al. 2007).

PRC1 complexes have been named after the PCGF member present. Thus, complexes with PCGF2/MEL18 or PCGF4/BMI1 were termed PRC1.2 and PRC1.4, respectively, and are considered the canonical PRC1 complex. Characteristically, these PRC1 complexes, but not others, contain Polyhomeotic-like paralogs (PHC1, PHC2, PHC3), proteins with a sterile alpha motif (SAM) widely used domain in protein–protein interactions (Qiao and Bowie 2005) which are instrumental in Polycomb repression (Isono et al. 2013); additional PRC1.2 and PRC1.4 subunits with SAM motifs are the Sex comb on midleg paralogs (SCML1,

SCML2), one of which (SCML2) also has a malignant brain tumor (MBT) motif, a binding domain for methylated histone H3K9 (Bonasio et al. 2010). Another feature of PRC1.2 and PRC2.4 is the presence of one or more paralogs of the CBX family of N-terminal chromodomain-containing proteins (CBX2/M33, CBX4/PC2, CBX6, CBX7 and CBX8), the homologs of Drosophyla Polycomb. Chromodomains, as MBT repeats, recognize histone methylated at lysines, and those in CBX proteins preferentially bind tri-methylated H3K27 (Bernstein et al. 2006b; Fischle et al. 2003).

While PCGF and RING1 proteins associate through their N-terminal RING-finger motifs, the C-terminal region of RING1 proteins interacts with a conserved Polycomb repressor box at the C-terminal region of CBX proteins (Satijn et al. 1997; Schoorlemmer et al. 1997). That same RING1 region binds the RING1 and YY1-binding protein (RYBP) (García et al. 1999) and its paralog YY1-associated factor 2 (YAF2) (Kalenik et al. 1997). RING1 proteins bind either CBX or RYBP exclusively (Wang et al. 2010). This probably explains why the other PRC1 complexes (PRC1.1, PRC1.3, PRC1.5, and PRC1.6) contain, instead of CBX subunits, RYBP or YAF2 subunits (Gao et al. 2012). The RING1-PCGF1/NSPC1 core is found with KDM2B (a DNA-binding protein) and BCOR paralogs (Gearhart et al. 2006; Sánchez et al. 2007); PRC1.6 contains RING1-PCGF6/MBLR; heterodimers DP1-E2F6 and MAX-MGA that bind DNA sequences for E2F sites and E2 boxes, respectively; the MBT-repeat protein l(3)mbt-like 2 (L3MBTL2) and other subunits (Ogawa et al. 2002); PRC1.3 and PCR1-5, finally, are defined by heterodimers RING1-PCGF3 and RING1-PCGF5 and contain, yet, additional subunits. Altogether, PRC1 complexes are far more heterogeneous than PRC2. PCGF subunits bind chromatin in partially overlapping patterns (Gao et al. 2012), suggesting distinctive activities for PRC1 complexes, although this remains largely unknown.

5.4.3 Other Complexes with Polycomb Subunits

While simplified PRC1 forms and PRC2 are recognizable in Drosophila, other complexes found in flies seem not to have corresponding homologs in mammals. A protein assembly recently isolated containing Sex comb on midleg with four MBT domains (SFMBT) homologs is proposed to be the counterpart of PHO-repressive complex (PHO-RC), a heterodimer of PHO and SFMBT proteins (Klymenko et al. 2006). The mammalian complex contains additional subunits, including well-known chromatin modifiers as LSD1 and COREST (Zhang et al. 2013). Analogously to PHO-RC, mammalian SMFBT complexes also interact with PRC1 (Zhang et al. 2013).

As mentioned earlier, Drosophila PR-DUB complex contains ubiquitin protease Calypso and ASX (Scheuermann et al. 2010). Calypso homolog in mammalian cells, BAP1, also associates with homologs ASXL1 and ASXL2, but unlike Drosophila PR-DUB, they form part of much diverse biochemical entities (Dey et al. 2012).

5.5 Targeting Polycomb Function

Transitions between cell states, from pluripotent to more differentiated cell types, are accompanied by changes in the genomic regions marked by Polycomb activity (Bracken et al. 2006; Mohn et al. 2008). In Drosophila cells, nucleosomes at Polycomb-targeted promoters are in a highly dynamic state (Mito et al. 2007) and steady-state histone modifications requires continued Polycomb recruitment. Indeed, Polycomb association with chromatin, as measured by live imaging (FRAP), shows very short residence times, within the same range as transcription factors (Steffen et al. 2012). Of note, exchange rates are highest at pluripotent cells and tend to slow down in more mature cells (Fonseca et al. 2012). During differentiation, Polycomb colonization of new sites is accompanied by eviction from sites destined to be derepressed, reflecting a different outcome of antagonic influences on Polycomb association at these sites. In contrast, at stably silenced regions, Polycomb presence probably is maintained by a lower rate of chromatin remodeling and the spreading of Polycomb-modified nucleosomes, thereby contributing to the developmental restriction that goes with cell differentiation (Zhu et al. 2013). In some cases, however, loci silenced by Polycomb progressively acquire a stably silent state maintained by Polycomb-independent means, generally involving DNA methylation (van Arensbergen et al. 2013).

How Polycomb complexes are directed to their targets is a subject of intense research. Seminal work with pluripotent mammalian cells has mapped PRC1 and PRC2 binding preferentially to promoters of loci encoding developmental regulators (Boyer et al. 2006; Lee et al. 2006). These promoters are located in a subset of specialized, methylation-free GC-rich sequences (CpG islands, CGI) (Ku et al. 2008; Mikkelsen et al. 2007). Nucleosomes at these sites are enriched in H3K4me3 and H3K27me3 marks, usually thought of as "activating" and "repressing" marks. In general, these loci show little or no expression in pluripotent cells. However, upon differentiation their status changes and promoters retain one or another mark depending on activation or silencing of the locus in the new cell state (Azuara et al. 2006; Bernstein et al. 2006a; Cui et al. 2009; Mikkelsen et al. 2007). Indeed, removal of H3K27 methylation through EED inactivation results in derepression of these promoters (Boyer et al. 2006); on the other hand, decreased H3K4 methylation at these promoters, upon downregulation of dpy-30 homolog (DPY30), a subunit of SET1/MLL complexes, interferes with transcriptional activation needed at genes induced during differentiation(Jiang et al. 2011a). It has been proposed that such a singular chromatin configuration (bivalent domains) (Bernstein et al. 2006a) allows genes encoding developmentally relevant transcription factors and signaling molecules to be silent while poised for activation. Polycomb regulation in Drosophila, however, occurs in the absence of CGIs or "bivalent domains." Instead, functionally similar regions are identified, bound by Polycomb and Trithorax (TrxG) products (some of which are MLL homologs). These regions are thought to be in a "balanced" state and—although enriched in H3K27me3—have no H3K4me3 marks (Gaertner et al. 2012; Schwartz

et al. 2010). Recently, ChIP studies in *D. melanogaster* showed that in addition to transcriptionally silent loci, PRC1 subunits also bind transcriptionally active promoters co-occupied by cohesins, where they participate in promoting expression from these loci (Schaaf et al. 2013b).

