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Inner workings and regulatory inputs that control Polycomb repressive complex 2

M. Maggie O'Meara and **Jeffrey A. Simon***
Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota 55455, USA

Abstract

Polycomb repressive complex 2 (PRC2) is a conserved multisubunit enzyme that methylates histone H3 on lysine-27. This chromatin modification is a hallmark of target genes transcriptionally silenced by the Polycomb system. At its core, PRC2 activity depends upon the SET domain active site of its catalytic subunit, EZH2, as well as critical stimulatory inputs from noncatalytic subunits, especially EED and SU(Z)12. We review recent progress on this core PRC2 machinery, including key features of the active site, control mechanisms that operate via EZH2 phosphorylation, and subunit elements and architectures that influence PRC2 function. Among these, we highlight work identifying an EED regulatory site that enables PRC2 to bind pre-existing methylated H3-K27 and stimulate enzyme output. These advances illuminate basic inner workings of PRC2 and also provide insights that could aid design of PRC2 inhibitors. The chromatin landscape that PRC2 encounters *in vivo* is decorated with many histone modifications that accompany active transcription, such as H3-K4 methylation. It has long been assumed that these "active" modifications oppose PRC2 at some level but, until recently, mechanisms of this antagonistic cross-talk have been elusive. We discuss new findings that illuminate how H3-K4 and H3-K36 methylation, H3-K27 acetylation, and H3-S28 phosphorylation each exert a negative impact on PRC2 function. The emerging picture presents PRC2 as a cooperative multipart machine, intricately outfitted to sense and respond to the local chromatin environment and other cues. This PRC2 design ensures flexibility and finetuning of its fundamental gene silencing roles in diverse biological contexts.

Keywords

chromatin; Polycomb; histone methyltransferase gene silencing; epigenetics

I. Introduction

Polycomb repressive complex 2 (PRC2) is an essential chromatin modifier conserved from plants to flies to humans (Pien and Grossniklaus 2007; Schuettengruber et al. 2007; Schwartz and Pirrotta 2007; Simon and Kingston 2009; Sawarkar and Paro 2010; Margueron and Reinberg 2011). Its central and best-studied function is to methylate histone H3 on lysine-27 (K27; Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002), with the tri-methylated reaction product, H3-K27me3, constituting a common feature of repressed chromatin (Cao and Zhang 2004b; Schwartz et al. 2006; Schuettengruber et al. 2009; Filion et al. 2010). Located at over 2,000 sites in the mouse genome (Ku et al. 2008), PRC2 and H3-K27me3 have widespread roles in developmental

*Corresponding author. Mailing address: 321 Church St. SE, Minneapolis, MN 55455, Phone: (612)-626-5097, Fax: (612)-626-6140, simon004@umn.edu.

processes of multicellular organisms and they are implicated in fundamental chromatin mechanisms that underlie stem cell regulatory circuits and cancer progression.

H3-K27 methylation is achieved through the highly cooperative action of four core PRC2 subunits (Fig. 1a). The catalytic subunit, E(Z) in *Drosophila* or EZH2 in humans, bears a SET domain which houses the enzyme active site (Rea et al. 2000; Dillon et al. 2005; Joshi et al. 2008). However, E(Z)/EZH2 is essentially inactive on its own and requires critical inputs from its partner subunits ESC/EED, SU(Z)12, and NURF55/RpAp48 (Cao and Zhang 2004a; Pasini et al. 2004; Ketel et al. 2005; Nekrasov et al. 2005). A major goal here is to review the inner workings and core organization of PRC2, including advances on understanding the PRC2 active site and mechanisms that control PRC2 activity through its catalytic or noncatalytic subunits. Comprehensive knowledge about PRC2 inner workings is essential to inform efforts to design small molecules that can specifically inhibit or modulate this key chromatin-modifying enzyme in cultured cells, animal models and, potentially, patients.

The Polycomb repressed state, enriched for H3-K27me3, is functionally opposed by actively transcribed chromatin, which preferentially features "activating" modifications such as H3-K4me3, H3-K36me3, and acetylation of several histone tail lysines. Indeed, impairment of H3-K4 or H3-K36 histone-methylating enzymes leads to excess H3-K27 methylation *in vivo* (Papp and Muller 2006; Srinivasan et al. 2008). Widespread distribution of these alternative states is reinforced by recent genome-wide studies that define distinct Polycomb-silenced and active chromatin domains (Filion et al. 2010; Schwartz et al. 2010; Kharchenko et al. 2011). Correspondingly, there is molecular crosstalk whereby PRC2 senses activating modifications in the local chromatin environment via direct biochemical contact, and is consequently down-regulated. We will emphasize recent studies that illuminate how several antagonistic histone modifications can dampen or inhibit PRC2 function.

In this review, we focus on advances in understanding basic mechanisms and inputs to PRC2 function. This includes the core PRC2 machinery that deposits H3-K27me3, inner mechanisms that coordinate these working parts, and extrinsic signals and chromatin features that modulate PRC2 by impinging on core components. Besides the four central subunits, PRC2 is also impacted by additional partner proteins, which can be considered regulatory subunits that associate with the core complex. Chief among these are fly PCL and its mammalian homologs (Nekrasov et al. 2007; Cao et al. 2008; Sarma et al. 2008; Savla et al. 2008; Walker et al. 2010) and JARID2 (Peng et al. 2009; Shen et al. 2009; Landeira et al. 2010; Li et al. 2010; Pasini et al. 2010a), which help target PRC2 to chromatin sites of action as well as influence PRC2 enzyme activity. Another key class of PRC2 binding partners are non-coding RNAs (ncRNAs), such as HOTAIR and XIST, which are also implicated in PRC2 targeting (Rinn et al. 2007; Pandey et al. 2008; Zhao et al. 2008; Zhao et al. 2010). Roles of these protein and ncRNA cohorts in PRC2 chromatin recruitment have been recently reviewed (Hekimoglu and Ringrose 2009; Simon and Kingston 2009; Bracken and Helin 2009; Margueron and Reinberg 2011), so this topic will be only tangentially covered here. For recent progress on PRC2 roles in more biological and clinical contexts, readers are referred to alternative review articles that cover developmental and stem cell functions of PRC2 (Sparmann and van Lohuizen 2006; Rajasekhar and Begemann 2007; Pietersen and van Lohuizen 2008; Ito and Sun 2009) and the role of PRC2 in cancer epigenetics (Simon and Lange 2008; Bracken and Helin 2009; Chase and Cross 2011).

