Functional analysis of the core subunits of Polycomb Repressive Complex 2

A Dissertation
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Adviser: Jeffrey Alan Simon

August 2012

Acknowledgements

At the onset, I would like to express my gratitude to Prof. Jeffrey A. Simon for providing support and guidance over the course of my graduate education. I would like to thank him for integrating me as a part of his lab and providing a home base to continue my graduate studies. I would like to take this opportunity to thank Prof. Haojie Huang and Dr. Shuai Chen for a very exciting and rewarding collaboration. I am also thankful to Prof. Jennifer Westendorf for providing excellent guidance and support during the first two years of graduate education.

I am thankful to my committee members, Prof. Michel Sanders, Prof. Dennis Livingston, Prof. Kathleen Conklin, Prof. Carol Lange and Prof. Vivian Bardwell for their constant support and encouragement throughout my graduate days. I take this opportunity to thank Darlene Toedter for making sure I was on track with all the graduate school requirements.

I would not have sustained my long winding path to graduation without the support and company of some special people I met in graduate school. I am in short of words to thank Ellen for always being there to listen and support me during my stay in the Simon lab. Thank you very much for coffee, African Violets, lots of “anti-Murphase”, and last but not the least help with flies and experiments. I would like to thank Liangjun for being a great friend and mentor, and willingness to always help trouble shoot experiments. My thanks to Maggie for working with me on the isolation of HeLa polynucleosomes, and “Bona-Starbucks”. Thanks to Kayla, the “Simon lab fairy” for constant supply of reagents. I would like to thank the former members of the Simon lab, Aidan for “Tubby on two” and lots and lots of help with fly genetics, Preeti for a being a great friend and fellow graduate student, Neal, for constant encouragement and elder-berry preserves, Dan for help with polytenes, and Mary for being a wonderful student. I would also like to thank Carrie Ketel and Erica Andersen, whose work formed the basis for this thesis. Thanks to all the 5th floor DBC labs for reagents and equipment, especially the O’Connor and Bardwell labs, and Arpan for help with embryo collections.

I would like to thank my parents for giving me the gift of education. Thanks to my brother for being a great friend. Last but not the least, my husband, Neeraj and my little girl, Anya for their unconditional love and support.

The work presented in Chapter 2 and Chapter 3 of this thesis was supported by NIH grants to Prof. Jeffrey A. Simon and Prof. Haojie Huang respectively.

Dedication

To Achan, Amma, Neeraj and Anya…

Abstract

Polycomb Repressive Complex 2 (PRC2) is a highly conserved multisubunit complex that methylates histone 3 on lysine 27 (H3K27). PRC2 function is implicated in diverse biological processes including stem cell regulation, cancer progression and genomic imprinting. This makes it critical to understand the mechanism by which PRC2 mediates gene silencing. In *Drosophila*, PRC2 consists of 4 core subunits, E(Z) with the SET domain, WD40 repeat proteins ESC and NURF55, and a structurally distinct SU(Z)12. E(Z), the catalytic subunit of PRC2 with SET domain possess the histone methyltransferase activity. A key feature of PRC2 is that E(Z) cannot methylate H3K27 without the help of its partner subunits SU(Z)12 and ESC. There is limited information on the structure and molecular function of SU(Z)12 in PRC2.

In Chapter 2 of this thesis, we performed a systemic analysis of *Drosophila* SU(Z)12 domains to understand its contribution to PRC2. We analyzed the three conserved domains of fly SU(Z)12 and assigned critical functions to these individual domains. Our studies conclude that the N- terminal conserved domain of SU(Z)12 has binding determinants for the subunit E(Z) in PRC2. We show that the conserved, centrally located non-canonical, non-DNA binding C_2H_2 zinc finger is dispensable for PRC2 enzyme function *in vitro.* However, its function is critical for PRC2 function *in vivo* as mutations in the conserved residues of this domain failed to rescue the strong null phenotype of $Su(z)12^{3}/Su(z)12^{4}$. We also find that the highly conserved C-terminal VEFS domain is critical for enzyme function as well as assembly of the PRC2 complex.

In Chapter 3 of this thesis, we highlight key findings on phosphorylation of EZH2 by Cyclin Dependent Kinases (CDKs), and their modulatory effect on PcG silencing.

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Chapter 1

Introduction

1.1 Polycomb Group Proteins

Polycomb group proteins [PcG)] are a family of pleiotropic epigenetic silencers implicated in growth and development, cancer progression and suppression, stem cell maintenance and regulation, and X-chromosome inactivation (Simon and Kingston, 2009).The differentiation of body segments along the animal anterior-posterior axis of vertebrates is determined by the accurate expression of homeotic [*Hox*] genes. PcG proteins were first identified in *Drosophila* as factors regulating the spatial expression patterns of *Hox* genes, as mutations in these proteins cause homeotic transformation, transformation of one body segment into another (Lewis, 1978; Struhl, 1981). In Drosophila, *Hox* gene expression is established during embryogenesis by the gap and pair rule genes, while PcG proteins maintain this pattern of gene expression providing cellular memory.

The PcG repressor family of proteins sort into four distinct complexes [Fig. 1] termed Polycomb Repressive Complex 1[PRC1], Polycomb Repressive Complex 2 [PRC2], Pho-Repressive Complex [Pho-RC] (Simon and Kingston, 2009) and Polycomb Repressive Deubiquitinase [PR-DUB] (Scheuermann et al., 2010) with different biochemical properties. These complexes are highly conserved between mammals and flies, making the fly system an excellent tool

to gain mechanistic insights into Polycomb complexes and their roles in gene silencing. In *Drosophila*, PcG complexes localize in specialized regulatory regions in target genes called Polycomb Response Elements [PRE] (Simon et al., 1993). However, such a region is not well defined in mammals. In the following sections, I will first briefly describe PRC1, Pho-RC and PR-DUB as well as discuss PRC2 and its core components in detail, which are the main subject of this thesis.

1.2 Polycomb Repressive Complex 1and related complexes.

In *Drosophila*, PRC1 contains four core subunits Polycomb [PC], Polyhomeotic [PH], RING/SCE [Sex Comb Extra] and PSC [Posterior Sex Comb] [Fig.1] (Shao et al., 1999; Francis et al., 2001). A recombinant four subunit version of fly PRC1 can bind nucleosome templates, inhibit nucleosome remodeling by SWI/SNF remodelers, compact nucleosome arrays as well as inhibit transcription *in vitro* (Shao et al., 1999; Francis et al., 2001; 2004). Similarly, human PRC1, purified from nuclear extracts can block nucleosome remodeling by SWI/SNF remodelers (Levine et al.).

The core subunits of PRC1 can serve different roles towards its function. *In vitro*, PSC play a central role in inhibiting nucleosome remodeling, transcription and chromatin compaction (Francis et al., 2001; King et al., 2002). PC can bind the repressive histone modification H3K27me3 [H3 lysine 27 trimethyl] through its chromodomain [Fig 1], while RING/SCE, with its RING finger domain possess E3 ligase activity for H2A lysine118 [H2AK118] and H2A lysine 119 [H2AK119] in

flies and mammals respectively. [Fig. 1] (Wang et al., 2004a). PSC also act as a co-factor to stimulate RING1-mediated E3 ligase activity. Taken together, PRC1 serve two distinct molecular functions, i) RING/SCE mediated H2AK118 ubiquitination and ii) PSC mediated block to SWI/SNF nucleosome remodeling and chromatin compaction. It is unclear which of the two activities are central to PRC1-mediated gene silencing.

PRC1 has different flavors in mammals and flies [Fig. 2A and B]. In flies, the two core components of PRC1, PSC and RING/SCE are found in another complex dRAF (*Drosophila* Ring-Associated Factor) along with the H3-K36 demethylase KDM2 [Fig 2B] (Simon and Kingston, 2009) . dRAF can also function as an E3 ligase to ubiquitinylate H2AK118. However, in mammals, PRC1 subunits are found in several distinct complexes [Fig. 2B]. One such flavor of PRC1 has BCOR co-repressor along with NSPC1, RING1, RING1B/RNF2, and RYBP as well as components of an SCF ubiquitin ligase, SKP1, and FBXL10 [Fig 2B] (Gearhart et al., 2006). Recently, yet another flavor of PRC1, RYBP-PRC1 was characterized in mouse ES cells comprising RYBP, RING1B and Mel-18/BM1 [Fig. 2B] (Tavares et al., 2012). To add to the complexity, six different versions of PRC1 were biochemically characterized with RING1 as the central component along with distinct PCGF subunits [mammalian homologs of PSC] (Gao et al., 2012). Unlike fly PRC1, mammalian PRC1 is more diverse and the functional roles of mammalian PRC1 and related complexes are still being sorted out.

Figure 1: Core subunit compositions of *Drosophila* PcG complexes.

The four biochemically characterized PcG complexes are illustrated with their biochemical functions indicated.

Pho-RC can bind to PRE via the sequence specific DNA binding activity of the PHO subunit. SFMBT is the binding partner for PHO in this complex. In flies, Pho-RC can interact with and recruit PRC2 to chromatin sites of action.

PRC2 consists of four core subunits, E(Z), ESC, SU(Z)12 and NURF55. The biochemical function of PRC2 is to deposit the trimethyl mark on H3K27 residue via the catalytic SET domain of the E(Z) subunit. The methyl mark is indicated by the red spot.

PRC1 consists of four core subunits, SCE, PSC, PH and PC. The PC subunit has chromodomain that can specifically recognize H3K27 trimethyl marks deposited by PRC2. The SCE/RING subunit provides the catalytic function to ubiquitinate H2AK118. The ubiquitin mark is indicated by blue star.

PR-DUB consists of Calypso and ASX. Its characterized biochemical role is to remove the ubiquitin marks from H2AK118. This function is provided by the subunit, Calypso.

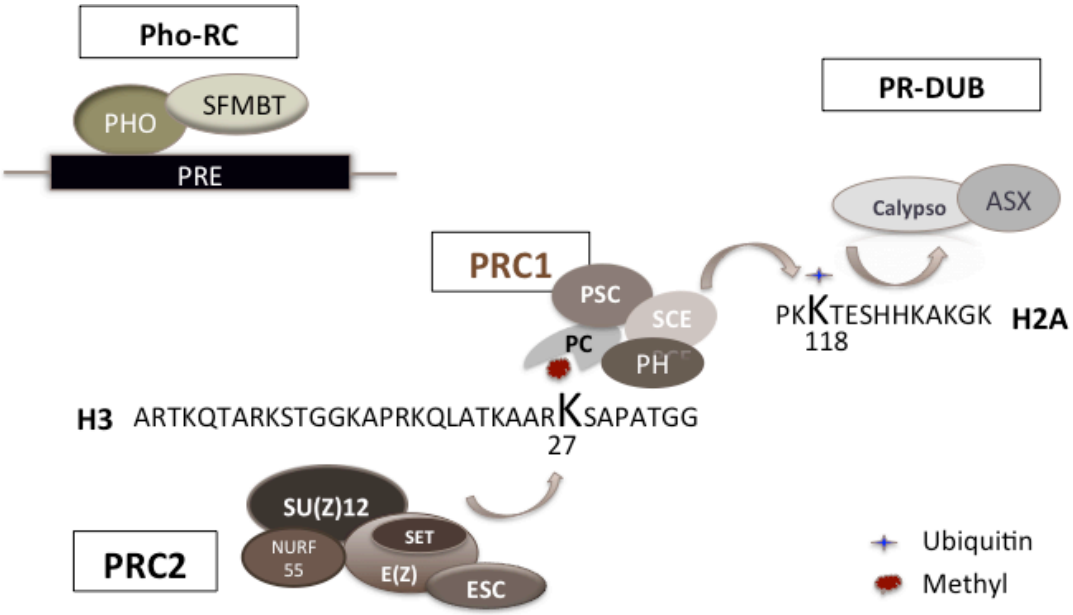
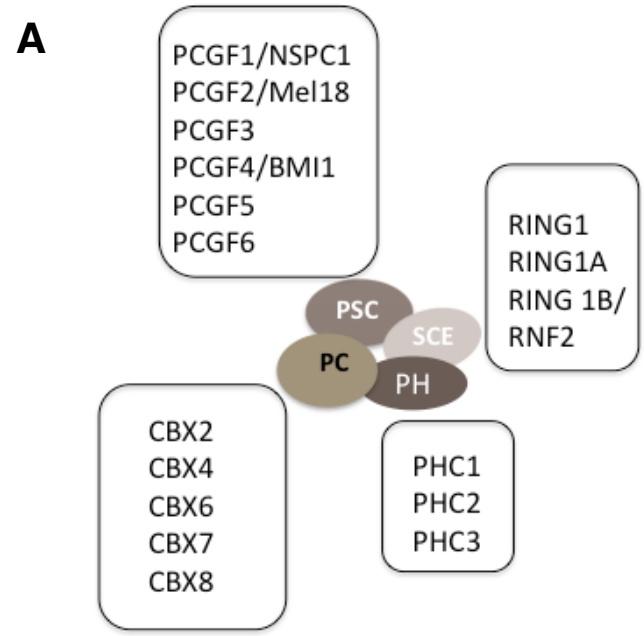