The association of Polycomb complexes with chromatin is influenced by DNA-binding proteins, noncoding RNAs, and interactions with resident proteins such as histones. It is conceivable that the nature of these associations and the possibility of their mutual reinforcement determine the overall avidity of binding. Therefore, while recruiting has been usually considered to be instructed, for instance, by proteins or RNAs recognizing specific DNA sequences, it is becoming increasingly accepted that Polycomb association with targets is a consequence of chromatin sampling, thereby being responsive to transcriptional status (Klose et al. 2013). First, I will discuss mechanisms that influence binding of Polycomb complexes to its targets and then their maintenance or eviction.

5.5.1 Polycomb Recruiting Through DNA-Binding Proteins

With the exception of Drosophila Pleiohomeotic (PHO) and its paralog (PHO-L) genetically defined Polycomb products lack ability to bind DNA (PHO-L) (Brown et al. 1998, 2003). PHO, PHO-L and its vertebrate homolog YY1 transcription factor (YY1) bind DNA through four conserved zinc-finger motifs (Brown et al. 1998). In mammals, however, evidence for YY1-dependent association of Polycomb proteins to targets is limited (Woo et al. 2010) and it appears likely that YY1 cannot be considered as a general Polycomb recruiter in mammals (Mendenhall et al. 2010). In Drosophila, Polycomb-repressive elements (PREs), genomic regions with sites for PHO and other DNA-binding proteins recruit Polycomb complexes and mediate repression of transgenic constructs and endogenous targets (Müller and Kassis 2006). Other DNA-binding proteins functionally linked to Polycomb silencing are GAGA factor (GAF), Dorsal Switch Protein 1 (DSP1), Pipsqueak (PSQ), Grayny Head-like (GRH), Zeste, and SPPS (a member of the Sp1/KLF family of zinc-finger proteins) (Ringrose and Paro 2007). Polycomb recruiting to PREs is most likely indirect, through subunits that interact with DNA-binding proteins, as illustrated by Polycomb (PC) association with PSQ and GRH (Strübbe et al. 2011). PRE-like sequences are hardly known in mammalian cells (Sing et al. 2009; Woo et al. 2010). However, comparative mapping of H3K27me3-marked regions and RNA transcripts in a neural differentiation model identifies intergenic sequences (Transcribed Intergenic Polycomb sites, TIPs) which might be analogous to intergenic PREs in Drosophila (Hekimoglu-Balkan et al. 2012). At any rate in Drosophila cells, in addition to PREs, PRC1 proteins bind, facilitated by cohesins, many promoters (Enderle et al. 2011), although in this case not for silencing functions (Schaaf et al. 2013b).

5.5.2 *Proteins Binding GC-Rich DNA as Recruiters of Polycomb Complexes in Vertebrates*

In mammalian pluripotent cells, EZH2 and SUZ12 occupy CGI regions (Ku'08), unusual genomic domains which are unmethylated genomic domains interdispersed in a landscape of methylated DNA (Deaton and Bird 2011; Illingworth and Bird 2009; Stadler et al. 2011). About 70 % of mammalian promoters, including many at intergenic sites are contained within CGIs (Illingworth et al. 2010). Gene expression, divergent transcription, RNA pol II pausing, and nucleosome destabilization, all of them features of a permissive chromatin state concur at CGIs (Blackledge and Klose 2011; Core et al. 2008; Deaton and Bird 2011; Fenouil et al. 2012). Recent work shows that CGI-like, non-methylated Polycomb marked regions are present throughout vertebrates and, therefore, are not unique to warm-blood vertebrates as previously thought (Long et al. 2013b). PRC1 subunits also locate to CGI, although co-localization with PRC2 products is restricted to the subset of larger size CGIs (Ku et al. 2008). Gene bodies of Polycomb-repressed genes in ES cells are marked by H3K27me3 and H2AUb1, but enrichment peaks map close to the transcription initiation site (TSS) (Brookes et al. 2012).

To test whether the prevalent location of Polycomb complexes at CGI is mediated by DNA-binding proteins, computational searches for binding motifs recognized by transcription factors yielded a reduced number of sites for repressors, mostly expressed in differentiated cells, i.e., nonfunctional in ES cells. Moreover, Polycomb-bound regions showed a remarkable absence of binding motifs for transcriptional activators (Ku et al. 2008). Thus, the best predictor for Polycomb association is a high content in GC sequences (Mendenhall et al. 2010). In an alternative approach, searching in pluripotent cells for transcription factors contained in Cbx-containing PRC1 complexes, the RE1-silencing transcription factor (REST) was identified (Dietrich et al. 2012; Ren and Kerppola 2011). This DNA-binding protein that also interacts with PRC2 (Dietrich et al. 2012) was among the very few transcription factors identified during a computational search of TF motifs in Polycomb-bound CGIs in ES cells (Ku et al. 2008). However, whether REST is directly recruiting Polycomb to their targets is not clear, since even though RING1B or SUZ12 is enriched among REST binding sites, RING1B occupies only a very small subset of REST motifs (Dietrich et al. 2012) and genes derepressed upon inactivation of REST overlap only partially with those upregulated in RING1B-deficient cells (Dietrich et al. 2012). Despite this, independent experiments showed REST motifs appearing in a different computational search that combined the occurrence of predicted binding sites for transcription factors with the dynamic changes in H3K27me3 occurring during neural differentiation of pluripotent cells (Arnold et al. 2013). This study also revealed motifs for members of the SNAIL family of transcription factors that together with REST motifs were found predictive of transient H3K27me3 marks taking place during differentiation of neural progenitors. Furthermore, DNA fragments containing REST or SNAIL binding sites confer H3K27m3 enrichment to linked sequences

in transgenes, demonstrating the ability of transcription factors to configure chromatin landscapes (Arnold et al. 2013).