II: PRC2 Core Complex

Among components of the four-subunit PRC2 core complex (Fig. 1a), the SET domain of E(Z)/EZH2 is essential for enzyme function (Cao et al. 2002; Muller et al. 2002; Joshi et al.

2008) and two of the noncatalytic subunits, ESC/EED and SU(Z)12, are also vital for activity (Cao and Zhang 2004a; Pasini et al. 2004; Ketel et al. 2005; Nekrasov et al. 2005). The mechanisms by which these two critical subunits boost catalysis are not fully understood (see below). The fourth stoichiometric subunit, NURF55/RbAp48, also contributes; however, PRC2 subcomplexes lacking this subunit maintain substantial enzyme function (Cao and Zhang 2004a; Ketel et al. 2005), so it appears less central to the core methyltransferase. Another PRC2 partner, PCL/PHF1, has been implicated in specifically boosting the efficiency of the H3-K27me2 to H3-K27me3 conversion (Nekrasov et al. 2007; Sarma et al. 2008). Since recombinant PRC2 can generate H3-K27me3 without PCL/PHF1 (Nekrasov et al. 2007; Joshi et al. 2008; Sneeringer et al. 2010), this appears to be a modulatory rather than essential input to the core enzyme.

The critical inputs of multiple PRC2 subunits, together with their striking conservation from plants to humans (Whitcomb et al. 2007; Ito and Sun 2009; Sawarkar and Paro 2010), indicate that PRC2 is an ancient chromatin-modifying machine whose subunit architecture has preserved a functionally robust output. The precise nature of the subunit contacts and mechanisms that potentiate PRC2 activity have not yet been revealed. This stems partly from paucity of direct structural information on the PRC2 active site and the key functional interfaces of E(Z)/EZH2 with its partners. Nevertheless, recent progress has illuminated several aspects of the core PRC2 machine, including active site requirements, modulation by subunit phosphorylation, and the key stimulatory influence of the EED subunit. We review knowledge and advances on PRC2 inner workings by considering each core subunit.

E(Z)/EZH2

E(Z)/EZH2 contains a C-terminal SET domain plus several additional domains implicated in PRC2 function (Fig. 1b). In the context of assembled PRC2, this SET domain can perform three successive methyl transfer reactions, ultimating producing H3-K27me3. This contrasts with other types of HMTases whose capacity for methyl transfer is more limited; for example SET7/9 can only monomethylate naive histone lysine substrates (Xiao et al. 2003) and G9a, a dimethylase, lacks the ability to trimethylate the histone tail (Wu et al. 2010). Interestingly, a single amino acid change in its active site can convert G9a into a trimethyltransferase, which lacks mono- and di-methylation activity (Wu et al. 2010). This differential methylation capacity is governed by the precise configuration of the lysine substrate binding pocket, with active site tyrosine residues frequently implicated as key determinants (Collins et al. 2005; Couture et al. 2008). Correspondingly, recent progress pinpoints an active site tyrosine in determining the methylation capacity of EZH2.

Role of active site residue Y641 in differential control of mono/di/tri-methylation

Although EZH2 over-abundance has long been associated with tumor tissue samples (Varambally et al. 2002; Kleer et al. 2003; Simon and Lange 2008), somatic missense mutations that alter EZH2 in cancer cells have only recently been described. Analysis of certain lymphoma subtypes reveals recurrent mutations at a single EZH2 residue, Y641, within the SET domain (Morin et al. 2010). Based on analogy to structurally solved SET domains (Dillon et al. 2005), this active site tyrosine contacts the -amino group of the substrate lysine. Strikingly, these Y641 alterations shift the methylation capacity of EZH2; specifically, H3-K27 mono- and di-methylation is reduced but there is a concomitant surge in tri-methyltransferase activity (Sneeringer et al. 2010; Yap et al. 2011). Thus, the Y641 mutations appear to act via a gain-of-function mechanism, resulting in excess H3-K27me3 in tumor tissues. How does Y641 mutant EZH2 produce excess H3-K27me3 if it cannot efficiently generate the precursors H3-K27me1 and H3-K27me2? The answer lies in genetic coupling between wild-type and Y641 mutant EZH2, with wild-type supplying the precursor moieties and the mutant boosting the final step to H3-K27me3 (Fig. 2a). Indeed, the Y641

alleles are invariably recovered in lymphoma tissues in heterozygous form along with a wild-type allele. This fascinating disease mechanism is wholly consistent with genetic observations on *Drosophila* E(Z) from 20 years ago (Jones and Gelbart 1990). The mutant allele that first identified E(Z) (Wu et al. 1989) is a missense mutation that alters precisely the same active site tyrosine as Y641 (Y655; Joshi et al. 2008). Significantly, the gain-of-function behavior of this fly Y655N *[E(z)^{1}]* allele requires wild-type *E(z)* activity *in trans* (Jones and Gelbart 1990), just as the oncogenic EZH2 Y641 mutations are functionally coupled with a wild-type EZH2 allele. This contrasts with another gain-of-function E(Z) active site mutation, R741K (*E(z)^{Trm}*; Bajusz et al. 2001), which produces excess H3-K27me3 without coupling to wild-type E(z) (Stepanik and Harte 2012).

These Y641 discoveries contribute to a unifying emerging view of the role of EZH2 and allied H3-K27 modifiers in cancer (Sneeringer et al. 2010; see Fig. 2). To date, cancer progression has been associated with the following related gain-of-function scenarios: 1) increases in EZH2 catalytic efficiency (Sneeringer et al. 2010; Yap et al. 2011), 2) EZH2 over-expression (Varambally et al. 2002; Kleer et al. 2003; Simon and Lange 2008), 3) overabundance of PCL family cofactors, such as PHF19, which promote H3-K27me3 accumulation (Wang et al. 2004), and 4) loss of the opposing H3-K27me3 demethylase, UTX (van Haaften et al. 2009). Thus, although diverse mechanisms are deployed, they all lead to the same outcome: abnormally elevated levels of H3-K27me3 in cancer cells.