Pho-RC
PHO
SFMBT
PRE
PR-DUB
Calypso
ASX
PRC1
PSC
SCE
PC
PH
PKKTESHHKAKGK
118
H2A
H3 ARTKQTARKSTGGKAPRKQLATKAARKSAPATGG
27
PRC2
SU(Z)12
NURF
55
SET
E(Z)
ESC
Ubiquitin
Methyl

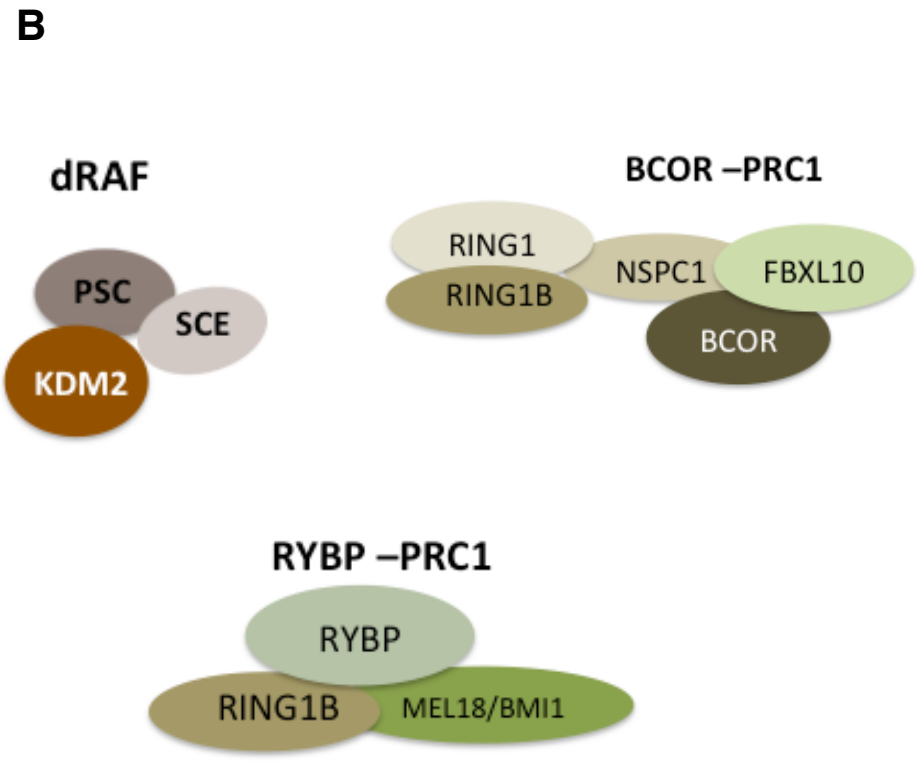
Figure 2: PRC1 and related complexes.

This figure is adapted from Sauvageau and Sauvageau, 2008, and Simon and Kingston, 2009.

A. PRC1 has different flavors in flies and mammals. The canonical fly PRC1 is represented, and the mammalian homologs of various subunits indicated in the box. In mammals, a wide range of PRC1 related complexes exist with different combinations of subunit paralogs (Gearhart et al., 2006; Gao et al., 2012; Tavares et al., 2012).

B. In Drosophila, dRAF (Ring Associated Factor) exists as a distinct complex from canonical PRC1. dRAF consist of KDM2, PSC and SCE/RING. In mammals, a dRAF-related complex exists with BCL6 co-repressor (BCOR). BCOR-PRC1 consists of RING1/B, NSPC1, BCOR and FBXL10 [KDM2B]. RYBP-PRC1 is a newer member of the PRC1 related complexes consisting of RYBP, RING1B and Mel18/BMI1.


A
PCGF1/NSPC1
PCGF2/Mel18
PCGF3
PCGF4/BMI1
PCGF5
PCGF6
RING1
RING1A
RING 1B/
RNF2
PSC
SCE
PC
PH
CBX2
CBX4
CBX6
CBX7
CBX8
PHC1
PHC2
PHC3


B
dRAF
PSC
SCE
KDM2
BCOR –PRC1
RING1
NSPC1
FBXL10
RING1B
BCOR
RYBP –PRC1
RYBP
RING1B
MEL18/BMI1

1.3 Polycomb Repressive Deubiquitinase (PR-DUB).

PR-DUB is a relatively new biochemically characterized member of the PcG family of complexes. PR-DUB is comprised of Calypso [BAP1], a ubiquitin C-terminal hydrolase, and ASX [ASXL1], a previously characterized non-catalytic PcG protein [Fig.1] (Scheuermann et al., 2010). In flies, PR-DUB localize to known PcG target genes and bind the well characterized PRE of the Hox gene, *Ubx* (Scheuermann et al., 2010). The Calypso-ASX complex can remove the ubiquitin mark on H2AK118 deposited by the RING subunit of PRC1 (Scheuermann et al., 2010). H2AK118 ubiquitination by PRC1 is implicated in PcG gene silencing (Wang et al., 2004a). Paradoxically, flies with mutations in Calypso that disrupt its deubiquitinase function show increase in H2AK118 ubiquitin levels as well as hyper-silencing of target genes (Scheuermann et al., 2010). How can ubiquitination and deubiquitination of H2AK118 have the same functional consequence? One possibility is that PR-DUB fine-tunes PRC1 mediated H2AK118 ubiquitin levels by striking a balance between H2AK118 ubiquitination and its deubiquitination to achieve optimal expression levels of PcG target genes.

1.4 PHO-Repressive Complex (PHO-RC)

PHO-RC, is the only PcG complex that has a subunit with sequence specific DNA binding activity [Fig. 1 and Fig. 4] (Klymenko et al., 2006; Müller and Kassis, 2006; Oktaba et al., 2008). The PHO [Pleiohomeotic] subunit possess C2H2 zinc fingers, and has been shown to play a critical role in targeting

PcG complexes to PRE (Klymenko et al., 2006; Müller and Kassis, 2006). Together, PHO and dSFMBT [*Drosophila* Scm-related gene containing four mbt domains], the DNA binding and methyl lysine binding subunits respectively, constitute the PHO-RC, essential for *Hox* gene silencing. Genome-wide studies in flies show co-localization of PHO with components of PRC1 and PRC2 (Oktaba et al., 2008). PHO, with its DNA binding domain is a major recruiter of PcG proteins to PRE. Mutations in PHO (Wang et al., 2004b) and mutations in DNA recognition sites of PHO (Klymenko et al., 2006) result in loss of PRC1 and PRC2 from target chromatin. To associate with other PcG complexes, PHO can interact with PRC2 subunits ESC and the cysteine rich CXC domain of E(Z) (Wang et al., 2004b; Oktaba et al., 2008). PHO-RC can also bind PRC1 subunits PC and PH directly *in vitro* (Mohd-Sarip et al., 2006). dSFMBT harbors four MBT repeat domains, spanning about 100 amino acids each, which together can bind mono and di-methylated lysine residues. However, binding specificity for a particular modified histone residue is yet to be determined (Klymenko et al., 2006; Grimm et al., 2009). SFMBT can interact with another MBT domain protein SCM [Sex Comb on Midleg] (Grimm et al., 2009; Wang et al., 2010), also required for the recruitment of PRC1 and PRC2 to its genomic targets (Mohd-Sarip et al., 2006; Wang et al., 2010). However, binding of PHO-RC to the PRE is independent of SCM.

SFMBT localizes with PHO in genome-wide studies (Oktaba et al., 2008; Grimm et al., 2009), and its association with PHO targets it to PREs of PcG

genes (Klymenko et al., 2006; Grimm et al., 2009). Although PHO-RC is critical for targeting PcG complexes to PRE in flies, targeting mechanisms in mammals are poorly defined.

1.5 Polycomb Repressive Complex 2

Polycomb Repressive Complex 2 (PRC2) is a highly conserved multisubunit protein complex that deposits the trimethyl mark on lysine 27 of histone 3 (H3K27me3), a major repressive chromatin modification [Fig.1] (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). Originally identified in *Drosophila* as silencers of *Hox* genes, PRC2 forms the most fundamentally conserved core of all known Polycomb repression machinery (Whitcomb et al., 2007; Sawarkar and Paro, 2010). PRC2 function is implicated in diverse biological processes including developmental decisions, stem cell regulation, cancer progression and genomic imprinting (Simon and Kingston, 2009). Genome-wide studies have identified PRC2 occupancy at and silencing of dozens of genes that control cell fate and developmental decisions (Boyer et al., 2006; Nègre et al., 2006; Tolhuis et al., 2006).

In *Drosophila*, PRC2 consists of four core subunits: the catalytic subunit enhancer of zeste [E(Z)], Extra Sex Combs [ESC], Suppressor of zeste 12 [SU(Z)12] and NURF55 [Fig.1 and Fig.3] (Czermin et al., 2002; Müller et al., 2002). Similarly, mammalian PRC2 consists of the catalytic subunit EZH2, SUZ12, EED and RbAp48/46 (Cao et al., 2002; Kuzmichev et al., 2002). A key

feature of PRC2 is that E(Z) cannot methylate H3K27 without its partner subunits SU(Z)12 and ESC (Ketel et al., 2005; Nekrasov et al., 2005). Loss or mutations in either of the two subunits compromises the activity of PRC2 holoenzyme. In the following sub-sections, I will discuss the core subunits of PRC2 and review the known properties and activities of these subunits.

1.5.1 E(Z)/EZH2

E(Z) is the catalytic subunit of PRC2. Its signature SET domain is commonly found in chromatin proteins that methylate lysine residues. The SET domain houses the active site for histone methyltransferase reaction. Though the three-dimensional structure of fly E(Z) is yet to be determined, the structural details about this conserved catalytic domain can be inferred from homology modeling and known crystal structures of other SET domain proteins.

When assembled into PRC2, E(Z) can perform three successive methyl transfer reactions leading to the H3K27me3 reaction product (O'Meara and Simon, 2012). However, E(Z)/EZH2 belongs to the SET domain family of proteins that cannot perform the methyl transfer function by itself (Pasini et al., 2004; Ketel et al., 2005; Nekrasov et al., 2005). Within the SET domain, there are several functionally conserved motifs, the CXG motif, that bind adenosylmethionine, the YXG motif in the catalytic site, the hydrophobic FLF motif that constitutes a part of the lysine binding pocket, and two C-terminal motifs that form a pseudoknot (Dillon et al., 2005; Joshi et al., 2008). A fully functional PRC2 requires the non-catalytic subunits SU(Z)12 ad ESC (Pasini et

al., 2004; Ketel et al., 2005; Nekrasov et al., 2005). The domain organization of E(Z) exhibits several conserved domains (Joshi et al., 2008; O'Meara and Simon, 2012). The SET domain that confer the catalytic function (Müller et al., 2002), an N-terminal ESC/EED interacting domain (Jones et al., 1998; Tie et al., 1998; Han et al., 2007), a conserved Domain-I shown to interact with Polycomb like (PCL) (O'Connell, 2001), Domain-II required for binding to the SU(Z)12 partner subunit (Ketel et al., 2005), and a CXC domain, which augments E(Z) mediated enzyme function (Ketel et al., 2005) [Fig. 3].

Different SET domain proteins have preferential capacities to mono, di or tri-methylate lysine residues (O'Meara and Simon, 2012). For example, SET7/9 mono-methylates lysine residues in the consenus [R/K] [S/T] [K] motifs of histone and non-histone proteins (Chuikov et al., 2004; Egorova et al., 2010). In contrast, G9a is a H3K9 dimethylase that lacks the inherent ability to trimethylate, but a single amino acid substitution in its active site confers ability to trimethylate histone (Wu et al., 2010). G9a recognize the ARKS motif similar to that recognized by PRC2, and interestingly, auto-methylates in a similar consensus motif within itself (Chin et al., 2007). Similar to G9a, the active site configuration of EZH2 is critical to provide substrate specificity (O'Meara and Simon, 2012). The Y641 residue in EZH2 is a critical determinant to specify the enzyme's ability to perform the mono, di or tri-methylation steps [discussed in section 1.8.1].

Figure 3: Domain organization of core PRC2 subunits in flies

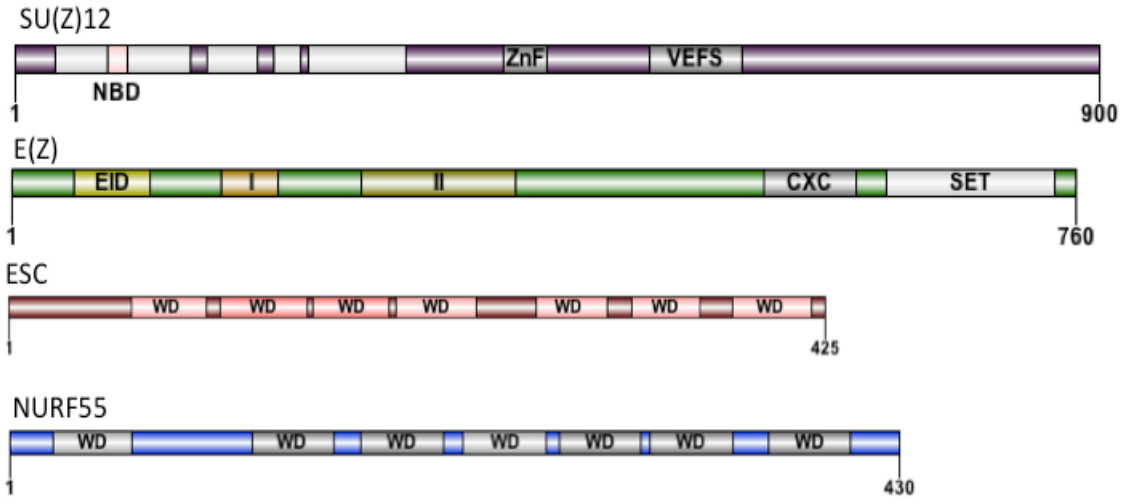
PRC2 consists of four core subunits E(Z), SU(Z)12, Esc and NURF55.