The PRC1 subunit FBXL10/KDM2b has also been shown to be involved in recruiting Polycomb complexes. FBXL10/KDM2b has a CXXC zinc-finger motif similar to that of other proteins known to bind non-methylated CpG sequences (Long et al. 2013a). ChIP studies show that most PRC2- and PRC1-bound sites in ES cells are also enriched in FBXL10/KDM2b (Farcas et al. 2012; He et al. 2013; Wu et al. 2013) and that binding depends on the CXXC domain (He et al. 2013; Wu et al. 2013). FBXL10/KDM2b interacts directly with RING1B (Sánchez et al. 2007) and PCGF1/NSPC1 (Wu et al. 2013). Although RING1B enrichment at Polycomb targets decreases modestly when FBXL10/KDM2b is downregulated, overall levels of total H2AUb1 are clearly reduced (Wu et al. 2013). CBX7 association, in contrast, is not affected, consistent with its ability to bind H3K27me3. Thus, Polycomb proteins bound at their targets in the absence of FBXL10/KDM2b account for poor derepression (Farcas et al. 2012; He et al. 2013; Wu et al. 2013). FBXL10/KDM2b is bound to most CGIs (Wu et al. 2013), suggesting that only binding to DNA is not sufficient for recruitment of this PRC1 complex. Whether the extended contacts offered by large Polycomb-bound CGIs or the activity of additional players help locating FBXL10/KDM2b Polycomb partners to CGIs remains to be established. The use of DNA-binding proteins that recognize non-methylated DNA may explain why in hypo-methylated cells H3K27me3 marks appear at ectopic sites while their presence decreases at Polycomb targets, which concomitantly are upregulated (Reddington et al. 2013). Therefore, the activity of DNA methyltransferases and the selective recognition of methylated/unmethylated DNA may be important during the establishment of Polycomb domains after epigenetic reprogramming at the earliest stages of development.

5.5.3 Other DNA-Binding Proteins as Polycomb Recruiters

At least two PRC2 subunits with potential for DNA binding may play a role in recruiting the complex to their targets. One, JARID2, was not found in initial isolates of PRC2 complexes. JARID2 has JMJC domain related to that found in histone demethylases, although it is catalytically inactive (Tsukada et al. 2006). Several groups found that JARID2 and EZH2 or SUZ12 co-occupy a large number of genomic sites (Landeira et al. 2010; Li et al. 2010; Pasini et al. 2010a; Peng et al. 2009; Shen et al. 2009). JARID2 inactivation is accompanied by decreased PRC2 binding. However, the effects on H3K27me3 levels differ among studies and are interpreted proposing HMTase-inhibiting (Peng et al. 2009; Shen et al. 2009) or activating (Li et al. 2010; Pasini et al. 2010a) roles for JARID2. These discrepancies remain unresolved and the existence of distinct PRC2 complexes, one without JARID, responsible for most H3K27 methylation, and another with JARID2, strongly bound to DNA, has been suggested by way of explanation (Herz and

Shilatifard 2010). In vitro, JARID2 binds DNA through its AT-rich interaction domain (ARID) (Li et al. 2010), but the in vivo effect of this possible binding has not been determined.

PRC2 subunit AEBP2 is a three zinc-finger protein binds to an unusual, CTT(N) 15-23cagGCC sequence. A very small collection of genomic sites bound in brain tissue by AEBP2 was also bound by SUZ12 (Kim et al. 2009). It is not clear if all AEBP2 bound depends on its DNA-binding activity and if this capacity would serve to target PRC2 or if, on the contrary, most AEBP bound to chromatin is a part of PRC2 targeted by other means.

PRC2 recruitment is affected by loss-of-function mutations in ASXL1 (Abdel-Wahab et al. 2012). The levels of H3K27me3 and derepression of Polycomb targets also are associated with ASXL1 inactivation. ASXL1 belongs to complexes with ubiquitin protease BAP1 (Dey et al. 2012; Scheuermann et al. 2010), and although it is not found in PRC2 complexes, it co-immunoprecipitates with the PRC2 subunit SUZ12 (Abdel-Wahab et al. 2012). There is no evidence for ASXL proteins binding DNA directly, although bioinformatic analysis identifies a N-terminal domain compatible with a winged helix-turn-helix fold found in other DNA-binding proteins (Aravind and Iyer 2012).

PRC1 recruitment through DNA-binding proteins has been described in hematopoietic cells. Co-occupancy of genomic sites bound by RING1B and the runt-related transcription factor 1 (RUNX1), a heterodimeric DNA-binding protein found as a fused product in acute myeloid leukemia, has been observed in hematopoietic cells (Yu et al. 2012). Moreover, upon RUNX1 deletion, RING1B occupancy is reduced, consisting with a role for RUNX1 in Polycomb recruiting. Biochemical analysis shows that this can occur through direct interaction between PCFG4/BMI1 and RUNX1 (Yu et al. 2012).

Finally, the PRC1 subunit RYBP which binds non-specifically DNA in vitro (Neira et al. 2009) has been proposed as a mediator of PRC1 recruiting independent of binding to H2K27me3 (Tavares et al. 2012). RYBP binds many genomic sites occupied by RING1B (Gao et al. 2012; Hisada et al. 2012; Morey et al. 2013; Tavares et al. 2012) and its association with chromatin, in contrast to that of RING1B, is not affected by EED depletion (i.e., lack of H3K27me3) (Hisada et al. 2012; Tavares et al. 2012). In the absence of H3K27me3, RING1B binding is very much decreased (Leeb et al. 2010; Tavares et al. 2012), and therefore, it is difficult to evaluate the actual contribution of RYBP to PRC1 recruitment in the presence of H3K27me3. After RYBP inactivation, the extent of PcG targets occupancy by PRC1 is affected mildly (Hisada et al. 2012; Morey et al. 2013) or more substantially (Tavares et al. 2012) at the same time that H2AUb levels decrease (Gao et al. 2012; Morey et al. 2013; Tavares et al. 2012).

In summary, it is clear that the association of PRC complexes with chromatin can be facilitated by DNA-binding proteins. Of these, proteins recognizing generic DNA features (i.e., CpG-rich sequences) play a more prevalent role than conventional transcription factors. However, within specific cell lineage or developmental time contexts, these may contribute effectively to PRC recruitment to specific targets.

5.5.4 Polycomb Association with Chromatin Through Interaction with Histones

Some Polycomb subunits recognize and bind specific sites in histones. As for many other chromatin modifiers, this represents opportunities to promote binding and stabilization of its association or, on the contrary, to repel contact. These activities can be determined not only by covalent modifications at histone tails, but also by nucleosome density.