Phosphorylation of EZH2 by cyclin-dependent kinases

An important emerging area concerns the mechanisms that modulate PRC2 activity, either positively or negatively, in response to intrinsic and extrinsic cellular cues (Sawarkar and Paro 2010). Among these cues, PRC2 activity has long been linked to cell cycle status through a role in promoting cell proliferation (Varambally et al. 2002; Bracken et al. 2003). Inputs to PRC2 activity by the cell cycle machinery have recently been described that operate via direct phosphorylation of EZH2 at multiple threonine residues (Chen et al. 2010; Kaneko et al. 2010; Wei et al. 2011; Wu and Zhang 2011). Specifically, cyclin-dependent kinases (CDKs) phosphorylate human EZH2 at T350 (T345 in mouse) and at T492 (T487 in mouse; Fig. 1b). The consequence of T350 phosphorylation is stimulation of H3-K27me3 deposition and hypersilencing at chromatin targets such as Hox loci (Chen et al. 2010). This occurs through increased PRC2 binding at target loci rather than by boosting intrinsic PRC2 enzyme activity (Chen et al. 2010; Kaneko et al. 2010). Consistent with this, T350 phosphorylation enhances interaction of EZH2 with ncRNAs, such as HOTAIR, implicated in PRC2 recruitment (Kaneko et al. 2010). Thus, CDK-mediated T350 phosphorylation positively impacts PRC2 function by augmenting its association with target chromatin. The relatively modest level of T350phos (only ~1% of EZH2 has T350phos in unphased cells) might suffice for ncRNAs to implement initial PRC2 targeting and H3-K27 methylation, which could prime subsequent binding of unmodified EZH2-PRC2 complexes that recognize and further propagate the repressive mark (Margueron et al. 2009; Kaneko et al. 2010; see below).

In contrast, EZH2 phosphorylation at T492 appears to negatively impact PRC2 function in cells (Wei et al. 2011). At the mechanistic level, T492 phosphorylation is reported to diminish PRC2 HMTase by disrupting EZH2 association with its required partner subunits, EED and SU(Z)12 (Wei et al. 2011). However, another group finds that a phospho-mimic change at the corresponding mouse residue (T487D) has no adverse effect on PRC2 assembly or HMTase (Kaneko et al. 2010). Another inconsistency is that CDK inhibition or knockdown was found to either increase (Chen et al. 2010) or diminish (Wei et al. 2011) Hox gene expression. Further work will be needed to reconcile these findings. It may be that the reported positive and negative influences due to phosphorylation on these two EZH2 residues operate in the same cell types but change dynamically, with one or the other

predominating, during cell cycle progression. Alternatively, the discrepancies could reflect distinct regulatory mechanisms that control PRC2 in different cell types under study.

A new wrinkle in the EZH2 phosphorylation story is the observation that CDK phosphorylation of mouse EZH2 at T345 and T487 fosters ubiquitylation and proteosomal degradation of EZH2 (Wu and Zhang 2011). How can these seemingly disparate consequences (increased PRC2 chromatin binding versus PRC2 elimination) be reconciled? One possibility is that these outcomes reflect a multistep mechanism that limits the duration of the response to transiently elevated CDKs. Initially, T350 phosphorylation could trigger a transient increase in chromatin binding, thereby boosting local H3-K27me3, but then a rapid turndown is necessitated as CDK levels drop and the next cell cycle transition ensues. This scenario resembles "hit-and-run" control of transcription factors whereby activation is quickly followed by ubiquitylation and factor removal to finetune the response to transient cellular signals (Muratani and Tansey 2003).

Phosphorylation of EZH2 by mitogen-activated protein kinase (p38)

In addition to intrinsic cell cycle cues, examples of extrinsic signaling pathways that impact PRC2 are also emerging. The original example is the Akt pathway, which triggers EZH2 phosphorylation at serine-21, leading to reduced histone methylation by PRC2 (Cha et al. 2005). Much remains to be determined about how serine-21 phosphorylation is deployed in a physiological context since diverse cellular inputs can activate Akt kinases. More recently, another EZH2 phosphorylation site, T372, has been identified as a target of the mitogen-activated protein kinase, p38 (Palacios et al. 2010). Here the biological context is well-defined; the p38 pathway is activated in muscle stem cells in response to inflammatory cytokines that signal tissue damage. This leads to muscle regeneration by shifting gene expression programs to stimulate differentiation and dampen proliferation. At the mechanistic level, EZH2-T372 phosphorylation promotes interaction with the YY1 targeting protein, which recruits PRC2 to repressed targets, such as Pax7, in muscle cells (Caretti et al. 2004; Palacios et al. 2010). This boost in PRC2 recruitment and gene silencing resembles the outcome of T350 phosphorylation (see above), except in this case a protein rather than ncRNA recruiter is utilized.

The burst of new discoveries on EZH2 control by phosphorylation at multiple sites (summarized in Fig. 1b) has done much to connect this key chromatin modifier to upstream regulatory networks. Interestingly, mammalian EZH1, which is 65% identical to EZH2, lacks the conserved CDK phosphorylation sites found in EZH2 (Zeng et al. 2011). This differential capacity to respond to CDKs makes sense since EZH2 predominates in proliferating cells whereas EZH1 is more abundant in non-dividing cells (Margueron et al. 2008; Shen et al. 2008). It seems likely that further efforts will reveal additional mechanisms that link PRC2 epigenetic outputs to intrinsic and extrinsic cellular stimuli.

Inputs to EZH2 from core partner subunits

Among characterized SET domain proteins, there is a continuum ranging from those that can function as lysine methyltransferases on their own to those that require assembly into multiprotein complexes. At one extreme, the viral SET (vSET) protein is an active HMTase that consists essentially of an isolated SET domain (Qian et al. 2006). PRC2 lies at the other extreme, with its three noncatalytic core subunits required to stimulate activity, particularly the two (EED and SUZ12) that directly contact EZH2 (Fig. 1a). How these subunits potentiate EZH2 HMTase remains largely an open question. An instructive example is provided by another SET domain methyltransferase MLL1 which, like EZH2, depends heavily on partner subunits for optimal activity (Dou et al. 2006). Efficient MLL1 methylation of its H3-K4 target requires, at minimum, its partners RbBP5 and ASH2. A

recently solved structure shows that, compared to stand-alone HMTases, the MLL1 active site is not properly configured for catalysis (Southall et al. 2009). Structural elements of the lysine substrate binding pocket are misaligned so that proper juxtaposition of methyl donor and -amino group acceptor cannot occur. Since addition of RbBP5 and ASH2 to the isolated MLL1 SET domain stimulates enzyme activity *in vitro*, the simplest explanation is that partner binding induces conformational changes that optimize the active site (Southall et al. 2009; Justin et al. 2010). Although comparable structural insight on the EZH2 active site is so far lacking, it seems reasonable to envision that similar mechanisms operate in PRC2. Indeed, recent work has revealed allosteric input of the EED subunit to PRC2 enzyme activity (see below).