SU(Z)12 contains an N-terminal domain with scattered stretches of conserved residues interspersed with regions of less conservation, a C2H2 zinc finger [ZnF] domain and a C-terminal VEFS domain. The N-terminal domain of SU(Z)12 harbors the NURF55 binding element [NBE] in flies.

E(Z), the catalytic subunit of PRC2 has a C-terminal SET and CXC domain, domains I and II, and an N-terminal ESC interacting domain (EID).

NURF55 and ESC belong to WD repeat family of proteins that fold into a beta-propeller structure.


SU(Z)12
NURF
55
SET
E(Z)
ESC


SU(Z)12
ZnF
VEFS
NBD
1
900
E(Z)
EID
I
II
CXC
SET
1
760
ESC
WD
WD
WD
WD
WD
WD
WD
1
425
NURF55
WD
WD
WD
WD
WD
WD
WD
1
430

1.5.2 ESC/EED

ESC/EED is a stoichiometric core subunit of PRC2 (Müller et al., 2002; Ketel et al., 2005; Nekrasov et al., 2005). ESC is a critical non-catalytic subunit shown to allosterically boost E(Z)-mediated H3K27 trimethylation (Ketel et al., 2005; Margueron et al., 2009). PRC2 lacking ESC shows significant reduction in HMTase function (Ketel et al., 2005; Nekrasov et al., 2005). ESC/EED belongs to the evolutionarily conserved family of WD40 repeat proteins, which adopts the classical beta propeller fold, providing a scaffold for protein-protein interactions (Han et al., 2007; Margueron et al., 2009; Xu et al., 2010). Like many proteins with the beta propeller fold, EED has a central cavity with an aromatic cage for binding ligands or partners. The aromatic cage in EED preferentially binds H3K27me3 and other repressive chromatin marks like H3K9me3 and H3K20me3 (Margueron et al., 2009) and this stimulates the overall PRC2 enzyme activity. Thus, interaction of EED with H3K27me3 via its aromatic cage provide a feedback mechanism to sense and propagate the methyl mark during cell cycle progression (Margueron et al., 2009). Aside from the aromatic cage, ESC/EED features top and bottom surface loops, and an N-terminal tail (O'Meara and Simon, 2012). The top surface loops provide residues critical for HMTase function of PRC2 (Ketel et al., 2005) while the bottom loops bind the N-terminal region of E(Z)/EZH2 (Tie et al., 1998; Han et al., 2007). The N-terminal tail of ESC/EED binds the histone fold domain of H3 (Tie et al., 2007). EED is also the subunit of PRC2 that mediates the interaction of PRC2 to HDACs (van der Vlag

and Otte, 1999).

During mammalian development, EED is required in early embryonic stages in particular for the establishment of mesoderm and anterior-posterior axis (Faust et al., 1998). EED null animals show reduced levels of H3K27me3, emphasizing the requirement for EED for PRC2 function (Montgomery et al., 2005). In flies, ESC is required for proper development as embryos lacking ESC arrest at first larval instar, with all segments transformed towards eighth abdominal segment (Struhl, 1981).

1.5.3 SU(Z)12 /SUZ12

Among the three core subunits of PRC2, the function and contribution of SU(Z)12 to PRC2 is the least characterized of all. Like ESC/EED, SU(Z)12 is required for the histone methyltransferase activity of PRC2 (Cao and Zhang, 2004a; Pasini et al., 2004; Ketel et al., 2005; Nekrasov et al., 2005). SU(Z)12 has three conserved domains the N-terminal domain, a C2H2 zinc domain, and the 76 amino acid long VEFS domain (Birve et al., 2001; Ketel et al., 2005). The VEFS domain of SU(Z)12 is highly conserved from plants through animals and plays a critical role in the assembly of PRC2 complex by mediating interaction with E(Z) (Ketel et al., 2005). The VEFS domain also has critical residues that potentiate E(Z) mediated H3K27 methylation, possibly by allosteric activation. Mutations of these particular VEFS residues have no impact on the integrity of the PRC2 complex (Ketel et al., 2005). This suggests two separate roles for the VEFS domain in PRC2 function, i) provide binding interface to interact with E(Z),

and ii) provide critical residues to boost enzyme function. Identification of individual residues in the VEFS domain that impact PRC2 function either by disrupting the enzyme function or assembly will be beneficial to the goal of exploiting SUZ12 for therapeutic purposes.

The second conserved domain of SU(Z)12, the C2H2 zinc finger is a non-canonical, non-DNA binding zinc finger domain with an unknown molecular function (Birve et al., 2001). Mutation in certain conserved residues of this domain did not alter the properties of PRC2 *in vitro* (Ketel et al., 2005). Functional analysis of this domain is a major subject of Chapter 2 of this thesis. The N-terminal domain of SU(Z)12 consists of conserved scattered domains with a lower degree of conservation in plants but highly conserved between flies and mammals. The N-terminal domain harbors the NURF55 binding epitope (NBE), which may help connect SU(Z)12 and NURF55 in the intact PRC2 complex (Furuyama et al., 2006; Schmitges et al., 2011). The N-terminal region of SUZ12 can bind ancillary subunits of PRC2 like JARID2 (Peng et al., 2009). This region also possess the only identified lysine residue in SUZ12 that is subject to SUMO modification, a modification that has no known functional consequence (Riising et al., 2008). The exact role and contributions of this domain towards PRC2 needs further investigation.

In flies, lack of SU(Z)12 results in larval lethality, while hypomorphs die as pharate adults (Birve et al., 2001). Parallel to this observation, SUZ12 knock out mice are embryonic lethal and die with defects in endoderm formation (Pasini et

al., 2004).

1.5.4 NURF55/ RbAp46/48

NURF55 is an evolutionary conserved stoichiometric, non-catalytic core subunit of PRC2, also found in many other chromatin remodeling complexes like Histone acetyl transferase 1 (HAT-1), histone deacetylase complexes, ATP-dependent chromatin remodeling complexes NURF and NuRD, and Chromatin Assembly Factor 1 (CAF-1) (Martínez-Balbás et al., 1998; Pasini et al., 2004; Furuyama et al., 2006). Similar to its mammalian homologues, Retinoblastoma associated proteins (RbAp46/48), NURF55 can also form complexes with the retinoblastoma protein in *Drosophila* (Lewis et al., 2004; Chen et al., 2008). Like ESC, NURF55 belongs to the WD40 repeat family of proteins with the signature beta propeller structure commonly used for interaction with other proteins. Aside from the beta-propellers, NURF55 has a distinct alpha helix in the N-terminal, and long connecting loops between the propellers (Song et al., 2008). Together, they provide the binding pocket for histone 4, making NURF55 structurally unique from other WD40 family proteins that use the central pocket for protein-protein interactions (Song et al., 2008). There is a high level of similarity in the solved crystal structures of NURF55 (Song et al., 2008) and RbAp46 (Murzina et al., 2008), underscoring the conservation of NURF55 function. NURF55 can associate with the histone H3 tail [1-15] residues through the top surface of the beta propeller, also called the canonical binding site [c-site] (Furuyama et al.,

2006; Song et al., 2008; Schmitges et al., 2011). NURF55 can also bind helix 1 of H4 or SU(Z)12 through its side pocket [S/H site] (Schmitges et al., 2011). Taken together, these data suggest that when NURF55 is assembled into PRC2, it precludes the binding of H4 (Furuyama et al., 2006; Schmitges et al., 2011). These findings also suggest that NURF55 can bind the H3 tail and SU(Z)12 at the same time. NURF55 binds to the N-terminus of SU(Z)12 (Furuyama et al., 2006; Schmitges et al., 2011), and together they form the minimal nucleosome binding unit within PRC2 (Nekrasov et al., 2005).

In *Drosophila*, loss of NURF55 is associated with concomitant loss of H3K27me3, its function critical for normal development, cell viability and proliferation (Furuyama, 2006; Anderson et al., 2011). However, subsequent studies refute this and the exact role of NURF55 *in vivo* is still uncertain (Wen et al., 2012). Fly NURF55 nulls are lethal and die before pupariation during the second instar stage (Wen et al., 2012). However, NURF55 minimally contributes to the enzyme function of PRC2 *in vitro* (Ketel et al., 2005; Nekrasov et al., 2005). In spite of the recent advances made, the molecular mechanism by which NURF55 contributes to chromatin regulation is still unclear. In some chromatin complexes, it may help interface with nucleosome, but its not clear whether this applies to PRC2.

1.6 Binding partners of PRC2

PRC2 is composed of a distinct core complex, as described in the

previous section. However, we can expect a wide array of modulatory components that help PRC2 achieve its functional output in response to various cellular cues. Indeed, PRC2 has several ancillary non-stoichiometric subunits that modulate its enzyme activity as well help recruit it to target genes. In the following subsections, I will describe some of the known binding partners and our current understanding of their contributions to PRC2 function.

1.6.1 Polycomb like

PCL binds core PRC2 components in flies and mammals

In flies, mutations in Polycomb like [PCL] display homeotic phenotypes similar to that of the PcG protein PC (Duncan, 1982). PCL has three mammalian homologs, PCL1 [PHF1], PCL2 [MTF2] and PCL3 [PHF19] (Margueron and Reinberg, 2011). In mammals, reduced levels of PCL2 results in less severe defects of the homeotic phenotype affecting gene expression primarily in the central nervous system and tissues of the mesoderm lineage (Walker et al., 2010). Evidence for PCL as a binding partner for PRC2 is based on observations that show PCL and SU(Z)12 co-localize at PREs of *Ubx* and *Abd-B* in flies (Nekrasov et al., 2007). PCL also co-purifies with E(Z) and core PRC2 subunits in sub-stoichiometric levels (Nekrasov et al., 2007). Similarly, mammalian homologues of PCL, PCL1 [PHF1] co-purified with PRC2 (Cao et al., 2008), and PCL 2 [MTF2] co-occupied several but not all PRC2 target sites and sites of H3K27me3 investigated in mouse ES cells (Walker et al., 2010). PCL2 can also interact with SU(Z)12 in mouse ES cells (Walker et al., 2010). PCL3 [PHF19],

was recently characterized as a PRC2 binding partner and co-localizes with SU(Z)12 in CpG islands (Hunkapiller et al., 2012).

PCL can modulate enzyme function and targeting of PRC2

There is evidence that PCL contributes to the function of PRC2 in two ways, i) by modulating enzyme function (Walker et al., 2010) and ii) by help recruiting PRC2 to target chromatin (Nekrasov et al., 2007; Savla et al., 2008). In *Drosophila,* PCL null mutants show decreased levels of H3K27me3 while maintaining stable accumulation of PRC2 subunits (Nekrasov et al., 2007; Hunkapiller et al., 2012). Concomitant with this observation, cells lacking PCL in imaginal discs had reduced levels of H3K27me3, albeit not as severe as in clones depleted of E(Z) and SU(Z)12 (Nekrasov et al., 2007). Similarly, reduced levels of mammalian PCL1 [PHF1], resulted in global reduction of H3K27me3 levels (Sarma et al.). Mammalian PCL1 [PHF1] can co-purify with PRC2 and enhance H3K27me3 activity by favoring a higher Vmax and lower Km for the histone methyltransferase reaction (Cao et al., 2008).

The role of PCL and its homologues in targeting PRC2 stems from studies in flies, where PCL mutants showed reduced binding of SU(Z)12 to a *Hox* gene PRE in wing imaginal discs (Nekrasov et al., 2007; Savla et al., 2008). In mammals, PCL2 recruits PRC2 to target genes in ES cells and sites of X chromosome inactivation (Walker et al., 2010; Casanova et al., 2011). Taken together, the decreased levels of H3K27me3 observed in mutants or cells lacking PCL can be attributed to a defect in targeting of PRC2 to genomic sites.

1.6.2 JARID2

JARID2, the founding member of the JmjC domain proteins, belongs to a larger family of demethylases called Jumonji proteins (Landeira et al., 2010) . Unlike other members of the JmjC family of proteins, JARID2 does not share lysine demethylase activity due to lack of conservation of critical co-factor binding sites within its active site (Landeira et al., 2010). JARID2 co-purifies with PRC2 core components in flies and mammals thereby establishing an undisputed role as a PRC2 binding partner (Peng et al., 2009; Shen et al., 2009; Landeira et al., 2010; Li et al., 2010). However, its exact role in the functional output of PRC2 is a conundrum.