Chromobox-containing subunits of PRC1 complexes recognize and bind in vitro tri-methylated H3K27 (Bernstein et al. 2006b; Fischle et al. 2003). For a long time PRC2-dependent recruitment of PRC1 has been considered to be essential for PRC1 targeting. Chromatin binding of chromobox CBX7 PRC1 subunit is severely affected in EED-deficient (without H3K27me3 marks) cells (Tavares et al. 2012), just as it is the association of RING1B (Leeb et al. 2010; Tavares et al. 2012), presumably due to its CBX7-dependent binding, indirectly, through CBX7. However, PRC1 subunits (or H2AUb1 marks) only co-localize partially with PRC2-bound/H3K27me3-enriched sites (Ku et al. 2008). Moreover histone H2AUb1 or RING1B recruitment to the silenced X-chromosome is little affected in cells without H3K27me3 (Leeb et al. 2010; Schoeftner et al. 2006; Tavares et al. 2012). Together, these observations support the existence of alternative means for PRC1 targeting.

As described above, the methylation status of specific residues of histone H3 influences PRC2 association as well as the catalytic activity of EZH2 (Margueron et al. 2009; Schmitges et al. 2011). Thus, methylated H3K4 and H3K36 are refractory to PRC2 association, while methylated H3K27 stimulates binding and methyltransferase activity. Most likely this indicates that such contacts are mainly mechanisms by which alterations in histone modifications spread to adjacent nucleosomes. Chromatin modifiers that participate in propagation of chromatin states often act through binding to the product of the activity of the catalytic subunit, thereby enhancing the processivity of the modification (Hathaway et al. 2012). However, there is an apparent inconsistency of PRC2 HMTase inhibition by H3K4me3 (Schmitges et al. 2011) and the coexistence of bound Polycomb at nucleosomes with H3K4me3 and H3K27me3 in bivalent domains. An explanation for this finding is that these modifications are on separate H3 tails in vivo and that PRC2 inhibition only occurs when K4 and K27 marks are in a nucleosomal symmetric fashion, but not if asymmetric (Voigt et al. 2012).

5.5.5 Noncoding RNAs as Polycomb Recruiters

Noncoding RNAs (ncRNAs) are a large collection of nuclear and cytoplasmic RNAs synthesized similarly to mRNAs and that engage in a variety of regulatory functions (Batista and Chang 2013; Guttman and Rinn 2012; Mercer and Mattick

2013). ncRNAs fold in stable high-order structures which determine their function. Often they are the product of divergent transcription, a characteristic of RNA pol II promoters (Core et al. 2008; Seila et al. 2008), in which the paired transcript is a protein-coding mRNA (Sigova et al. 2013). Biochemical analysis shows molecular interactions between some ncRNAs and chromatin modifiers, including Polycomb products (Guttman et al. 2011; Khalil et al. 2009; Zhao et al. 2010).

The idea that ncRNAs may recruit Polycomb complexes to targets originated in studies about the function of a ncRNA expressed from the HOXC gene cluster, HOX Antisense Intergenic RNA (HOTAIR). Its inactivation correlates with upregulation of a segment of the HOXD cluster encoding the late-expressing genes HOXD8 to HOXD13 (Rinn et al. 2007). Moreover, this derepression is accompanied by loss of H3K27me3 and reduced SUZ12 occupancy. Since HOTAIR binds SUZ12 and EZH2, it was suggested that ncRNAS could target Polycomb-dependent repression in trans (Rinn et al. 2007). In agreement with this idea, ectopic HOTAIR expression in epithelial tumor cells results in altered distribution of H3K27me3 and PRC2 occupancy of new sites (Gupta et al. 2010). Other examples of Polycomb recruiting through ncRNAs are found at the silenced X-chromosome and some imprinted loci on mouse chromosomes 7 and 12. For example, RepA, a ncRNA encoded in the Xist locus (Zhao et al. 2008a), or ncRNAs from Kcnq1ot1 or Meg3 loci (Pandey et al. 2008; Zhao et al. 2010) also bind PRC2 products and are required for sustained H3K27me3 levels and locus silencing. In all cases, targeting occurs in cis, unlike HOTAIR which operates in trans. Binding of PRC1 complexes to other ncRNAs has also been described (Guttman et al. 2011; Yap et al. 2010). The best studied, ANRIL, an antisense transcript overlapping the Ink4 locus in human cells (encoding tumor suppressors), recruits CBX7 in cis (Yap et al. 2010). Polycomb binding to ncRNAs occurs through RNA sequences folded in complementary stem-loop structures (Zhao et al. 2008a). Rather than restricted to a few ncRNAS, a large number of them are found in pull-down assays with anti-SUZ12 and anti-EZH2 antibodies (Khalil et al. 2009; Zhao et al. 2010). In addition, many short ncRNAs, $\simeq$50–200 nt in length, associated with CGI regions, contain sequences with potential stem-loop folding that bind SUZ12 (Kanhere et al. 2010). These short ncRNAs use TSSs distinct from those of mRNAs, are expressed independently of Polycomb, and are lost from loci derepressed during differentiation (Kanhere et al. 2010). It is not known whether, as longer ncRNAs (Guttman et al. 2011; Tsai et al. 2010), they also bind other chromatin regulators.

Specific protein domains involved in ncRNA binding have not been defined, except for the chromobox of CBX7, which binds ANRIL although through residues not involved in H3K27me3 recognition (Yap et al. 2010). On the other hand, EZH2 affinity for HOTAIR is affected by cyclin-dependent kinase 1 (CDK1) phosphorylation (Kaneko et al. 2010).

Despite the known cases of ncRNA-mediated Polycomb targeting to specific genes, it is not clear whether this is a general mechanism for specific recruiting. HOTAIR activity, for instance, is not restricted to the HOXC cluster; instead many other sites are found to bind HOTAIR as identified by a Chromatin Isolation by RNA Purification (ChIRP) method (Chu et al. 2011). On the other hand, recruitment

appears coordinated with other chromatin modifying activities, since a single ncRNA is able to bind at the same time Polycomb subunits and other chromatin regulators (Guttman et al. 2011; Tsai et al. 2010). It is likely that if short ncRNAs are going to act as Polycomb recruiters, they would function as a way to sense transcriptional state, rather than to identify specific targets.

5.5.6 *Switching Transcriptional States at Polycomb-Regulated Targets*

CGIs are genomic regions conducive to transcription initiation (Deaton and Bird 2011) and are focal points of the competition between Polycomb activity and effective transcription (Lynch et al. 2011). Histone modifications unfavorable to Polycomb residence or the recruitment of transcriptional activator complexes will switch a previously Polycomb-silenced promoter to an active state. Likewise, transcription cessation or active repression would set up a scenario for incoming Polycomb complexes to take over as silencing agents.