ESC/EED

EED is a WD repeat protein (Fig. 1b) that folds into a seven-bladed β-propeller (Han et al. 2007; Margueron et al. 2009; Xu et al. 2010). β-propellers occur in functionally diverse proteins, with the donut-like structure typically providing a scaffold for interactions with partner proteins and effectors. Recent work shows that EED is equipped with a central pocket atop its β-propeller that binds specifically to trimethylated lysines (Margueron et al. 2009; Xu et al. 2010). Such a binding site is consistent with affinity for H3-K27me3 retained by PRC2 lacking a SET domain (Hansen et al. 2008). The EED top pocket, featuring a conserved aromatic cage, is configured for preferential binding to repressive chromatin marks, including H3-K27me3, H3-K9me3 and H4-K20me3, as opposed to "activating" marks such as H3-K4me3. This discrimination relies on residues flanking the methylatable lysine, thereby exploiting the ARKS motifs at repressive positions K9 and K27. PRC2 binding to tri-methylated H3 peptides boosts its HMTase activity, with the H3-K27me3 peptide delivering the most robust stimulation (Margueron et al. 2009; Xu et al. 2010). Thus, EED harbors a site for allosteric PRC2 stimulation via binding the H3-K27me3 reaction product. Importantly, this may provide a mechanism to maintain or propagate H3-K27 methylation in chromatin regions where this repressive mark pre-exists (Hansen et al. 2008; Margueron et al. 2009). Consistent with this, mutational disruption of the aromatic cage in the fly homolog, ESC, reduces H3-K27me3 levels *in vivo* (Margueron et al. 2009).

Several observations suggest that the EED/ESC subunit does more than just sense and convey local H3-K27me3 concentration. First, whereas PRC2 activity is boosted 3 to 7-fold by addition of H3-K27me3 peptides (Margueron et al. 2009; Xu et al. 2010), complete loss of the EED/ESC subunit causes quantitatively more severe impairment of PRC2 HMTase (Ketel et al. 2005; Nekrasov et al. 2005). A basic stimulatory role is further suggested by the requirement for ESC in PRC2 catalysis upon recombinant substrates lacking pre-methylated H3-K27 (A. Rai and J.A.S., unpubl. results). Second, (Xu et al. 2010) show that an alternative histone modification, H1-K26me3, also binds the EED aromatic cage but that, in this case, the impact is to inhibit PRC2 HMTase. Although H1-K26 methylation by PRC2 has been described (Kuzmichev et al. 2004), its potential impact on PcG silencing remains to be determined. Thus, more work is needed to fully reveal contributions of the ESC/EED subunit to PRC2 activity and regulation. To date, structure/function studies have established at least these three modules within ESC/EED: 1) the β-propeller top pocket that binds H3-K27me3 (Margueron et al. 2009; Xu et al. 2010), 2) another interface on the bottom of the β-propeller that binds an N-terminal portion of EZH2 (EID in Fig. 1b; Han et al. 2007), and 3) an N-terminal tail, outside the β-propeller, that can bind the histone-fold domain of H3 (Tie et al. 2007).

SU(Z)12

The domain organization of SU(Z)12 is depicted in Fig. 1b. The C-terminal VEFS domain mediates stable binding to EZH2/E(Z), thereby promoting PRC2 assembly. This VEFS

interaction is strikingly conserved from plants to flies to mammals (Chanvivattana et al. 2004; Yamamoto et al. 2004; Ketel et al. 2005). Beyond simply providing subunit contact, the VEFS domain also likely plays an instructive or allosteric role in controlling PRC2 enzyme activity. Alteration of a conserved and highly charged subelement of the SU(Z)12 VEFS domain (Fig. 1b) disrupts PRC2 HMTase while preserving complex assembly (Ketel et al. 2005). This suggests that the VEFS domain has stimulatory influence. Furthermore, recent findings implicate SU(Z)12 in allosteric inhibition of PRC2 HMTase by H3-K4me3 (Schmitges et al. 2011; see below). This enzyme antagonism is attributed to a C-terminal portion of SU(Z)12 encompassing VEFS. Thus, the VEFS domain provides a key surface for EZH2 contact that could help optimize the PRC2 active site and also mediate regulatory inputs that control enzyme output. Structural information on VEFS domain architecture and the nature and consequences of its contact with EZH2/E(Z) should do much to illuminate underlying mechanisms.

Other SU(Z)12 domains include a single C_2H_2 zinc finger of unknown molecular function and an extended N-terminal region bearing scattered conserved subelements (Fig. 1b). Consistent with these conserved blocks, the N-terminal domain appears to subdivide into several functional modules; the region spanning amino acids 79–91 of fly SU(Z)12 provides a binding surface for the NURF55 subunit (Schmitges et al. 2011) and residue 274 is altered in the partial loss-of-function fly *Su(z)12*^{2} mutant (Birve et al. 2001).

Besides its contributions to core PRC2, there is also evidence that SU(Z)12 mediates interactions with PRC2 cofactors such as Jarid2 (Peng et al. 2009). Moreover, SU(Z)12 is the PRC2 subunit with the strongest affinity for a set of short ncRNAs emanating from the 5' ends of repressed target genes (Kanhere et al. 2010). Further investigation of these SU(Z)12 interactions, particularly *in vivo*, is required to reveal how they contribute to PRC2 function and regulation.