Genome-wide studies in flies and ES cells show that PRC2 and JARID2 co-occupy PRC2 targets (Peng et al., 2009; Shen et al., 2009; Landeira et al., 2010; Li et al., 2010). However, the role of JARID2 in modulating PRC2 enzyme function is still under debate. In ES cells, there is one report that suggests that JARID2 is required to achieve high levels of H3K27me3 (Li et al., 2010) while two other reports suggest an inhibitory role for JARID2 (Peng et al., 2009; Shen et al., 2009). Adding to the complexity is a recent study in flies, showing that JARID2 is not required for targeting PRC2 to genomic targets. Although JARID2 co-localized with SU(Z)12 genome wide, upon loss of JARID2 in mutant flies, SU(Z)12 and H3K27me3 levels were not altered on polytene chromosomes compared to wild-type controls (Herz et al., 2012).

Though a context -dependent modulatory role for JARID2 in PRC2 readout

can be assigned, as lack of JARID2 showed a slight increase (Peng et al., 2009; Shen et al., 2009) as well as decrease in H3K27me3 levels (Li et al., 2010). However, the complexity of JARID2 action warrants thorough analysis of its function in different cell types and contexts. Possibly, in flies with defined PRE sequences, JARID2 is dispensable for targeting PRC2. Also, JARID2 with its DNA binding domain may have evolved as a recruiter of PRC2 in mammals where PREs are not well defined at the DNA sequence level. Another possibility is that JARID2 is a part of larger chromatin machinery with PRC2 and loss of JARID2 can be compensated by one or more modulatory proteins in the context of PRC2 function. Evidence for this stems from the observation that JARID2 can interact with known ancillary subunits of PRC2 like PCL2 [MTF2] and fly homolog of AEBP2, JING [discussed in the following section].

1.6.3 AEBP2/JING

AEBP2, a Gli-Kruppel Cys2-His2 zinc finger protein, can bind PRC2 components both *in vivo* and *in vitro* as well as boost PRC2 enzyme function *in vitro* (Cao and Zhang, 2004b). AEBP2 is critical for development as mice homozygous for AEBP2 are embryonic lethal (Kim et al., 2009). Genome wide studies identified the AEBP2 DNA binding motif, CTT(N)15-23cagGCC, very close to the known genomic targets of PRC2 (Kim et al., 2009). Consistent with this observation, SU(Z)12 co-occupied most AEBP2 binding sites. Interestingly, the fly homolog of AEBP2, JING, was recently shown to co-purify with JARID2 (Herz et al., 2012).

Based on this observation, together JING and JARID2 may modulate PRC2 function. Possibly, PRC2 exists in transient complexes with JARID2 and JING. A transient complex would explain why reduced levels of JARID2 did not show any significant effect on the recruitment of PRC2 [discussed in the previous section]. JING could possibly compensate for the loss of function of JARID2 and target PRC2 to chromatin sites of action. Hence, reduced levels of JARID2 in flies may not have direct effects on the recruitment of PRC2.

1.6.4 Association with DNMTs and HDACs

In addition to the proteins described above, PRC2 is shown to localize and interact with DNA methyltransferases [DNMTs] and histone deacetylases [HDACs]. PRC2 subunits EZH2 and EED can directly interact with DNMT1, DNMT3A and DNMT3B, to maintain CpG levels (Viré et al., 2005). It is unknown whether DNMTs are recruited through direct contact with EZH2 or indirectly by other factors. However, the recruitment of EZH2 to target genomic sites does not require DNMTs (Simon and Lange, 2008). Subsequent studies in colon cancer showed that genes marked for hypersilencing by DNA methylation are frequently occupied by EZH2 and marked by H3K27me3 (Schlesinger et al., 2007). However, the exact contribution of PRC2 to DNA methylation is an area of active investigation with a lot of interest in developing drugs for combined epigenetic therapy (Kelly et al., 2010).

EZH2 also interacts synergistically with HDACs (Simon and Lange, 2008).

The binding of HDACs to PRC2 is mediated via the subunit EED (van der Vlag and Otte, 1999). The H3K27 residue is subject to acetylation as well as methylation. Interaction with HDACs is a key step in deacetylating the K27 residue to prime it for methylation by PRC2 (Tie et al., 2009). The histone acetyltransferase [HAT] CREB-binding protein [CBP] deposits the acetyl mark on H3K27 (Tie et al., 2009). The interaction of PRC2 with HDAC has immense clinical significance as HDAC inhibitors are in clinical trials for several different types of cancer. The only known indirect inhibitor of PRC2, DZNep lack specificity as its is a known S-adenosylhomocysteine (AdoHcy) hydrolase inhibitor, which indirectly inhibit S-adenosyl-methionine (AdoMet) dependent reactions (Miranda et al., 2009). Many methyltransferases are known to carry out this reaction, questioning the specificity of DZNep. Also, only 140 out 751 target genes activated by DZ-Nep were PRC2 targets (Tan et al., 2007). This underscores the importance of developing therapeutics that specifically take out PRC2 function either alone or in combination with other epigenetic targets. For example, LBH589, an HDAC inhibitor is reported to decrease the HMTase function of EZH2 in acute leukemia (Fiskus et al., 2006). HDAC inhibitors and DNMT inhibitors alone and in combination are in clinical trials (Kelly et al., 2010) and are great targets for combined epigenetic therapy.

1.7 PcG targeting to chromatin

During the last decade several models have been proposed for the

recruitment of PRC2 and PRC1 to target sites in the genome. However, the exact mechanism or mechanisms by which PRC1 and PRC2 are targeted to chromatin remains under study. Some proposed mechanisms and recent advances are discussed below.

1.7.1 Polycomb response elements

In *Drosophila*, Polycomb Response Elements (PRE) are regions in chromatin depleted of nucleosomes that serve as a scaffold or landing pad for several protein-DNA and protein-protein interactions involving PcG proteins and their recruiters (Mishra et al., 2001; Müller and Kassis, 2006). PREs were originally identified as *cis*-regulatory sequences required for PcG silencing in *Drosophila* (Simon et al., 1993). The core subunits of PRC2 have no known classical DNA binding domains. Therefore, these complexes require the help of trans-acting factors to attract them to PREs of target genes (Simon and Kingston, 2009).

One of the original models [Fig.4] proposed that PRC2 is targeted to a PRE by DNA binding factors of the Pho-RC (Müller and Kassis, 2006; Simon and Kingston, 2009) as well as transcription factors such as GAGA factor (Strutt et al., 1997; Schwendemann and Lehmann, 2002; Mulholland et al., 2003), pipsqueak (Huang et al., 2002; Schwendemann and Lehmann, 2002), and zeste (Mulholland et al., 2003). In this model, once PRC2 is recruited to the PRE, it deposits the H3K27me3 mark, which in turn can serve as a docking site for

PRC1 via the chromodomain of PC [Fig.4]. Consistent with this, loss of PRC2 often results in loss of PRC1 at target sites (Cao et al., 2002). Also, PHO binding PREs were found approximately 1 kb upstream of the transcription start site in genome-wide studies (Oktaba et al., 2008), providing an attractive mechanism to target PcG proteins to PREs that are located proximal to the promoter of target genes. However, this model has been called to question by several reports pointing towards PRC2-independent recruitment of PRC1(Schoeftner et al., 2006; Pasini et al., 2007; Vincenz and Kerppola, 2008; Yu et al., 2012), and models favoring PRE-independent recruitment of PRC2 to genomic sites in flies and mammals (Schoeftner et al., 2006; Herz et al., 2012). Though a substantial number of PREs show co-localization of PHO-RC, PRC1 and PRC2 (approximately 45%), most PREs do not, suggesting alternate mechanisms of targeting PcG to chromatin (Oktaba et al., 2008).

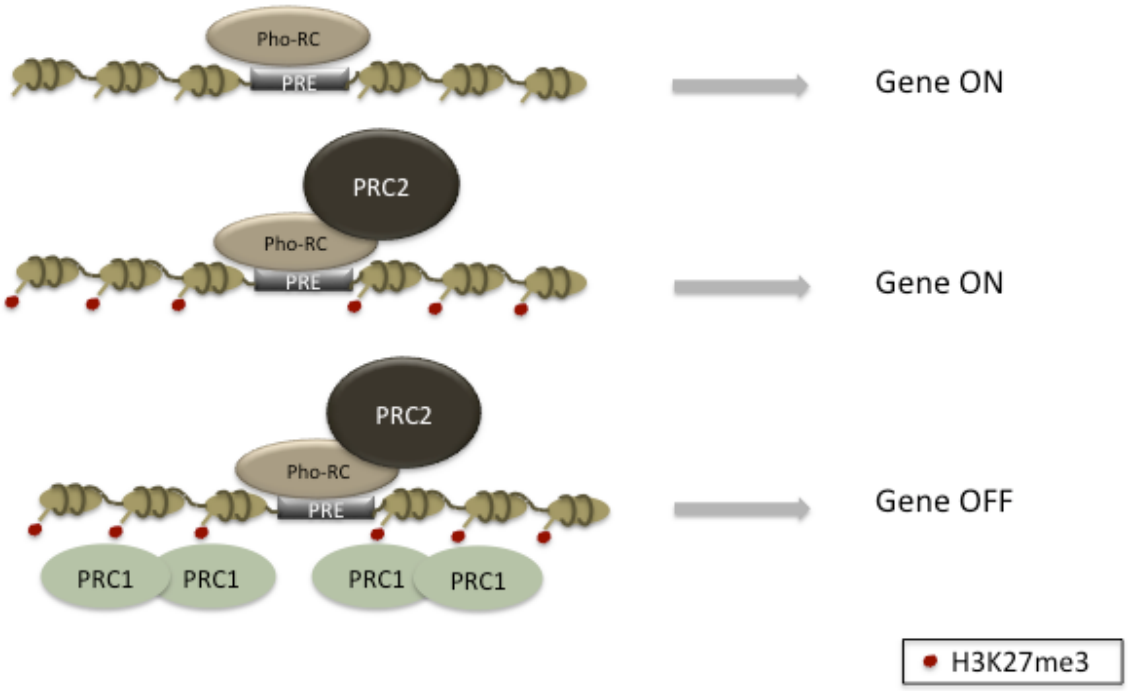
Despite major advances in the understanding of PRE and its significance in *Drosophila,* PREs are not well defined in mammals. Polycomb binding sites were reported for the HOX-D cluster that bound BMI1, YYI and EED (Woo et al., 2010). Yin Yang 1(YY1), the mammalian ortholog of PHO, is implicated in recruiting PcG proteins to target loci. YY1 was shown to silence a PcG reporter in *Drosophila*, very similar to PHO, via its 25 amino acid long REPO domain (Atchison et al., 2003; Wilkinson et al., 2006). However, unlike its fly counterpart PHO, YY1 is not a major recruiter of PcG proteins in mammals. GATA-1 binding to PRC2 is important for silencing of a subset of target genes in terminal erythroid

maturation (Yu et al., 2009). In mammals, CpG islands attract mammalian PRC2 suggesting that CpG islands might provide a potential platform for the recruitment of several chromatin remodelers to silence transcription (Ku et al., 2008; Mendenhall et al., 2010). There are some indications that mammalian PRE harbor a GAGA motif (Sing et al., 2009; Woo et al., 2010). However, in mammals, the exact nature of these DNA elements is still unclear.

In mammals lacking a defined PRE, long non-coding RNAs are emerging as recruiters of PcG complexes to chromatin sites of action. With the identification of GA rich motifs that recruit long non-coding RNA like HOTAIR [discussed in section 1.7.2], RNA molecules likely recognize these motifs along with transcription factors, and help recruit PcG proteins to PREs (Chu et al., 2011). The precise mechanism of PcG targeting warrants more mechanistic studies in the context of whole genome as well as individual PcG target genes.

Figure 4: The hierarchal model for the recruitment of PcG complexes to target genes in *Drosophila*.

In this model, Pho-RC binds to the PRE of target gene via the DNA binding activity of the subunit PHO. PHO can interact with E(Z) and ESC (Wang et al., 2004b) in PRC2 and recruit it to chromatin sites of action. The recruitment of PRC2 is followed by trimethylation of H3K27 residues by E(Z). The H3K27 trimethyl mark is sensed by the chromodomain of PC subunit in PRC1. PRC1 can inhibit nucleosome remodeling and compact chromatin (Shao et al., 1999; Francis et al., 2001; 2004) resulting in gene silencing. The red spots represent the trimethyl mark on H3K27 residue. However, it is unclear whether a similar hierarchy exists in mammals.