Polycomb function in Drosophila is antagonized by TrxG complexes (Schuettengruber et al. 2011). A TrxG subunit that provides a clue about how this may occur is the CREB-binding protein (CBP, CREBBP), a histone acetyltransferase which acetylates H3K27 (Tie et al. 2009). Its homolog in mammalian cells, CREBBP/KAT3A and the HAT E1A-binding protein p300 (Ep300) have been found to acetylate histone H3K27 (Pasini et al. 2010b). H3K27 acetylation prevents its methylation by EZH2, thus facilitating reversal of Polycomb-dependent repression. An indication of the effects caused by alterations in the relative levels of antagonic modifiers of H3K27 is the increase in H3K27ac in pluripotent cells lacking PRC2 subunit SUZ12 (Pasini et al. 2010b). Conversely, hyperactive mutant E(Z) results in reduced H3K27ac and inappropriate silencing in Drosophila embryos (Stepanik and Harte 2012). Interestingly, acetylation of histone H3K27 is a feature of active enhancers (Creyghton et al. 2010) possibly underlying Polycomb eviction associated with enhancer activation (Vernimmen et al. 2011).

Activation of Polycomb-repressed genes is often a response to developmental signals transduced through kinases (Sawarkar and Paro 2010). Some of these environmental cues are transmitted through histone phosphorylation events mediated by members of the mitogen- and stress-activated kinases (MSK), both in Drosophila and in mammalian cells. Under mitogenic stimulation, or retinoic acid-induced differentiation, MSK1 and 2 phosphorylate histone H3K27me3 at serine 28 (Gehani et al. 2010; Lau and Cheung 2011). Such a modification is accompanied by Polycomb eviction and acquisition of H3K27Ac marks. A similar activity is seen in Drosophila, where recruiting of JIL1, a MSK homolog, correlates with the establishment of H3K27acS28ph marks at promoters and enhancers (Kellner et al. 2012). Polycomb displacement resulting from H3S28

phosphorylation is effective not only in interphase, but also during prometaphase and mitosis, as seen by in vivo imaging of PC (Fonseca et al. 2012). However, the detailed mechanism of the reversion of a Polycomb-silenced state remains to be elucidated. In the likely sequence of events, early phosphorylation would promote Polycomb eviction. It is not certain that histone demethylases would play a role in this switch, at least in mammalian early development, because loss of methyltransferase and loss of demethylase correlate with phenotypes at distinct developmental times (Shpargel et al. 2012). Moreover, combined action of distinct demethylases would be required in order to fully demethylate H3K27 (Kooistra and Helin 2012) to an acetylation substrate.

For gene-specific switching to a Polycomb-repressed state, deacetylation of histone H3K27ac may be a first step. This has been documented in ES cells, where recruitment of the NuRD complex to its targets results in concurrent deacetylation and subsequent methylation of H3K27 (Reynolds et al. 2012). Alternatively, PRC2 complexes could also be recruited to transcriptionally active, H3K36me3-marked, sites, through binding of containing Polycomb-like PCL subunits via their TUDOR domains (Ballaré et al. 2012; Brien et al. 2012; Cai et al. 2013). At least in one case, H3K36me3 demethylase NO66 associated with PCL protein PHF19 (Brien et al. 2012) would initiate the transition of an active state to Polycomb-repressed state. In addition, chromatin compaction after transcription termination stimulates the HMTase activity of PRC2 (Yuan et al. 2012) and therefore assists in the establishment of a repressed state.

5.5.7 Maintenance of Histone Marks on Polycomb-Modified Nucleosomes

Specific gene expression and chromatin states are perpetuated throughout cell divisions, thereby ensuring the stability of differentiation stages. During DNA replication, the incorporation in nucleosomes of newly synthesized histones necessitates the deployment of mechanisms that propagate histone marks patterns to daughter cells (Zhu and Reinberg 2011). Maintenance processes are also demanded by nucleosome turnover that occurs at transcribed genes and active DNA regulatory elements during interphase (Henikoff 2008). Preserving histone modifications in relation with replication-independent turnover of nucleosomes could occur at least in two ways: deposition of pre-marked histones and residence of histone modifiers at the turnover site. Here, adjacent histone H3K27me3 could serve as an anchor (and catalytic activator) of Polycomb HMTase (Margueron et al. 2009; Yuan et al. 2012). Additional factors, in analogy with the ATRX helicase linking DAXX histone chaperon-dependent assembly of histone H3.3 nucleosomes (Eustermann et al. 2011), could also be involved.

In proliferating cells, H3–H4 tetramers do not dissociate during genome replication. Thus, daughter DNA strands contain both newly synthesized histones and

those from parental origin (Xu et al. 2010). As parental histones contain specific modifications which are bound by complexes containing specific modification-recognition modules (i.e., EED for H3K27me3), the catalytic module (EZH1, EZH2) of such complexes would reinstate these modifications in the nucleosome. Alternatively, the association of a histone modifier with the replication machinery could ensure the modification of reformed nucleosomes. The interaction of chromatin modifiers with elements of the replicating machinery such as PCNA (Rowbotham et al. 2011) or the CAF1 chaperone (Loyola et al. 2009) has indeed been demonstrated. Similarly, EZH2 has been shown to co-localize with BrdU-labeled foci (Hansen et al. 2008), and, in Drosophila embryos, PRC2 and PRC1 subunits are in close proximity to replisome components (Petruk et al. 2012). Also, in assays in vitro, PRC1's subunits PSC, PC, and SCE are found stably associated with replicating DNA (Follmer et al. 2012; Francis et al. 2009). However, no tri-methylated H3K27 or H3K4 are found on nucleosomes repositioned some time after passage of the replication fork in Drosophila embryos (Petruk et al. 2012). This observation is consistent with those of studies in mammalian cells showing that H3K27 tri-methylation starts at S-phase and is completed only after mitosis, during G1 phase (Zee et al. 2012). Also in mammalian cells, approximately half of H3K27me3 on newly synthesized histone H3 is produced from unmodified K27 in S and G2 phases, whereas the remaining modification takes place in G1 from histones in di-methylated form (Zee et al. 2012). The stepwise nature of Polycomb HMTase action suggests that the maintenance of transcriptional states may be compatible with fluctuations at histone marks (Huang et al. 2012). PRC1-dependent modification of histone H2A also takes place during the G1 phase, after USP16-driven global deubiquitination wave in G2 and mitosis (Joo et al. 2007).