NURF55/RpAp48

Like EED, NURF55 is a WD repeat protein that forms a seven-bladed β-propeller (Song et al. 2008). However, NURF55 is functionally distinct from the other three PRC2 subunits in two major ways. First, whereas EED, EZH2, and SUZ12 appear dedicated to PcG silencing, NURF55 is present in many chromatin-modifying complexes with diverse functions. Besides PRC2, NURF55 appears in an ISWI-class nucleosome remodeling complex (NURF), a CHD-class remodeling complex (NuRD), chromatin assembly factor 1 (CAF1), and in histone acetyltransferase and deacetylase complexes (Suganuma et al. 2008). Second, NURF55 is not essential for robust PRC2 histone methyltransferase. Loss or impairment of any of the other three PRC2 subunits causes dramatic reduction of K27 methylation *in vitro* (Cao and Zhang 2004a; Pasini et al. 2004; Ketel et al. 2005; Nekrasov et al. 2005), whereas removal of NURF55 yields a trimeric complex that retains robust activity, estimated at merely ~2 to 3-fold less than intact core complex on polynucleosome substrates (Cao and Zhang 2004a; Ketel et al. 2005). This dispensability for catalytic function, together with widespread deployment in other chromatin modifiers, underscores a long-standing puzzle: just what does NURF55 do in the PRC2 complex?

One long-appreciated feature of NURF55 is its ability to bind histone H4 through contact with helix 1 of the histone fold (Verreault et al. 1997). Details of this interaction were revealed by a co-crystal structure, which defines a specialized side pocket of the NURF55 β-propeller that binds this H4 helix (Song et al. 2008). Although the NURF55 side pocket contributes to activity of a HAT complex (Song et al. 2008), it was not clear how it could function in PRC2 since H4 helix 1 is not accessible when packaged in nucleosomes. This conundrum appears resolved by more recent structural studies demonstrating that this same NURF55 side pocket also binds to an N-terminal portion of SU(Z)12, in a manner that likely

precludes H4 interaction (Nowak et al. 2011; Schmitges et al. 2011). Thus, in PRC2, the NURF55 side pocket is likely used for SU(Z)12 contact and complex assembly rather than histone binding.

WD repeat proteins commonly feature cavities or channels sculpted on the β-propeller top surface for protein interactions. In WD repeat chromatin proteins, these top pockets are often configured to bind histone tails (Suganuma et al. 2008), as exemplified by WDR5, which functions in H3-K4 methyltransferases (Ruthenburg et al. 2006), and EED (Margueron et al. 2009; discussed above). With satisfactory understanding of the specialized NURF55 side pocket achieved, attention has shifted to the more commonly utilized top surface. Recent structural and biochemical studies reveal a NURF55 top channel that binds to the histone H3 tail peptide, residues 1-15 (Nowak et al. 2011; Schmitges et al. 2011). Occupancy of the top and side pockets are independent such that, in PRC2, NURF55 can simultaneously bind the extreme H3 tail and SU(Z)12. What role could NURF55 binding to H3(1-15) play in a complex that methylates more proximally at H3-K27? Surprisingly, disruption of NURF55-H3 tail interaction has little or no effect on either PRC2 histone methyltransferase or overall affinity for nucleosomes (Schmitges et al. 2011). This emphasizes the intricate engineering of the PRC2 machine, which likely has several nucleosome-contacting surfaces and clearly contains multiple inputs that impact HMTase. Currently, the best guess about NURF55-H3 tail binding is that it might help PRC2 sense substrates bearing inhibitory H3-K4me3 (Schmitges et al. 2011; see below), but functional tests have yet to substantiate this.

These recent advances provide key pieces of the NURF55 puzzle. We now appreciate that NURF55 provides a multifunctional platform for histone and PRC2 subunit contacts. How these and potentially other NURF55 elements are integrated for function within PRC2 remains to be determined. *In vivo* tests will be needed to complement the structural and biochemical insights. In this regard, recently described fly NURF55 alleles (Anderson et al. 2011), one of which likely disrupts the SU(Z)12-binding side pocket, could be useful.

III. Control of PRC2 by Antagonistic Histone Modifications

Antagonism by H3-K4 and H3-K36 methylations

Histone methylation at positions H3-K4 and H3-K36 are hallmarks of actively transcribed genes. Their distributions along gene bodies tend to differ, with H3-K4me3 enriched at promoter/transcription start site regions, H3-K36me2 most abundant in 5'-coding regions, and H3-K36me3 peaking towards the 3' ends of coding regions (Barski et al. 2007; Bell et al. 2007; Barrand et al. 2010). H3-K36me2/3 accumulation in coding regions parallels association of H3-K36 methyltransferase with elongating RNA pol II (Li et al. 2003). There is abundant evidence that these K4 and K36 methylations oppose Polycomb silencing. Indeed, *Drosophila* trithorax (TRX), a K4-methyltransferase (Smith et al. 2004) and ASH1, a K36-methyltransferase (Tanaka et al. 2007; Yuan et al. 2011), were established as genetic suppressors of Polycomb mutations long before their enzyme functions were defined (Kennison and Tamkun 1988; Shearn 1989). Moreover, genetic and genome-wide studies imply that *Drosophila* TRX and ASH1 are dedicated antagonizers of PcG silencing, operating specifically at PcG target sites rather than as general activators of transcription (Klymenko and Muller 2004; Schwartz et al. 2010).

Two new studies shed light on molecular mechanisms underlying this antagonism (Schmitges et al. 2011; Yuan et al. 2011). The basic finding is that PRC2 methylation at H3-K27 is inhibited on substrates with pre-existing H3-K4me3 or H3-K36me2/3 (see Fig. 3a). Importantly, pre-installed H3-K4me3 does not impair PRC2 binding to substrate nucleosomes, suggesting an allosteric inhibitory mechanism that instead dampens catalytic efficiency (Schmitges et al. 2011). Another key finding is that PRC2 inhibition requires that

the K4 and K36 methylations appear on the same histone tail subject to K27 methylation (Schmitges et al. 2011). Consistent with this *cis* requirement, mass spectrometric analysis shows that K27me3 rarely co-exists with K4me3 or K36me2/3 on the same histone tails *in vivo* (Young et al. 2009; Yuan et al. 2011). The *cis* requirement also distinguishes this inhibitory mechanism from the stimulatory effect of K27me3 on PRC2 (see above), which can operate *in trans* (Margueron et al. 2009). Although the PRC2 modules that mediate K4me3 and K36me2/3 inhibition have not yet been pinpointed, the evidence so far suggests that both inhibitory inputs operate through SU(Z)12 (Schmitges et al. 2011).