Pho-RC
PRE
Gene ON
PRC2
Pho-RC
PRE
Gene ON
PRC2
Pho-RC
PRE
Gene OFF
PRC1
PRC1
PRC1
PRC1
H3K27me3

1.7.2 RNA dependent recruitment of PcG complexes

In mammals, non-coding RNAs (ncRNA) may have evolved as a major component of the PcG targeting machinery. Emerging data has drawn parallels between transcription factors and ncRNA. Similar to transcription factors, ncRNA bind to selective regions in the genome like promoters and enhancers in a DNA sequence specific manner (Chu et al., 2011). The long ncRNA HOTAIR is known to recruit PRC2 to target chromatin (Rinn et al., 2007; Gupta et al., 2010). HOTAIR has recently been shown to bind a GA rich polypurine motif (Chu et al., 2011). Consistent with this observation, genome wide studies identified a significant overlap between sites of PRC2 and HOTAIR occupancy, substantiating the involvement of HOTAIR in PRC2 mediated gene silencing (Chu et al., 2011). In the same study, the authors showed that HOTAIR can bind target sites independent of the PRC2 subunit EZH2, validating the role of HOTAIR as a potential recruiter of PRC2 to target genes, not just providing a scaffold for chromatin machinery as reported previously (Tsai et al., 2010). Similar to mammals, a ncRNA in plants, called COLDAIR can directly bind PRC2 orthologues (Heo and Sung, 2011). The repertoire of ncRNA implicated in developmental gene silencing and disease progression is increasing and mechanisms that regulate the orchestrated co-ordination of chromatin regulators and ncRNA need more study.

1.7.3 Direct recruitment of PRC1 to target genes: is H3K27me3 required or dispensable?

Recent studies in *Drosophila* suggest that PRC2 and the H3K27me3 marks are dispensable for the recruitment of PRC1 to target genomic loci (Herz et al., 2012). A temperature sensitive allele of E(Z), E(Z) ^{61} when shifted to the restrictive temperature (29^0C) results in loss of E(Z) and a concomitant loss of SU(Z)12 binding and H3K27me3 levels in known PRC2 target loci. When polytene chromosomes were analyzed for the localization of PRC1 components after shifting the larvae to 29^0C, lack of PRC2 did not result in detectable changes in the localization of the PRC1 component PC (Herz et al., 2012). This raises the question about the exact role of H3K27me3 mark in PcG silencing. One possibility is that once established, PC binding is stable at target loci and it takes more than a mere shift in temperature for 24 hours to make it drop off the chromatin. However, this study is in agreement with an emerging view that questions the hierarchical model of sequential PRC2, H3K27me3 and PRC1 recruitment. Prior evidence that PHO can bind PRC1 directly also supports PRC2-independent recruitment of PRC1 (Mohd-Sarip et al., 2006).

Furthermore, in embryonic stem cells, PRC1 is recruited to X chromosome by *Xist* RNA in a PRC2-independent manner (Schoeftner et al., 2006). The fact that PRC1 type complexes can directly interact with several transcription factors (Gearhart et al., 2006; Yu et al., 2012) provides an alternate model for the recruitment of PRC1, independent of PRC2 and H3K27me3. In murine

thymocytes, PRC1 directly interacts with transcription factor RUNX1 and is recruited to PcG targets independent of PRC2 (Yu et al., 2012). Also, in cultured *Drosophila* S2 cells and wing discs of flies, knockdown of E(Z) does not affect the recruitment of PRC1 to PREs (Papp and Müller, 2006). The recently identified RYBP-PRC1, consisting of RYBP, RING1B, and MEL-18/BMI-1 show considerable overlap with PRC2 target genes. However, genome wide studies in EED null ES cells, showed the retention of RYBP-PRC1 despite of lack of PRC2 components and H3K27me3 (Tavares et al., 2012). Taken together, these data suggest the existence of PRC1 recruiters other than the trimethyl mark on H3K27.

1.8 PRC2 and cancer

Many recent studies correlated increase in PRC2 and associated H3K27me3 with invasive and metastatic forms of cancer. EZH2 levels are commonly elevated in aggressive forms of solid tumors (Simon and Lange, 2008). The prevailing view on the role of PRC2 in cancer directly links it to hyper-silencing tumor suppressors and critical cell cycle regulatory genes (Simon and Lange, 2008). The cohort of studies, including a few discussed in the following section, show EZH2 overexpression in metastatic forms of cancer and suggest that EZH2 is a proto-oncogene (Varambally et al., 2002; Kleer et al., 2003; Ding and Kleer, 2006). However, in the wake of recent studies showing loss of function mutations in leukemia and myeloid disorders (Ntziachristos et al., 2012; Score et

al., 2012), core PRC2 subunits and its ancillary binding partners can also behave as bona-fide tumor suppressor genes. A few advances are discussed below.

1.8.1 PRC2 as an oncogene

Initial studies implicating PRC2 in cancer progression showed that EZH2 levels are elevated in tumor samples as compared to normal tissues (Varambally et al., 2002; Kleer et al., 2003). In differentiated tissues, EZH2 levels are very low. However, EZH2 levels are high in cancers of many tissues including prostate, breast, colon and bladder. Increased expression of EZH2 is associated with increased levels of H3K27me3 levels (O'Meara and Simon, 2012). PRC2-mediated cancer progression associated with increased H3K27me3 is mostly the result of gain of function mutations in EZH2 [Y641] and [A677] (Morin et al., 2010; Sneeringer et al., 2010), overexpression of EZH2 (Varambally et al., 2002; Kleer et al., 2003; Ding and Kleer, 2006), increased expression of modulatory subunits like PHF19 (Wang et al., 2004b), decreased expression of negative regulators of EZH2 like miRNA-101 (Varambally et al., 2008; Cao et al., 2010) or decreased expression of the demethylase UTX as a result of inactivating mutations (van Haaften et al., 2009; Gui et al., 2011). The initial implications for PRC2 in cancer came from studies showing overexpression of PRC2 subunit EZH2 in metastatic forms of prostate and breast cancer (Varambally et al., 2002; Kleer et al., 2003). Overexpression of EZH2 can be correlated with the aggressive nature of cancer, and is predictive of poor prognosis. Subsequent studies identified an over

abundance of PRC2 subunits in different kinds of cancer including cancer of skin, colon and lung to name a few (Simon and Lange, 2008; Mills, 2010). Increased levels of EZH2 can also be correlated with increase in hyper-silencing of key tumor suppressors by DNA methylation [discussed in section 1.6.4].

Recently, mutations in the Y641 residue of EZH2 were identified in large B-cell and follicular lymphoma (Morin et al., 2010). This mutation was originally thought to result in loss of function of the PRC2 enzyme. However, mechanistic studies assigned a gain of function property that alters the ability of PRC2 to tri-methylate but not mono or di-methylate H3K27 (Sneeringer et al., 2010; O'Meara and Simon, 2012). Instead, these mutant proteins work together with wild-type EZH2 to use premade H3K27me1 and H3K27me2, causing a significant boost in tri-methyl H3K27 levels (Sneeringer et al., 2010; O'Meara and Simon, 2012; Yap et al.). Mechanistically, this residue compares to missense mutations in the corresponding active site Y655 residue of E(Z) in flies that behaves like a gain of function mutant in trans with wild type E(Z) (O'Meara and Simon, 2012). Recently, gain of function mutations were identified in the A677 residue of EZH2, also shown to increase levels of H3K27me3 (McCabe et al., 2012). However, the consequence of this mutation was slightly different from mutations in Y641 in that it retained the substrate preference for unmethylated, mono and di-methylated H3K27 (McCabe et al., 2012). Aside from providing mechanistic insights, these studies underscore the relevance of translational approaches to understand PRC2 mediated cancer progression. In addition, elevated levels of SUZ12, EED

and RbAp48 were observed in colon and breast cancers (Kirmizis et al., 2003). Oncogenic roles of SUZ12 is further substantiated by the finding that SUZ12 is up regulated in tumors derived from germinal cells, melanomas, pituitary and parathyroid adenoma and skin carcinoma (Martín-Pérez et al., 2010). Also, the 17q11.2 locus that contains *SUZ12*, is frequently amplified in mantle cell lymphoma (Martín-Pérez et al., 2010).

1.8.2 PRC2 as a tumor suppressor

In solid tumors, EZH2 is commonly overexpressed with concomitant increase in H3K27me3 levels. However, some malignancies of the hematopoietic lineage mostly result from inactivating mutations of PRC2 components. One of the first reports identifying a tumor suppressor function for EZH2 identified several deletions, mutations and frame-shifts of EZH2 in cases of myelodysplastic syndromes (Nikoloski et al., 2010). Missense mutations identified in this study were mostly located in the conserved domain II, required for binding to SUZ12 and CXC-SET domain required for HMTase function (Nikoloski et al., 2010). T-cell acute lymphoblastic leukemia (T-ALL) is commonly associated with activating mutations in NOTCH1. A recent study (Ntziachristos et al., 2012), in an attempt to identify chromatin factors that cooperate with oncogenic NOTCH1 mutations in T-ALL, observed several loss of function mutations in PRC2 subunits EZH2 and SUZ12. The authors showed that loss of H3K27me3 was significantly reduced in the promoters of NOTCH1 target genes

suggesting a model where NOTCH1 and PRC2 are mutually exclusive in the promoters of NOTCH1 target genes. Therefore, any inactivating mutation in EZH2 or SUZ12 sets the stage for enhanced NOTCH1 signaling and associated uncontrolled cell proliferation. This effect is elevated in scenarios where oncogenic NOTCH1 mutations are found in conjunction with loss of function mutations in EZH2 and SUZ12 (See chapter 3 for more information and further progress).

These studies warrant extreme caution while designing therapeutic molecules as increase in H3K27me3 levels correlate with metastatic forms of cancer and loss of the H3K27me3 mark results in certain tumors of haematopoietic origin. To avoid secondary malignancies, it is critical to understand the underlying mechanisms in different types of cancer before the advent of any treatment regimen.

1.9 Demethylases that act upon H3K27me3.

For a long time, the identity of H3K27 demethylases remained a mystery. In a developing organism, cell fate decisions require dynamic changes in H3K27me3 levels to dictate the expression of differentiation-specific genes. This scenario is true for embryonic stem cells, which maintain most of their target genes to respond to either transcriptional activation or silencing depending on lineage commitment (Bernstein et al., 2006). This suggested the existence of a specialized tool to demethylate H3K27me3 on polycomb targets whose

expression is switched off upon differentiation. The identity of such specialized demethylases were revealed independently by four different groups (Agger et al., 2007; De Santa et al., 2007; Lan et al., 2007; Lee et al., 2007) as UTX and JMJD3, with highly conserved jumonji demethylase active site domains. These studies also showed that UTX and JMJD3 remove methyl groups from H3K27me3 and H3K27me2 but not H3K27me1. In response to differentiation-specific cues, UTX was recruited to *Hox* gene promoters with a concomitant decrease in the levels of H3K27me3 and PRC2 components (Agger et al., 2007; Lee et al., 2007). However, UTX occupancy was limited to a few members of the *Hox* gene cluster, suggesting the presence of alternate demethylases in controlling the expression of PRC2 targets (De Santa et al., 2007). Recent studies identified inactivating mutations of UTX that correlated with increase in H3K27me3 levels (van Haaften et al., 2009).

1.10 Thesis Summary

In the following chapters, I describe two pieces of work performed to understand the contributions of PRC2 subunits to the function of PRC2 holoenzyme.

Chapter 2 describes the detailed analysis of the highly conserved domains of *Drosophila* SU(Z)12. We provide insights into the function of the conserved zinc finger domain of SU(Z)12. Although dispensable for *in vitro* enzyme function, we establish a requirement for zinc finger *in vivo*, which appears to be needed for

optimal binding of PRC2 to chromatin targets. We also performed mutational analysis of the N-terminal domain and extended the functional analysis of the VEFS domain with focus on key conserved residues commonly mutated in malignancies of haematopoietic origin.

Chapter 3 highlights some key findings on Cyclin dependent kinase [CDK] mediated regulation of EZH2 function and my contributions to define the mechanism of PRC2 modulation in response to this CDK mediated phosphorylation.

Chapter 2

Domain and functional analysis of *Drosophila* SU(Z)12: A functional role for the enigmatic zinc finger.

2.1 Introduction

Epigenetic modifications work together with genetic mechanisms to regulate gene expression during growth and development in normal tissues. In disease states like metastatic forms of cancer, one or more of these mechanisms often go awry. Both genetic and epigenetic markers have prognostic value. However, unlike genetic changes epigenetic modifications of histones and DNA are reversible, making them attractive targets for drug discovery and therapeutics. There is great interest in developing novel inhibitors that target specific epigenetic modifications with the goal of increasing specificity and reducing toxicity (Kelly et al., 2010).

Many recent reports have linked PRC2 and H3K27me3 deregulation to many types of cancer. PRC2-mediated cancer progression is associated with increased H3K27me3 (O'Meara and Simon, 2012), mostly as a result of gain of function mutations in EZH2 [Y641] and [A677] (Morin et al., 2010; Sneeringer et al., 2010; McCabe et al., 2012), overexpression of EZH2 (Simon and Lange, 2008), increased expression of modulatory subunits like PHF19 (Wang et al., 2004b), decreased expression of negative regulators of EZH2 like miRNA 101 (Varambally et al., 2008; Cao et al., 2010) or decreased function of the

H3K27me3 demethylase UTX as a result of inactivating mutations (van Haaften et al., 2009; Gui et al., 2011) . Oncogenic roles of SUZ12 are based on the finding that SUZ12 is up regulated in tumors derived from germinal cells, melanomas, pituitary and parathyroid adenoma and skin carcinoma (Martín-Pérez et al., 2010). Also, the 17q11.2 locus which houses *SUZ12*, is frequently amplified in mantle cell lymphoma (Martín-Pérez et al., 2010). Taken together these studies suggest that EZH2 and SUZ12 are proto-oncogenes, favoring a model where increased H3K27me3 results in the silencing of essential tumor suppressor genes.