5.6 Mechanisms of Polycomb-Dependent Repression

How Polycomb impacts transcriptional activity is still an unresolved issue. Linking Polycomb abilities, i.e., catalytic activities and protein–protein interactions with gene control mechanisms has proven to be difficult. For some time, it was accepted that Polycomb repression was related to "chromatin compaction," analogous to the largely absent gene expression within "closed" heterochromatic regions. However, this turned out to be not true. Rather than being simple ON/OFF switches, Polycomb act in a dynamic fashion just as is being realized for other chromatin modifiers (Reynolds et al. 2013). In cells with a developmental potential, Polycomb complexes act on genes still capable of changing their expression state by fine-tuning their transcription status by a variety of mechanisms, while a less dynamic scenario may be at play on the large inactive Polycomb domains of differentiated cells. A summary of Polycomb complexes, biochemical activities of their subunits, and major functions is shown in Fig. 5.2.

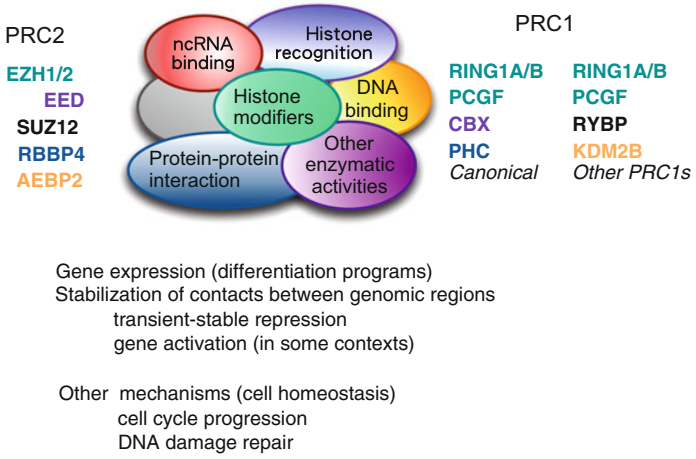

Fig. 5.2 Summary of Polycomb complexes and their activities. A hypothetical, unifying, Polycomb-repressive complex is shown, indicating a possible core of subunits and their biochemical activities. In association with targets (by histone recognition, DNA contacts, ncRNAs), reinforcing and maintenance (histone modifications) of their clustering (protein–protein interactions), and ability to become dissociated, Polycomb complexes regulate gene expression. Core subunits of PRC2 and PRC1 complexes are shown, together with their major associated activity. Generation of cell diversity and maintenance of cell homeostasis functions are categorized under transcriptional and non-transcriptional mechanisms

5.6.1 Polycomb Function and RNA Polymerase II Activity

A first hint about Polycomb action at transcription initiation was drawn from transgenic studies in Drosophila. Here, PRE repression of a heat shock promoter (known to bind paused RNA pol II prior to induction) was found to occur even in the presence of recruited RNA pol II and TFIID (general transcription factor essential for initiation). The repressed transgene was unable to produce mRNA (Dellino et al. 2004). Additional evidence, in Drosophila, pointing at a possible link between Polycomb function and RNA pol II pausing is PRC1 enrichment at stalled, proximal promoters that produce short sense transcripts in Drosophila cells (Enderle et al. 2011; Kharchenko et al. 2010; Muse et al. 2007; Nechaev et al. 2010; Zeitlinger et al. 2007). By studying muscle tissue during Drosophila embryogenesis, it was found that paused RNA pol II associates with muscle-specific promoters in a stage-specific, but not tissue-specific manner and that the repressed state correlated with tissue-specific Polycomb targeting (Gaertner et al. 2012). In this case, it would appear that polymerase release from pausing was restricted by Polycomb, although by unknown mechanisms. In *extra sex combs* embryos (mutation in the gene that encodes PRC2 subunit ESC), RNA pol II occupancy increases at many promoters, including those not bound by paused polymerase in wild type embryos (Chopra et al. 2011).

Loss of histone H2AUb1 in pluripotent ES cells after inactivation of RING1 proteins correlates with an increase in total RNA pol II bound at Polycomb-repressed promoters (Endoh et al. 2012; Stock et al. 2007). In a different experimental model, elongation inhibition was associated with the H2A ubiquitylating activity of hRUL138/DZIP3 (Zhou et al. 2008). Transcriptionally engaged RNA pol II in mammalian cells, as identified by genome-wide sequencing of run-on transcripts, peaks only at the TSS of PRC2-occupied promoters, whereas at PRC2 and PRC1 (bivalent) promoters the levels are very low (Min et al. 2011). Detailed studies of RNA pol II associated with Polycomb-repressed genes in ES cells finds a variant phosphorylated at S5 but not at S2 or S7. This RNA pol II species accumulates at TSSs, but it is also found throughout the entire transcriptional unit up to the transcription end site (Brookes et al. 2012). Loci with this unusual chromatin configuration lack H3K36me3 marks (a sign of active transcription elongation) and produce no mature mRNA. Unfortunately, molecular characterization of these promoters has not clarified yet how Polycomb would act through transcriptional pausing.

Unexpectedly, recent studies in Drosphila, however, support a role for PRC1 complexes assisting the pausing factors NELS and DSIF in polymerase modification at promoters for effective transcription (Schaaf et al. 2013b). In these studies, it was found that, in addition to the expected location on silent, H3K27me3-marked loci, PRC1 was found also on active, H3K27me3-free, genes which were also bound by cohesin (Schaaf et al. 2013b). Cohesins are known PRC1 interactors (Strübbe et al. 2011) and are required for PRC1 recruitment to active Drosophila promoters (Schaaf et al. 2013b). In addition, while cohesins associate with genes with promoter-proximal transcriptional pausing they do not, with a few exceptions, bind Polycomb-repressed loci (Schaaf et al. 2013a). For Polycomb-silenced genes, PRC1 down-regulation resulted in increases of the elongating form or RNA pol II (S2P) RNA pol II and of mRNA, in agreement with the release of a gene repression function. In contrast, active genes showed, upon PRC1 inactivation, decreased levels of total and S2 RNA pol II at gene bodies, with a concomitant reduction of mRNA levels, suggesting that PRC1 and pausing factors work together for effective transcription (Schaaf et al. 2013b).

5.6.2 Nucleosome Compaction by Polycomb

Reconstituted Polycomb complexes condense nucleosomal arrays in vitro, as determined by electronic microscopy (Francis et al. 2004). Thus, similar to HP1, high mobility proteins and others, Polycomb subunits could be categorized as chromatin architectural proteins (Luger et al. 2012; McBryant et al. 2006).