Taken together, these recent findings reveal that PRC2 is equipped to sense its chromatin environment and adjust activity accordingly (Margueron et al. 2009; Schmitges et al. 2011; Yuan et al. 2011); if PRC2 senses actively transcribed chromatin then its methyltransferase is inhibited and, conversely, repressed local chromatin stimulates PRC2. This built-in feedback mechanism is presumably circumvented or dampened in the case of "bivalent" chromatin domains, described in embryonic stem cells, which feature concomitant accumulation of H3-K4me3 and H3-K27me3 (Bernstein et al. 2006; Mikkelsen et al. 2007; Min et al. 2011).

Antagonism by H3-K27 acetylation

Since acetylation of lysine side chains is biochemically incompatible with their methylation, a theoretically simple way to impede PRC2 is via direct H3-K27 acetylation of substrate nucleosomes. Indeed, H3-K27 acetylation is generally enriched in promoters and coding regions of active genes in metazoans (Garcia et al. 2007; Wang et al. 2008). New findings now establish that H3-K27 acetylation does functionally oppose PcG silencing in both fly and mammalian systems (Tie et al. 2009; Pasini et al. 2010b; Schwartz et al. 2010).

In *Drosophila*, the major HAT that targets H3-K27 is CREB-binding protein (CBP; Tie et al. 2009). Similarly, among 17 mammalian HATs tested, the two orthologs CBP and p300 were preferentially implicated in H3-K27 acetylation (Pasini et al. 2010b). This K27 HAT identification then enabled loss and over-expression studies, which generally reveal inverse accumulations of H3-K27Ac and H3-K27me3 *in vivo*. For example, CBP knockdown depletes K27Ac and increases K27me3 (Tie et al. 2009) whereas PRC2 loss depletes K27me3 with concomitant gain of K27Ac (Tie et al. 2009; Pasini et al. 2010b). There is also intriguing complementarity of these dueling K27 modifications during *Drosophila* development, with K27Ac peaking in early embryos and then declining, whereas H3-K27me3 gradually accumulates as embyogenesis proceeds (Tie et al. 2009). The opposing nature of K27Ac and K27me3 is further revealed by ChIP analysis of selected PcG targets during ES cell differentiation (Pasini et al. 2010b) and in a more extensive genome-wide analysis of alternative chromatin states (Schwartz et al. 2010).

These advances on K27 acetylation may also impact some long-standing issues in field. First, histone deacetylases (HDACs) have previously been implicated in PcG silencing (van Der Vlag and Otte 1999; Tie et al. 2001) but their mechanistic contributions have not been defined. One straightforward role for HDACs, then, would be to remove acetyl groups from K27 side chains to render them methylatable by PRC2 (Fig. 3b). Second, the first-characterized fly TRX complex, called TAC1, has both CBP HAT and H3-K4 methyltransferase activities (Petruk et al. 2001; Smith et al. 2004) and physical interaction of mammalian TRX (MLL) with CBP has long been described (Ernst et al. 2001). This raises the intriguing possibility that delivery of K4me3 and K27Ac, which both oppose PRC2, could be molecularly coordinated.

Antagonism by H3-S28 phosphorylation

H3-K27 is immediately flanked by serine-28 (S28), which is known to be phosphorylated during mitosis and also during interphase (Dunn and Davie 2005; Dyson et al. 2005). This proximity begs the question of whether S28 phosphorylation impacts PRC2 function and PcG silencing. By analogy, at the similarly arranged K9-S10 module of histone H3, S10 phosphorylation can displace heterochromatin protein 1 (HP1) recruited to the H3 tail via affinity for neighboring methylated K9 (Fischle et al. 2005; Hirota et al. 2005). Two recent studies now establish that S28 phosphorylation can profoundly disrupt PcG silencing and identify the kinase(s) responsible (Gehani et al. 2010; Lau and Cheung 2011).

The development of an antibody that recognizes doubly modified K27me3-S28phos showed that S28 phosphorylation accumulates on PcG targets in response to mitogen- and stress-activated kinase pathways (Gehani et al. 2010). This chromatin response is triggered by several pathway inputs, including retinoic acid and mitogenic stimuli, with MSK1 and MSK2 implicated as the kinases that modify S28. The molecular consequence is displacement of PRC2 and its partnering PcG complex, PRC1, with concomitant desilencing of PcG targets.

These findings are reinforced by complementary studies of an engineered MSK1 directly tethered to reporter constructs and endogenous gene targets (Lau and Cheung 2011). Here, MSK1 targeting also leads to S28 phosphorylation, displacement of PRC1 and PRC2, and gene desilencing. Since kinase-dead MSK1 cannot trigger this response, the transcriptional activator function of MSK1 is directly tied to its phosphorylation of H3-S28. Moreover, MSK1-mediated S28 phosphorylation correlates with conversion of K27me3 to K27Ac (Lau and Cheung 2011), suggesting that S28phos and K27Ac are coupled to oppose PRC2 and PcG silencing. In this scenario, K27me3-S28phos could be an intermediate which, followed by K27 demethylation and acetylation, produces an active chromatin state marked by K27Ac-S28phos. Conversely, one might also expect to find a nuclear phosphatase that removes S28phos, thereby synergizing with PRC2 in target gene silencing.

Taken together, these studies suggest a molecular switch that deploys S28 phosphorylation to convert silenced PcG targets from OFF to ON. It is tempting to speculate that such a switch could operate during ES cell differentiation since both activated MSK1 and S28 phosphorylation occur in human ES cells (Gehani et al. 2010). The mechanism by which S28phos impacts PRC2 remains to be defined. Since structural studies imply that S28 is not a key determinant of H3 peptide interactions with the EED top pocket (Margueron et al. 2009; Xu et al. 2010), it seems likely that other PRC2 sites, perhaps within the SET domain itself, are deployed to sense and respond to S28 phosphorylation.