In contrast, two recent studies identified several somatic loss of function missense mutations in PRC2 subunits EZH2 and the VEFS domain of SUZ12 in T -cell acute leukemia [T-ALL] (Ntziachristos et al., 2012), and myeloproliferative neoplasms (Score et al., 2012). Ntziachristos et al., 2012, also identified frequent deletions of *SUZ12,* suggesting a previously unknown tumor suppressor function for SUZ12 in malignancies of hematopoietic origin. More recent studies in mice (Simon et al., 2012) show loss of EZH2 and reduction in H3K27me3 in T-ALL. PRC2 mutations were also found in high frequencies in pediatric cases of T- ALL (Zhang et al., 2012). However, the exact mechanism by which these mutations cause a failure in PRC2 function is still unknown. With data implicating PRC2 gain of function in many solid tumors and lymphomas and PRC2 loss of function in certain types of leukemia, any therapeutic intervention that targets reduction in H3K27me3 could lead to secondary malignancies of hematopoietic origin. This

warrants caution while designing the rationale to develop novel drugs that target PRC2 in cancer.

To date, there are no drugs in clinical trials that target PRC2. The only relevant inhibitor currently in preclinical trials is the inhibitor of S-Adenosylhomocysteine hydrolase called 3 Dezaneplanocin A [DZnep] (Kelly et al., 2010). DZNep lacks particular specificity for PRC2 as its is a known S-adenosylhomocysteine (AdoHcy) hydrolase inhibitor, which indirectly inhibits all S-adenosyl-methionine (AdoMet) dependent reactions (Miranda et al., 2009). This reaction is common to all the SET domain proteins with histone methyltransferase activity. DZnep lacks specificity and acts as an inhibitor of global histone methylation as only 140 out 751 genes activated by DZnep were EZH2 target genes (Tan et al., 2007). Considering that EZH2 shares the highly conserved SET domain with over 20 other human proteins, it will be challenging to develop an inhibitor that specifically targets the catalytic activity of EZH2. A key feature of PRC2 is that EZH2 on its own cannot efficiently methylate H3K27 (Ketel et al., 2005; Nekrasov et al., 2005). It absolutely requires its non-catalytic subunits EED and SUZ12 for enzyme function, making the core non-catalytic subunits attractive candidates to develop novel inhibitors. SUZ12 is unique in that it does not appear to be a member of a larger conserved family of proteins. The conserved domains in SU(Z)12 are shared only with its homologues in plants and mammals (Birve et al., 2001), making it a suitable candidate to develop potential inhibitors that can specifically disrupt PRC2 function either by itself or in

combination with other therapeutic agents. This makes it critical to gain insight into the structure and function of SUZ12 and the mechanism by which it potentiates PRC2 function. The only structural information currently available on SUZ12 is the crystal structure of a small peptide of *Drosophila* SU(Z)12 [residues 71-99] that includes a NURF55 binding epitope [NBE]/SU(Z)12 [77aa -93aa] that lies within the N-terminal domain of SU(Z)12 (Schmitges et al., 2011). Fly PRC2 is very similar to human PRC2 (Birve et al., 2001; Cao et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002) and thus provides an excellent model of conserved epigenetic machinery. In this study, we used *Drosophila* SUZ12 to gain insight into its contribution to PRC2 function with the goal of dissecting this machinery. To begin with, there is a paucity of SU(Z)12 alleles reported that are missense mutations in the conserved domains of SU(Z)12 that alter PRC2 function (Birve et al., 2001).

In this study, we took a systematic approach to study SU(Z)12 function by targeted disrupted of its conserved domains. *Drosophila* SU(Z)12 is highly similar to mammalian SUZ12 and has three conserved domains. These are, a highly conserved VEFS domain, a C2H2 zinc finger domain and the less conserved N-terminal domain, with short stretches of highly conserved blocks interspersed with less conserved stretches (Birve et al., 2001; Ketel et al., 2005). The VEFS domain of SU(Z)12 mediates binding to E(Z) as well as provides key inputs to boost the activity of PRC2 holoenzyme (Ketel et al., 2005). The function of the highly conserved zinc finger domain of SU(Z)12 is unknown. Here we show that

this SU(Z)12 zinc finger domain is important for PRC2 function *in vivo*, and provide evidence that this involves targeting PRC2 to genomic sites of action. We also provide evidence that the N-terminal domain has E(Z) binding determinants whose loss cause a modest defect in the HMTase function of PRC2. Finally, we define molecular mechanisms by which VEFS domain missense mutations implicated in myeloid leukemia disrupt PRC2 function.

2.2 Materials and Methods

2.2.1 Chromatin Immunoprecipitation (ChIP) and quantitative real-time PCR (qRT-PCR)

ChIP was performed based on a modified protocol from Upstate Biotechnology [Millipore Life Sciences] and as previously described (Ratnaparkhi et.al., 2008). In brief, *Drosophila* S2 cells were treated with 1% formaldehyde for one hour at 24^{0}C by gentle mixing in a nutator. Formaldehyde was removed by rinsing the cells in ice cold PBS twice, and cells were lysed in SDS lysis buffer with protease inhibitor cocktail (Roche). DNA was sheared to approximately 500 bp using Sonic Dismembrator 500 (Fischer Scientific) for 2 min total (cycles of 30 sec on 30 sec off) at 20% amplitude. 10% of the input was saved at -80^{o}C. The remaining extract was diluted 1:10 in ChIP dilution buffer and pre-cleared in 50ul of Protein-A agarose beads (Roche) for I hour at 4^{0}C. 30 ul of Anti-Flag M2 beads (Sigma), pre-washed twice in ChIP dilution buffer, was added to the pre-cleared extract. Immunoprecipitation was performed overnight at 4^{0}C, and the

immunoprecipitate was washed and eluted as per the instructions in the manufacturer's [Upstate] protocol. Input and immunoprecipitated samples were reverse cross-linked at 65^{o}C overnight and DNA purified by QIAquick PCR purification kit (Qiagen). Quantitative real time PCR was performed using Platinum SYBR green qPCR Supermix (Invitrogen, Life Technologies) to detect the amount of immunopreciptated DNA in each sample as described previously (Wang et al., 2010). Primer pairs were used to amplify regions in the *bxd* region of the *Hox* gene *Ubx* encompassing the major upstream PRE as described previously (Wang et al., 2004b; 2010).

2.2.2 Transient transfection in S2 cells

Drosophila S2 cells were split into 2 x 10^6 cells/ml and transfected with 2ug of pAc5.1 empty vector, pAc5.1 Flag- SU(Z)12-wt or pAc5.1 Flag-SU(Z)12-Znf Δ using Fugene HD-6 reagent (Roche) as per the manufacturer's instructions. Cells were harvested 3 days post transfection for western blot analysis and ChIP. Anti-Flag M5 antibody (1:5000) [Sigma] was used to detect expression of Flag-tagged transfected proteins.

2.2.3 Baculovirus constructs and site directed mutagenesis

Full length cDNA clones of E(Z), ESC, SU(Z)12, NURF55, FLAG-ESC and FLAG-E(Z) were inserted into pFastBac 1([Invitrogen] as previously described (Müller et al., 2002; Ketel et al., 2005). In-frame deletions of the SU(Z)12 zinc finger domain in pFastbac –SU(Z)12 parent vector [pFastBac-SU(Z)12 -ZnF Δ] were generated using a single primer PCR based strategy as described

previously (Makarova et al., 2000) followed by sequencing to confirm intact open reading frames. In-frame HA tagged N-terminal deletions of SU(Z)12 were generated using a tailed primer PCR strategy. Point mutations in the N-terminal domain, zinc finger and VEFS domain of SU(Z)12 were generated using the QuikChange site-directed mutagenesis kit [Agilent Technology] as per manufacturer's instructions.

2.2.4 Recombinant protein purification and detection

Recombinant protein complexes were purified from Sf-9 cells using Invitrogen's Bac to Bac system as previously described (Müller et al., 2002; Ketel et al., 2005). All purifications were performed in at least duplicates for all mutant and wild-type complexes analyzed in this study. Mutant PRC2 complexes were checked for assembly in parallel to wild-type controls by analyzing on 8%-10% SDS-PAGE, followed by staining in Coomasie Blue staining.

2.2.5 Embryo extracts and western blot analysis.

Embryo extracts were prepared as described previously (Jones et al., 1998){Jones:1998ui}. 0-12 hr embryos were dechorionated in freshly prepared 50% bleach for 2 minutes, washed extensively in embryo wash buffer [0.7% NaCl, 0.03% Triton –X-100], and homogenized in Buffer E [40 mM HEPES [pH 7.5], 350mM NaCl, 0.1% Tween, 10% glycerol, 100 ug/ul PMSF, 1mM benzamidine, 2 ug/ul aprotinin, 2 ug/ul leupeptin, 1ug/ul pepstatin A] using a Wheaton tight and loose homogenizer [Kimble Chase]. The homogenate was centrifuged for 30 minutes at 4^0C at 13000 rpm, collected in fresh tubes and

protein concentration determined by Bradford reagent [Biorad]. 50ug of the extract was used for analysis by10% SDS-PAGE. Anti-HA antibody [Cell Signaling] at 1:200 was used to detect the expression of HA tagged transgenic proteins. Anti-tubulin [1:1000] [Sigma] was used to determine protein loading.

2.2.6 Co-Immunoprecipitation

Immunoprecipitations were performed as previously described (Jones et al., 1998) with modifications. In brief, 50ul of Protein-A agarose beads per IP (Roche) were washed twice and the final slurry volume was brought to 50ul in IP buffer [10 mM HEPES (pH 7.5), 50 mM Nacl, 10% glycerol, 0.1%Triton-X-100, 1mM Dithiothreitol (DTT), 1uM $ZnSO_4$, 1mM phenylmethylsulfonyl fluoride (PMSF), 2 ug/ml leupeptin, 2 ug/ml aprotinin, 1ug/ml of pepstatin A]. Antibody-bead conjugates were produced by adding 15ul of crude anti E(z) serum [kind gift from Prof. Richard S. Jones, Southern Methodist University, TX] to 50 ul of pre-washed Protein-A beads [Roche] and the volume was brought up to 200 ul in IP buffer. The antibody bead mixture was incubated at 4^0C for one hour and washed 5 times in ice-cold IP buffer to remove unbound antibody. Embryo extracts were pre-cleared in 20 ul of Protein-A beads for one hour at 4^0C. 300 ug of pre-cleared extract was added to 50 ul of antibody Protein-A slurry and incubated overnight at 4^0C on a rotator. Bead precipitates were recovered by centrifugation at 2500 rpm at 4^0C and washed 5 times in two volumes of IP buffer using spin columns [Boca Scientific]. The precipitates were eluted in 30 ul of 2X SDS sample buffer. Precipitated proteins were analyzed on 10% SDS-PAGE and

transferred to nitrocellulose membrane. Anti-HA antibody [1:200] (Cell Signaling) was used for the detection of immunoprecipitated proteins.

2.2.7 Histone methyltransferase (HMTase) assay and substrates

HMTase assay was performed as previously described (Müller et al., 2002; Ketel et al., 2005) in triplicate using independent batches of purified mutant and wild-type recombinant PRC2 complexes purified in parallel. HeLa polynucleosomes were prepared as previously described (Herrera et al., 2000; Ketel et al., 2005).

2.2.8 SU(Z)12 transgene rescue constructs

SU(Z)12 genomic DNA including its normal promoter [3L: 19911150-19916900 (Flybase)] was amplified from *yDf(1)w*^{67c23} adult flies using Expand Long Range Polymerase (Roche) and tailed primers with restriction sites *Not* I and *Xba*I. This 5.8 kb genomic region was inserted into the P-element based pCaSper-4 germline transformation vector (pCaSper-4 gSU(Z)12) and pBlueScript vectors (pBS- gSU(Z)12). The pBlueScript version was constructed for manipulations involving site directed mutagenesis. An HA-tag was inserted in-frame directly upstream of the translational start site and downstream of the native SU(Z)12 promoter in the pBS-gSU(Z)12 construct by a single primer site directed mutagenesis strategy using *Pfu* Turbo Polymerase [Agilent technology] (Makarova et al., 2000).