Evidence for chromatin compaction has been gathered for subunits of PRC1 complexes and also for the PRC2 subunit EZH1. In a first observation, a Drosophila PRC1 complex was shown to compact chromatin as assessed by a decrease in the

average internucleosomal distances in preassembled arrays. This activity locates to the C-terminal region of PSC and is independent of DNA sequence and histone tails (Francis et al. 2004). However, the Drosophila C-terminal PSC region is not conserved in plants or metazoans. Nevertheless, a reconstituted mouse PRC1 complex, in a CBX2/M33-dependent manner, was shown to act similarly to PSC (Grau et al. 2011). Structural studies, however, determined that a conformationally disordered, highly charged region identified in chromo domain-containing and RING-finger-containing PRC1 subunits is sufficient for nucleosomal compaction (Beh et al. 2012; Grau et al. 2011). In vivo, PRC1 repression through DNA compaction has been shown for clustered Hox genes in ES cells. Here, fluorescent in situ hybridization shows that following RING1B/RNF2 depletion, Hox genes at the end of the cluster are activated and move away from the compact structure formed by the rest of silent genes (Eskeland et al. 2010). L3MBTL2, a MBT-domain PRC1 subunit, is also able to compact nucleosomal arrays in vitro. In contrast to its requirement for methylated H3 or H4 histone N-tails, chromatin compaction activity, just as that of PSC or CBX2/M33, does not require histone tails (Trojer et al. 2011).

Reconstituted PRC2 complexes containing EZH1, but not those containing its paralog EZH2, are highly active compacting chromatin in vitro but only as part of the complex (Margueron et al. 2008). Another difference with PRC1 compaction is that histone tails are needed. A single PRC2-EZH1 aggregate brings together three/four nucleosomes. In tissue culture cells, chromatin accessibility (measured as sensitivity to DNAse) at reporter constructs and endogenous genes decreased when bound by EZH1, in line with in vitro activity. Interestingly, transcriptional repression through PRC2-EZH1-mediated chromatin compaction maybe uncoupled from H3K27me3 (Margueron et al. 2008).

An in vitro effect of PRC1-dependent nucleosome compaction is the inhibition of ATP-dependent chromatin remodelers (Shao et al. 1999). Some in vivo evidence for this activity can be inferred from gain or loss of Polycomb occupancy at targets, depending on downregulation or ectopic expression of SNF5/SMARCB1, a core component of subunit of chromatin remodeler SWI/SNF (Kia et al. 2008; Wilson et al. 2010). However, in a different model (ES cells), no relationship could be found between SNF5 and Polycomb repression (You et al. 2013). Thus, the overall relevance of chromatin compaction in Polycomb function remains largely unknown. And yet, correlative evidence would suggest that the large increase in H3K27me3-marked nucleosomes observed in differentiated but not in pluripotent cells is due to diminished chromatin remodeling activity compared to that of cells with high developmental potential (Hawkins et al. 2010; Meshorer et al. 2006; Zhu et al. 2013).

5.6.3 *In Polycomb Bodies, Away from Transcription Factories*

Polycomb complexes form large macromolecular assemblies within the cell, so-called Polycomb bodies. In apparent contradiction with its chromatin compaction function, Polycomb bodies appear to localize to perichromatin, the interface between interchromatin regions and condensed chromatin (Cheutin and Cavalli 2012; Cmarko et al. 2002). Polycomb bodies in Drosophila include silent Polycomb targets, in particular large genomic regions enriched in H3K27me3-marked nucleosomes and characterized by high occupancy of Polycomb subunits (Cheutin and Cavalli 2012). In the microscope, these regions are seen as very large speckles. However, smaller Polycomb domains do not form stable bodies. The data are consistent with contacts between Polycomb-bound sites (Bantignies et al. 2011; Sexton et al. 2012) and suggest that these bodies form at sites of high Polycomb density rather than as coalescent points where genes locate for repression (Cheutin and Cavalli 2012). PRE-containing transgenes co-localize to Polycomb bodies when repressed (Bantignies et al. 2003; Grimaud et al. 2006) However, detailed studies with transgenes indicate that such co-localization depends on insulator elements rather than on PREs and Polycomb complexes (Li et al. 2011). Transgenes containing enhancers localize to different nuclear domains called transcription factories and this association is also dependent on insulator function (Li et al. 2013). Thus, for effective repression, Polycomb proteins seem to, in a reversible manner, stabilize gene location at transcriptionally silent sites.

Polycomb-related gene repositioning phenomena can also involve ncRNAs as exemplified by transcriptional units in human tissue culture cells controlled by the cell cycle regulator E2F1. Under proliferating conditions, these genes are transcribed and localize to interchromatin granules at nuclear bodies identified by the presence of splicing factors (Mao et al. 2011), whereas in quiescence, they are silent and localize to Polycomb bodies. A PRC1 chromobox protein, CBX4/PC2, co-localizes to these promoters through E2F1 association. Importantly, however, the residence of loci in transcriptionally inactive (Polycomb bodies) or active (interchromatin granules) environments depends on CBX4/PC2 associating with distinct ncRNAs, TUG1 and NEAT2, respectively (Yang et al. 2011). Selective affinity for one or the other is determined by posttranslational modification of CBX4/PC2, in this case methylation by the well-known HMTase SUV39H1. In the presence of mitogens, cell cycle kinases inactivate SUV39H1; CBX4/PC2 is demethylated by histone demethylase JARID1A/KDM4c; demethylated CBX4/PC2 loses affinity for TUG1 ncRNA and gains affinity for NEAT2 ncRNA at interchromatin granules. Relocation to a transcriptionally conducive environment is accompanied by recruitment of CDCA7L, a RING-class E3 ubiquitin ligase that mono-ubiquitylates H2B through binding to SUMOylated E2F1 (by CBX4/PC2) (Yang et al. 2011). This example demonstrates that we have barely scratched the surface of the complexities of how Polycomb is involved in regulating the balance between active and inactive gene expression states.

5.6.4 *Sometimes, Polycomb Subunits Participate in Gene Activation*

Although the best know functions of Polycomb are those concerning PRCs, activities as individual subunits are also reported, for instance in gene activation events. In prostate cancer cells, EZH2 HMTase activity is needed for gene expression (Xu et al. 2012). As no H3K27me3 is involved, it is suggested that other regulators, probably the androgen receptor in this case, may be a substrate for EZH2 catalytic activity. Likewise, EZH1 inactivation in a tissue culture model of skeletal differentiation results in defective RNA pol II occupancy and activation of myogenic genes. In this case, EZH1 interacts with RNA pol II and acts as a positive regulator of transcriptional elongation (Mousavi et al. 2012). Finally, Cbx8, in a complex with HAT TIP60/KAT5 and MLL-AF9, is necessary for transcriptional activation associated with a MLL-AF9-triggered leukemogenic program (Tan et al. 2011).