Conclusions: Towards inner workings of PRC2 brake and accelerator mechanisms

In summary, recent progress has identified key features of the chromatin landscape that modulate PRC2 function; we now appreciate that H3-K4me3, H3-K36me2/3, H3-K27Ac, and H3-S28phos can directly antagonize PRC2 whereas pre-existing H3-K27me3 can stimulate PRC2 activity. These advances contribute to a more complete picture of how PcG target chromatin is configured for robust silencing, full activation, or more balanced states in between (Papp and Muller 2006; Schuettengruber et al. 2009; Filion et al. 2010; Schwartz et al. 2010). Furthermore, biochemical and structural studies are shedding light on the precise mechanisms by which these chromatin marks, and other extrinsic cues, apply the brakes or engage the accelerator within the PRC2 machine. It is clear that the EED and SU(Z)12 subunits are crucial cogs and that they help execute slowdowns and speedups as required by cellular conditions and local chromatin terrain. Nevertheless, important puzzles remain. To fully reveal operations under the hood, we need to know the three-dimensional architecture

of the EZH2 active site as well as functional elements of key EED-EZH2 and SU(Z)12-EZH2 interfaces that presumably influence this active site. Protein dynamics studies should also address how newly identified PRC2 allosteric sites could deliver functional impact via potential conformational changes. The intricate workings of PRC2 have been finetuned by more than a billion years of evolution (Whitcomb et al. 2007; Sawarkar and Paro 2010). With recent advances, the prospects are favorable for viewing a high-resolution parts diagram and operations blueprint in the not-too-distant future.

Prospectus: Consequences of PRC2 output

Despite major progress on deciphering upstream inputs that control PRC2, the mechanistic consequences of its chromatin output, H3-K27me3, remain largely unresolved. Precisely how does acquisition of H3-K27me3 on local nucleosomes lead to gene silencing? One longstanding idea is that H3-K27me3 could help create a landing pad to recruit the partnering PcG complex, Polycomb repressive complex 1 (PRC1), to local chromatin (reviewed in Simon and Kingston 2009; Margueron and Reinberg 2011). In this scenario, the central job of PRC2 could be viewed as paving the way for PRC1 association which, in turn, implements the key events in gene silencing. However, it is far from clear that PRC1 recruitment is the sole or even main function of H3-K27me3. Alternative roles for H3-K27me3 in gene silencing could include: 1) preventing accumulation of H3-K27Ac and other "activating" histone acetylations (Tie et al. 2009; Pasini et al. 2010b), 2) blocking deposition of other activating modifications, such as H3-K4me3 or H3-K36me2/3, or 3) antagonizing the functions of remodeling complexes (Wilson et al. 2010).

Ultimately, to define precise mechanisms, discrete steps in the transcription cycle impacted by PRC2 and H3-K27me3 will need to be identified (Fuda et al. 2009). Thus, mid-gene RNA pol II elongation could be impeded by H3-K27me3-modified nucleosomes and/or polII could be impacted in 5' regions at the steps of promoter binding, initiation, promoter escape, or promoter-proximal pausing. These are not mutually exclusive possibilities and there could be inhibitory effects at several steps. Indeed, the broad domains of H3-K27me3 accumulation (Schwartz et al. 2010), together with PRC2 inhibition by both promoter-enriched (H3-K4me3) and 3'-region enriched (H3-K36me3) modifications (Schmitges et al. 2011; Yuan et al. 2011; see above), suggest that PRC2 is functionally deployed along the entire extent of gene bodies.

Recently, highly regulated genes in metazoans have been shown to accumulate promoter-occupied nucleosomes and promoter-proximal paused RNA pol II (Gilchrist et al. 2010), suggesting these two features as commonly used control points. Since most PcG target genes likely belong to this class, this chromatin architecture presents a baseline for considering mechanisms of PcG silencing complexes. Indeed, recent studies in *Drosophila* and mammalian cells have begun to dissect the relevant transcription cycle steps, with evidence emerging for PRC2 impact upon both polII promoter occupancy and post-initiation events (Chopra et al. 2011; Min et al. 2011). These new *in vivo* studies, which exploit powerful combinations of genetic, genomic, and molecular approaches, provide key steps forward in the quest to reveal PcG silencing mechanisms. With progress in this field now poised for rapid advances, further insights on both inputs and outputs of PRC2 should do much to define its ancient and fundamental roles in epigenome regulation.

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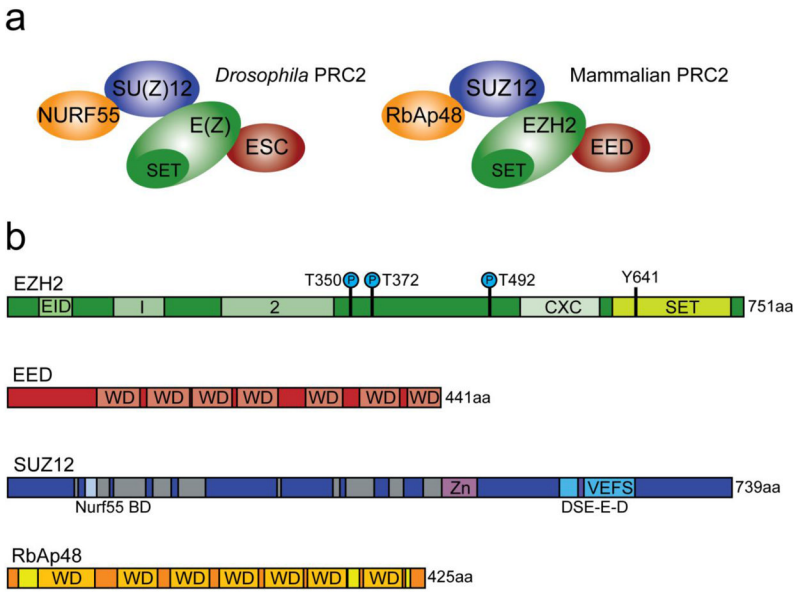

Fig. 1. PRC2 core complex

a) Models of core fly and human PRC2 complexes. Subunit compositions and established contacts between subunits are depicted. Variants of human PRC2 resulting from alternative subunit usage are described elsewhere (Simon and Kingston 2009; Margueron and Reinberg 2011). b) Domain organizations of human PRC2 subunits. EZH2 contains a C-terminal SET domain, an adjacent cysteine-rich CXC domain, and additional conserved regions as indicated. EID denotes EED-interacting domain. EZH2 phosphorylation sites and the Y641 mutation discussed here are indicated. Both EED and RbAp48 are β-propeller proteins built from an array of seven WD repeats. RbAp48 regions in yellow contribute to the side pocket that can bind SUZ12 or histone H4. SUZ12 contains a C-terminal VEFS domain, C_2H_2 zinc finger, and an extended N-terminal region with conserved blocks (gray) from plants to human. An N-terminal SUZ12 region that binds NURF55 (Schmitges et al. 2011) is indicated. DSE-E-D indicates a conserved and highly charged subelement within the VEFS domain (Ketel et al. 2005).