The native SU(Z)12 promoter-HA - genomic DNA fragment was then sub-cloned into the patt-B vector for the generation of a hybrid SU(Z)12 construct

for use in PhiC31 site-directed transgenesis. To this end, pFastBac-SU(Z)12 cDNA was digested with *Stu*I, which cuts in exon 1 and *Xba*I, flanking the 3'UTR in the polylinker, thereby generating a cDNA fragment that can be inserted in-frame into exon-1 of the pattB SU(Z)12 -HA-SU(Z)12 genomic construct digested with *Stu*I- *Xba*I. The resulting hybrid construct with the upstream SU(Z)12 genomic sequence, HA tag and a downstream SU(Z)12 cDNA was used for the generation of PhiC31 based site-directed transformant lines. Mutant rescue constructs with in-frame deletions and point mutations in the zinc finger of SU(Z)12 were generated using the same strategy except that pFastBac Su(Z)12 Zinc finger Δ and pFastBac SU(Z)12 P414AW415A were used to generate the cDNA fragment, respectively. Rescue constructs for N-terminal domain deletions were generated in the pBlueScript native promoter-HA-cDNA construct using a single primer PCR strategy (Makarova et al., 2000) and sub-cloned into patt-B vector.

2.2.9 Generation of germline transformants and SU(Z)12 genetic rescue

P-element based germline transformants were generated using pCaSper-4 gSU(Z)12 in the *yDf(1)w*^{67c23} host strain [Genetic Services, Sudbury, MA] (Peterson et al., 2004). Transformant flies were selected using the w^+ eye color and independent lines established. Multiple lines were generated and the insertion sites mapped to either 2nd or 3rd chromosome. Transgenic lines with SU(Z)12 inserted on Chromosome 2 were made homozygous and used for rescue tests.

A two step cross strategy was used to test rescue of the SU(Z)12 null phenotype *Su(z)12^{3}/Su(z)12^{4}*. Males from lines homozygous for transgene inserted in the second chromosome were crossed to virgin females of the *w; Su(z)12^{3} FRT2A h th e/ TM6C, cu Sb e Tb ca* genotype. F1 progeny with *w^{+},* non-stubble, non-ebony phenotype were selected and crossed to virgin females of the *w; Su(z)12^{4} FRT2A h th e / TM6C, cu Sb e Tb ca* genotype. Rescue is indicated by the survival of *w^{+},* non-stubble, ebony flies. The rescued flies also displayed the recessive hairy and thread phenotypes, confirming their *Su(z)12^{3}/Su(z)12^{4}* third chromosome genotypes.

PhiC31 mediated transgenic lines were generated as previously described (Groth et al., 2004) using the *VK37* host strain and 2L: 22A, a genomic landing site on the left arm of chromosome 2 at the cytological location 22A. patt-B mutant and wild-type SU(Z)12 constructs described in the previous section were used for the generation of transgenic lines and rescue tests of transgene inserts were performed as mentioned above.

2.3 Results

2.3.1 The zinc finger domain of SU(Z)12 is dispensable for E(Z) mediated histone methyltransferase activity of PRC2 *in vitro*.

The zinc finger of SU(Z)12 is a non-canonical, non-DNA binding domain (Birve et al., 2001) with an unknown function. Apart from binding to specific DNA sequences, C2H2 zinc fingers are generally implicated in binding to proteins and

RNA. Hence, one possible role of the zinc finger domain is to mediate stable interactions between PRC2 subunits. However, missense mutations in particular conserved residues of the zinc finger did not compromise either assembly or enzyme activity of PRC2 (Ketel et al., 2005). The mutants tested previously may not have disrupted the zinc finger domain.

To more definitively elucidate the function of the zinc finger domain, we generated an in-frame deletion that removes it completely and two double point mutants [C413A C416A and P414A W415A] that disrupt the Cys 2 motif of the zinc finger [Fig. 1A]. We used the Sf-9 baculoviral expression system to generate and purify wild-type and mutant recombinant PRC2 complexes *in vitro*. Reconstituted complexes were tested for the ability to assemble into a stable complex and for HMTase activity. We observed that the double mutants [Fig. 1B] and the in-frame deletion of the zinc finger domain [Fig. 1D] retained the ability to assemble into four-subunit PRC2 complexes *in vitro*. When tested for enzyme function *in vitro,* via histone methyltransferase reaction using HeLa polynucleosomes as the substrate, we observed that the zinc finger missense mutants and PRC2 lacking zinc finger domain entirely showed comparable catalytic efficiency to wild-type [Fig.1, C and E, respectively]. These results indicate that the zinc finger domain of SU(Z)12 is neither required for the stability and integrity of the PRC2 complex nor critical for the E(Z) mediated enzymatic function of PRC2. The results suggest a role for the conserved zinc finger that cannot be discerned *in vitro*. Hence, we decided to test the function of this

domain *in vivo* using a genetic rescue approach.

Figure 1 A: The conserved domains of SU(Z)12 as defined by conservation between flies and mammals. The shadowed region depicts residues in the zinc finger domain conserved between flies and mammals. Site-directed mutations in the conserved residues of the zinc finger domain used in this study are indicated. The shortened version of SU(Z)12 bearing an inframe deletion of the zinc finger domain is illustrated at the bottom.

A

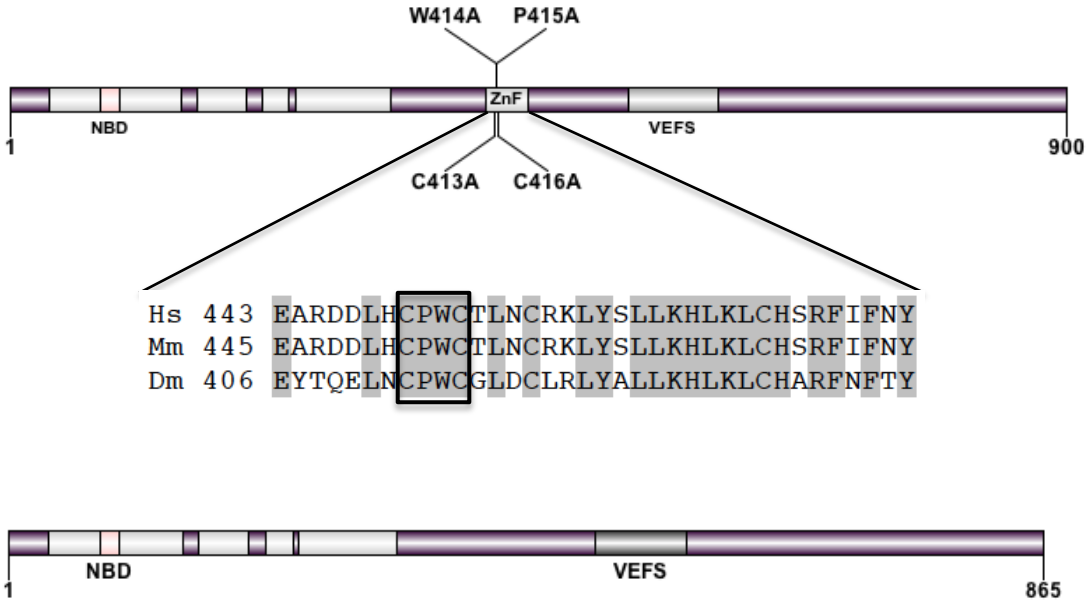
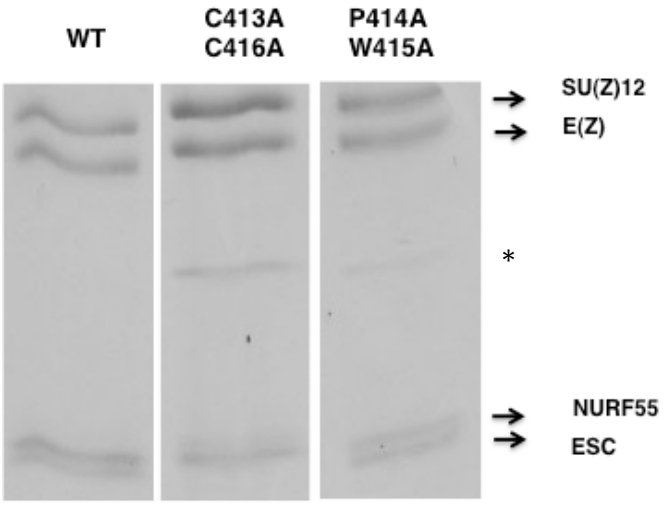

W414A
P415A
ZnF
NBD
VEFS
1
900
C413A
C416A
Hs 443 EARDDLHCPWCTLNCRKLYSLLKHLKLCHSRFIFNY
Mm 445 EARDDLHCPWCTLNCRKLYSLLKHLKLCHSRFIFNY
Dm 406 EYTQELNCPWCGLDCLRLYALLKHLKLCHARFNFTY
NBD
VEFS
1
865

Figure 1 B: Effects of mutations in the zinc finger domain of SU(Z)12. Assembly of reconstituted PRC2 complexes with double missense mutations C413A C416A and P414A W415A in the zinc finger domain of SU(Z)12 purified in parallel to the wild-type [WT] PRC2 complex. Mutant and wildtype PRC2 components were co-infected via baculovirus in Sf-9 insect cells and affinity-purified using Flag-ESC. Purified complexes were analyzed on 10% SDS-PAGE and detected by Coomasie blue stain. (*) indicates hsp70 which variably co-purifies with proteins overexpressed in Sf-9 cells (Müller et al., 2002).

Figure 1C: *In vitro* histone methyltransferase activity of the reconstituted double mutants of the zinc finger domain C413A C416A and P414A W415A using HeLa polynucleosome susbstrates and ^{3}H labelled S-adenosyl methionine.

B



Flag tag: ESC

C

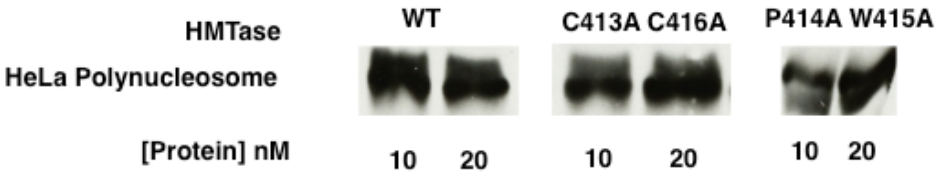
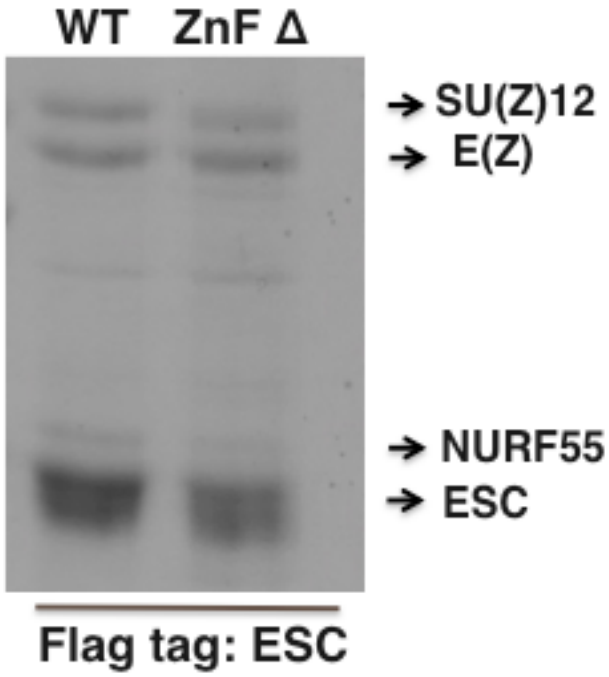


Figure 1 D: Tests for assembly of reconstituted PRC2 complexes with in-frame deletion of the zinc finger domain purified in parallel to the wild-type PRC2 complex using the SF-9 baculoviral expression system. Flag-tagged ESC was used to affinity purify wild-type and mutant PRC2 complexes lacking zinc finger. Purified complexes were analysed on an 8% SDS-PAGE and visualized by Coomasie blue staining.

Figure 1E: Tests for enzyme function. *In vitro* histone methyltransferase activity of reconstituted PRC2 complex with in-frame deletion of the SU(Z)12 zinc finger compared to wild-type PRC2. HeLa polynucleosomes were used as substrates in this assay with ^{3}H labelled S-adenosyl methionine as a co-factor.