5.7 Non-transcriptional Functions of Polycomb

Besides its role in transcriptional regulation, Polycomb directly influences also other important cellular functions such as DNA damage repair (Gieni et al. 2011; Vissers et al. 2012) and cell cycle progression. The latter does not include repression of proliferation inhibitors such as well-known Polycomb targets Cdk2nb/p15, Cdkn2a/p16, that encode cyclin-dependent kinase inhibitors that halt the cell cycle by impeding entrance in S-phase.

Roles for Polycomb in DNA damage have been inferred from the higher sensitivity of mutant cells to agents that induce DNA breaks (Chagraoui et al. 2011; Ginjala et al. 2011; Ismail et al. 2010; Pan et al. 2011; Wu et al. 2011). PRC1 and PRC2 subunits are rapidly recruited to sites of induced DNA damage after laser or ultraviolet irradiation (Chou et al. 2010; Hong et al. 2008). How this occurs exactly is obscured by contradictory evidence: for instance, BMI1/PCGF4 recruitment was found to be dependent and independent of poly(ADP-ribosyl) polymerase (PARP) activity (Chagraoui et al. 2011; Ginjala et al. 2011). Distinct contributions by several mechanisms acting, in a context-dependent manner, may be at the basis of these discrepancies. Co-localization of BMI1/PCGF4 with DNA-damage foci occurs before full H2AX phosphorylation (γH2AX), which is an early event occurring at sites of DNA damage that acts as a docking element for recruitment of the repair machinery (Papamichos-Chronakis and Peterson 2012; Soria et al. 2012). In fact, BMI1/PCGF4 and RING1B/RNF2 have been found to mono-ubiquitylate γH2AX as a step prior to the assembly of DNA repair proteins (Ginjala et al. 2011; Pan et al. 2011; Wu et al. 2011). Perhaps as a consequence of impaired DNA repair by homologous recombination, BMI1/PCGF4-deficient cells accumulate at G2/M (Ginjala et al. 2011). In the case of nucleotide excision repair, histone H2A ubiquitylation occurring upon ultraviolet

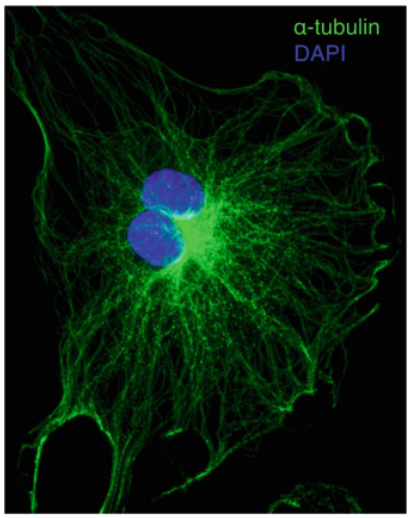

Fig. 5.3 Non-transcriptional functions of Polycomb proteins. Mitotic defects in cells lacking RING1A and RING1B. Example of binucleated cell, appearing in a culture of primary fibroblasts after RING1 protein inactivation, probably a consequence of failed cytokinesis

irradiation is also RING1B/RNF2 dependent (Bergink et al. 2006). However, the precise mechanism by which Polycomb complexes influence DNA repair still remains to be elucidated.

In addition to the contribution to DNA damage repair, Polycomb influences cell cycle progression via posttranslational modifications of cell proliferation regulators. For instance, loss of Drosophila PRC1 subunit PSC results in cells that accumulate at the G2/M phase. In contrast, inactivation of other PRC1 products, such as PC or SCE has no effect (Mohd-Sarip et al. 2012). PSC is found in complexes other than PRC1 and is associated with cell cycle regulators such as CDK1/CDC2, cyclin B (CCNB) and subunits of the Anaphase Promoting Complex (APC). CDK1-CCNB phosphorylates a collection of proteins involved in the transition form interphase to mitosis, including nuclear membrane breakdown and mitotic spindle assembly. Mitotic segregation defects seen in PSC-deficient cells correlate with decreased levels of poly-ubiquitylated CCNB, which appear to depend on PSC (Mohd-Sarip et al. 2012). The observation is surprising, considering that APC activity is directed to destroy CCNB towards the end of mitosis. It is possible that PSC modification of CCNB may therefore not be related to its proteasomal degradation. Mutations in other Drosophila PRC1 subunits showed no proliferative defects but inactivation of RING1 paralogs in mammalian fibroblasts results in mitotic aberrations as indicated by the presence of micronuclei and binucleate cells (Fig. 5.3). Another proliferative defect associated with Polycomb-dependent posttranslational modifications is the accumulation of geminin, a negative regulator of replication through inhibition of licensing factor CDT1. It is thought that defective poly-ubiquitylation in cells deficient in PRC1 subunit PHC1 results in unscheduled geminin stabilization and quiescence (Ohtsubo et al. 2008).

5.8 Concluding Remarks

The Polycomb field has exploded in the last few years and while we still tend to talk of two "types" of complexes (PRC1 and PRC2) the real situation is far more complicated. While their main functions are as transcriptional repressors, this article shows that Polycomb proteins are part of a dynamic and extensive protein network that performs diverse tasks in a number of different contexts and is also regulated by external signals. The different subunits of Polycomb complexes can be modified, exchanged, and associated with diverse types of other proteins and bind even to noncoding RNA and all of this in a cell type- and cell stage-specific fashion. System-wide studies are now urgently needed to link the epigenetic function of Polycomb complexes with the proteome. At the mechanistic level, as for other chromatin modifiers, there are still many gaps in our understanding of the molecular mechanisms by which Polycomb represses transcription. However, without such mechanistic insights we will not be able to counteract situations where Polycomb function is aberrant, as outlined in Chap. 6 about the role of Polycomb in leukemia. Much recent work has examined the location of genomic sites bound by Polycomb products and the associated histone marks. Future efforts should now attempt to put these linear maps of chromatin states into three-dimensional regulatory spaces and investigate the impact of Polycomb-dependent changes in nuclear architecture on transcription regulation. Single-cell approaches need to be established that provide access to details that are lost in cell population analyses and inform of the dynamic, rather than static, nature of the system. A great interest exists in translating new knowledge on Polycomb function into therapeutic/diagnostic possibilities, be in harnessing the power of these complexes in regulating self-renewal of stem cells for regenerative medicine or in taming/suppressing transformed cells. At any rate, we still have a long way to go until we understand the workings of such an evolutionary successful system in the generation of cell diversity and tissue homeostasis. There is still much scope for exciting and satisfying research.

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