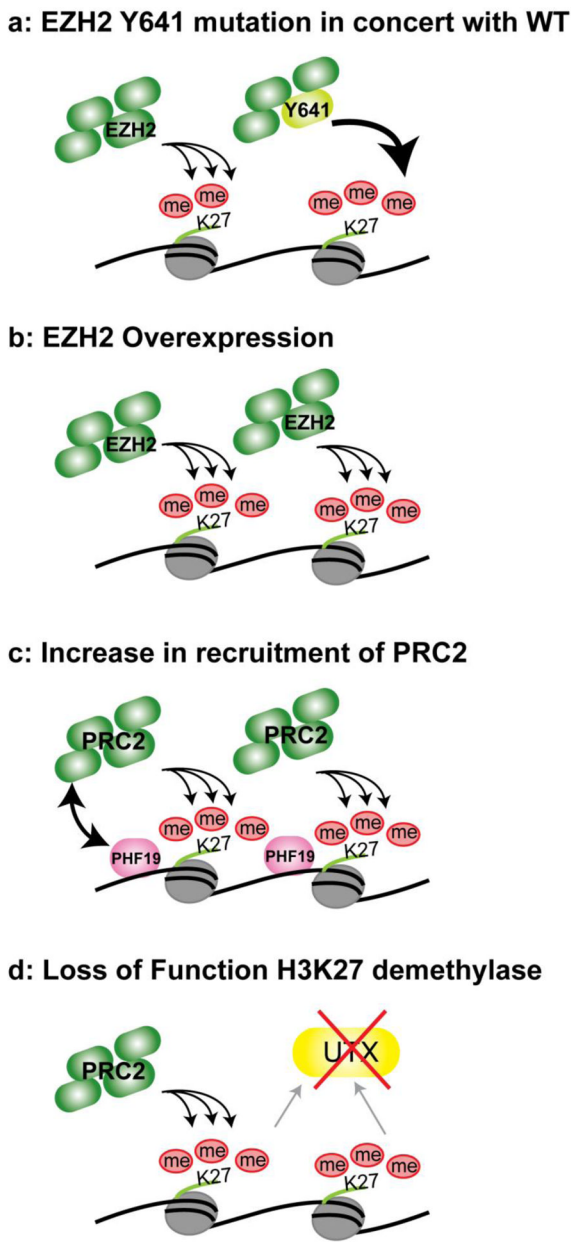

Fig. 2. Mechanisms that elevate H3K27me3 levels in cancer cells

a) While deficient in mono- and di-methylase activity, PRC2 bearing EZH2-Y641 mutations has enhanced capacity for H3K27 tri-methylation. Acting *in trans* with wildtype PRC2, which can perform all three methyltransferase reactions, the Y641 gain-of-function leads to higher overall H3K27me3 levels (Sneeringer et al. 2010; Yap et al. 2011). b) Overexpression of the EZH2 catalytic subunit leads to higher overall H3K27me3 levels. As EZH2 alone is inactive, this is presumably due to a net increase in assembled PRC2 complexes. c) Overexpression of PHF19/PCL3 (Wang et al. 2004) or an interacting non-coding RNA (not shown, see Gupta et al. 2010) enhances recruitment of PRC2 to targets, leading to increased H3K27me3. d) Loss of function mutation of the demethylase UTX results in higher H3K27me3 levels (van Haaften et al. 2009). All four illustrated mechanisms can raise H3K27me3 levels and potentially contribute to hypersilencing of target genes in cancer.

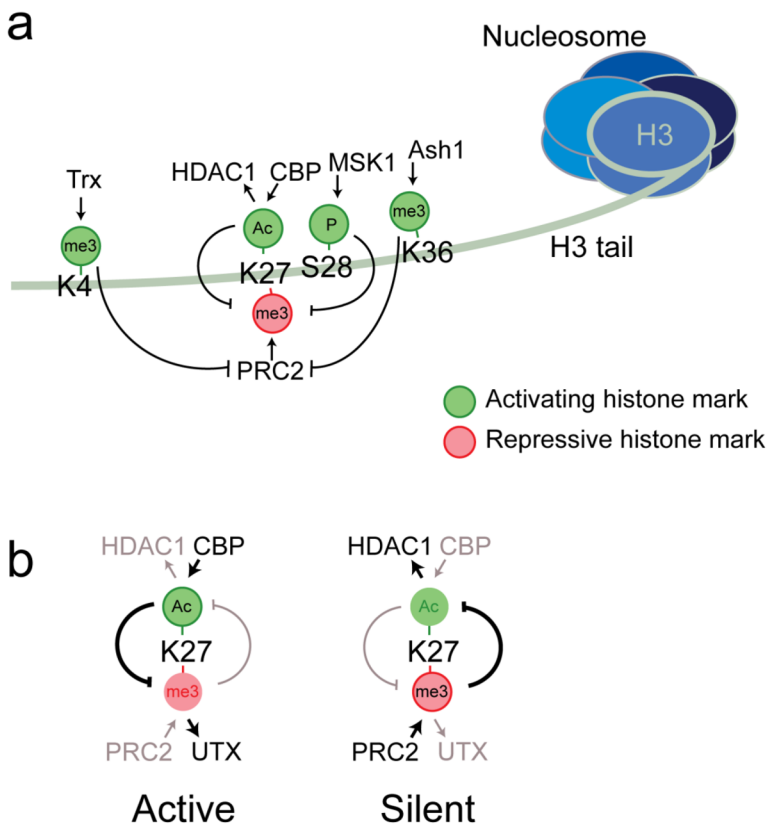

Fig. 3. Histone H3 tail modifications and crosstalk that impact PRC2

a) PRC2 tri-methylates K27, whereas CBP acetylates K27. MSK1 phosphorylates S28, which antagonizes PRC2 and may work in concert with CBP to acetylate K27. H3K4me3 deposited by Trithorax and H3K36me2/3 deposited by Ash1 inhibit PRC2 activity. b) Methylation and acetylation of K27 is mutually exclusive. The diagram on the left depicts K27 in its active state, with CBP delivering K27Ac. UTX is required to remove K27me3 before this acetylation can occur. The diagram on the right depicts K27 in its silent state, with PRC2 delivering K27me3. HDAC1 is required to remove K27ac before PRC2 can trimethylate it.