D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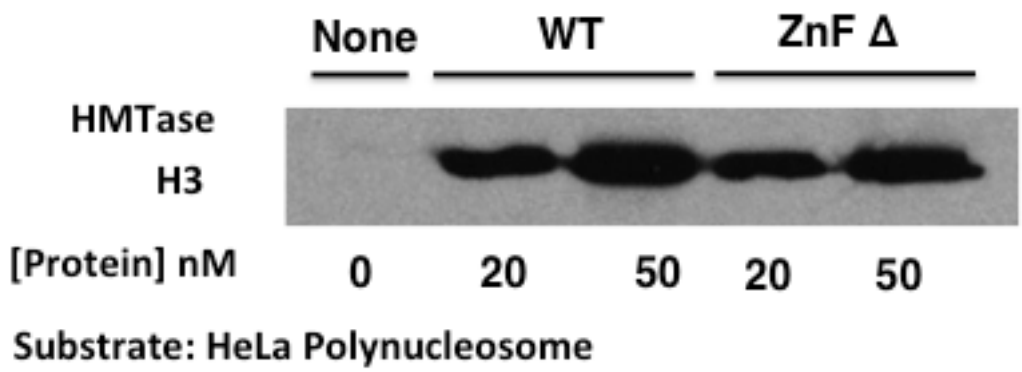
ZnF Δ
SU(Z)12
E(Z)
NURF55
ESC
Flag tag: ESC

E


None
WT
ZnF Δ
HMTase
H3
[Protein] nM
0
20
50
20
50
Substrate: HeLa Polynucleosome

2.3.2 The zinc finger domain of SU(Z)12 is required for PRC2 function but not assembly of the complex *in vivo*.

Considering the high degree of conservation of this domain and the failure to assign a function for this domain using our *in vitro* tests, we assessed the function of this domain *in vivo* using a genetic rescue assay. To define the genomic region necessary to rescue the null phenotype of $Su(z)12^{3}/Su(z)12^{4}$ trans heterozygotes (Birve et al., 2001), we first generated a P-element based 5.8 kb genomic SU(Z)12 rescue construct inclusive of the 5' and 3'UTR and driven by the native SU(Z)12 promoter [Fig. 2A]. *Su(z)12* is tightly flanked by neighboring genes, which delimits the extent of transgene design. We included approximately 800 base pairs of upstream sequence from the translational start site inclusive of the 5'UTR and approximately 900 base pairs downstream of the stop codon and spanning the 3'UTR. We tested three different transgenic lines with the transgene inserted in the 2nd chromosome for complementation and rescue of lethality in SU(Z)12 nulls [Table 1]. All three lines rescued the larval lethal phenotype of the SU(Z)12 null allelic combination, suggesting that one copy of the 5.8 kb genomic region tested was sufficient for full functional rescue. These results established the upstream region of SU(Z)12 genomic DNA inclusive of its native promoter that is sufficient to rescue $Su(z)12^{3}/Su(z)12^{4}$ larval lethality.

To control for position effects and variability in expression levels observed after P-element mediated random insertions and for the efficient comparison of

mutants, we designed a new rescue construct for use in phiC31 based site-directed transgenesis. The new version had an HA-tag inserted directly upstream of the translational start site of SU(Z)12 cDNA driven by the native SU(Z)12 promoter. This makes it convenient way to track the transgenic protein as well as insert mutated versions of SU(Z)12 to be tested and compared in the same genomic context [Fig. 2B]. The transgenes were inserted at a specific location in the 2nd chromosome for direct comparison. We generated two mutant zinc finger transgenic lines containing either P414A W415A or the in-frame zinc finger deletion using this approach, along with the wild-type transgenic line in parallel for direct comparison. Anti-HA western blot analysis was performed on embryo extracts to detect the stable accumulation of transgenic proteins. The transgenic zinc finger mutated proteins were expressed at comparable levels to wild-type SU(Z)12 [Fig. 2C]. When tested for rescue, a single copy of wild-type HA-SU(Z)12 could rescue the larval lethality of *Su(z)12^{3}/Su(z)12^{4}* in complementation tests. On the other hand, SU(Z)12 mutants lacking the zinc finger domain did not rescue the larval lethal phenotype. Rescue tests also showed that P414A W415A failed to rescue SU(Z)12 null lethality. However, some survival to late pupal stages [pharate adults], not shown, suggested that the P414A W415A mutants retains weak partial function. Together, these data suggests that the zinc finger domain of SU(Z)12 is critical for the function of PRC2 as well as normal development and viability in flies. We next assessed whether the inability to rescue was the result of failure to assemble *in vivo*. To this end, we performed

co-immunoprecipitation assays using embryo extracts from wild-type and mutants. Embryo extracts were immunoprecipitated with anti-E(Z) to recover PRC2 complexes, and tested by western blots with anti-HA for co-assembly of wild-type or zinc finger mutant forms of SU(Z)12. These results suggest that SU(Z)12 zinc finger mutants retain the ability to associate with PRC2 proteins [Fig. 2D]. The transgenic mutated and wild-type proteins assemble into PRC2 complex at comparable levels. The inability of the zinc finger mutants to provide SU(Z)12 function, together with their *in vitro* enzymatic function suggest an alternate *in vivo* role, such as targeting to chromatin sites of action.

2.3.3 Zinc finger domain of SU(Z)12 is required for targeting PRC2 to PREs.

The best characterized PRE is a 0.5 kb fragment located approximately 25 kb upstream of the HOX gene *Ubx* [Fig. 3A] (Wang et al., 2004b). To determine if the zinc finger of SU(Z)12 is required for targeting PRC2 to PREs, we generated Flag-tagged versions of wild-type SU(Z)12 [Flag-SU(Z)12] and the in-frame zinc finger deletion mutant [Flag-SU(Z)12 ZnF Δ] and constitutively expressed them under the control of an actin promoter after transfection in *Drosophila* S2 cells. Western analysis was performed to assess the stable accumulation of wild-type and mutant proteins [Fig. 3B]. Similar to observations made in embryos, co-immunoprecipitations demonstrated that both wild-type and mutant SU(Z)12 associated with E(Z) in S2 cells [Fig. 3C]. To assess the zinc finger is critical for targeting to a PRE, anti- Flag chromatin IP [ChIP] was performed on cells

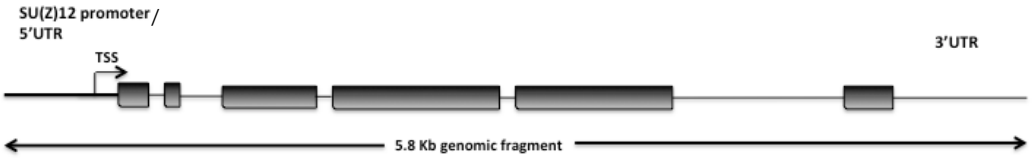
expressing Flag-SU(Z)12 and Flag-SU(Z)12 ZnF Δ. We found that SU(Z)12 lacking its zinc finger showed dramatically reduced ability to target to PRE [Fig. 3D]. A region approximately 2000 base pairs downstream of the PRE was used as a negative control in these experiments.

The zinc finger of SU(Z)12 does not bind DNA (Birve et al., 2001). However, recent studies in plants (Heo and Sung, 2011) and mammals (Kanhere et al., 2010)suggest that SU(Z)12 may mediate binding to non- coding RNAs. In mammals, non-coding RNA can aid target PRC2 targeting to genomic sites. However, interactions of PRC2 with defined non-coding RNAs have not yet been demonstrated in *Drosophila*. It is possible that the SU(Z)12 zinc finger binds non-coding RNA in both flies and mammals to target PRC2 to the promoters of target genes. The accessory binding partners of PRC2, like PCL and JARID2, also aid recruitment of PRC2 to target chromatin and commonly localize with SU(Z)12 in mammals and flies (Wang et al., 2004b; Peng et al., 2009; Li et al., 2010; Herz et al., 2012). In mammals, JARID2 directly interacts with SU(Z)12 via the N-terminal domain and an isolated SU(Z)12 zinc finger fails to interact with JARID2 (Peng et al., 2009). It is unknown if this JARID2 direct binding exists for the fly complex. An alternate possibility is that fly PRC2 interacts with PCL via the SU(Z)12 zinc finger domain. Further tests are required to assess such potential interactions.

Figure 2 A: Schematic representation of the 5.8 kb SU(Z)12 genomic region used to generate transgenic wild-type SU(Z)12 fly lines by P-element mediated transgenesis. TSS=Transcription start site, Exons are represented by blocks and introns by thin lines.

Figure 2 B: Schematic representation of the hybrid SU(Z)12 genomic promoter-HA-SU(Z)12 cDNA constructs used to generate transgenic fly lines with wild-type, P414A W415A, and in-frame zinc finger deletion of SU(Z)12 by PhiC31-mediated site-directed transgenesis. Arrows indicate exons and HA represents the haemagglutinin tag inserted in-frame directly upstream of the translational start site.

A


SU(Z)12 promoter /
5'UTR
TSS
3'UTR
5.8 Kb genomic fragment

B

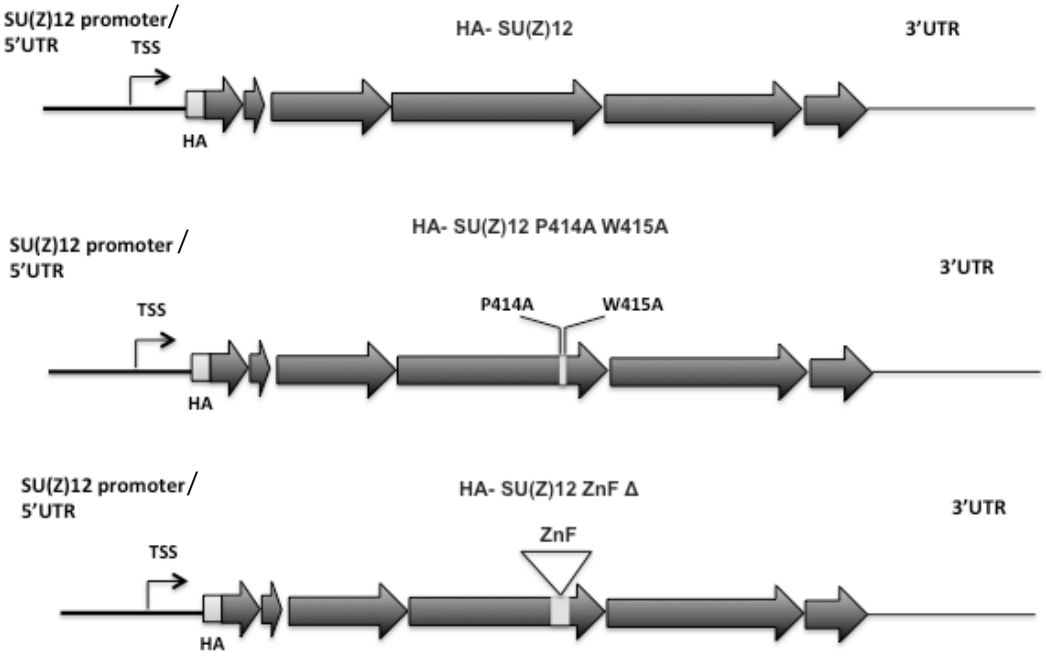

SU(Z)12 promoter/
5'UTR
TSS
HA- SU(Z)12
3'UTR
HA
HA- SU(Z)12 P414A W415A
SU(Z)12 promoter /
5'UTR
3'UTR
TSS
P414A
W415A
HA
SU(Z)12 promoter/
5'UTR
HA- SU(Z)12 ZnF Δ
3'UTR
ZnF
TSS
HA

Table 1 : Rescue of SU(Z)12 lethality with a 5.8kb genomic construct delivered by P-element mediated transgenesis. Three independent lines with inserts on the second chromosome were tested for rescue of *Su(z012^{3}/Su(z)12^{4}* null phenotype. The rescue tests were performed twice for each line. The expected ratio of full rescue is 0.125.

Table 2 : Rescue of SU(Z)12 lethality by an HA-tagged *Su(z)12* cDNA delivered by PhiC31 site-directed transgenesis. HA-tagged wild-type and mutant *Su(z)12* DNA were inserted in the same site on the second chromosome by PhiC31 site-directed recombination. (-) indicates the failure to rescue. The expected ratio of full rescue is 0.125. VK37 parental lines were analysed in parallel as a negative control. Rescue tests were performed in triplicate to confirm the failure to rescue.

Table 1

Line	Rescued Adults	Total Adults scored	Observed ratio
SUZ 48.1 h	28	211	.132
SUZ 74 h	22	220	.100
SUZ 88 h	20	262	.076

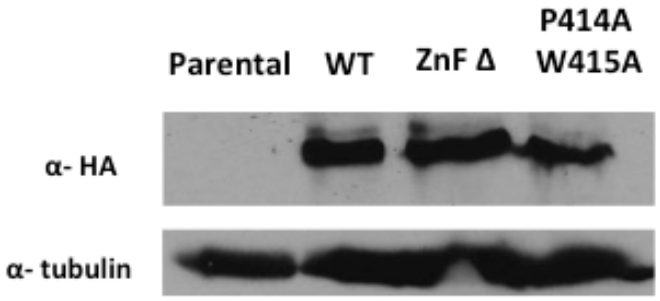
Table 2

Line	Rescued Adults	Total Adults scored	Observed ratio
HA-SU(Z)12	88	928	.095
HA- ZnfΔ	0	769	-
HA-P414AW415A	0	875	-
Parental (no transgene)	0	879	-

Figure 2C: Tests for the stability of transgenic wildtype and zinc finger mutant proteins *in vivo*. Anti-HA western analysis on 0-12 hr embryo extracts expressing transgenic HA- SU(Z)12, HA-SU(Z)12 ZnF Δ, or HA- P414A W415A. The *VK37* parental line lacking the transgene was used as a negative control. Anti-tubulin westerns were performed on the same blot to control for loading.

Figure 2D: Interaction of transgenic HA-SU(Z)12, HA-SU(Z)12 ZnF Δ and HA-P414A W415A with endogenous E(Z). Anti-E(Z) co-immunoprecipitations were performed on 0-12 hr embryo extracts expressing HA-tagged wild-type or mutant SU(Z)12 proteins. Input lanes represent 1/6 of the samples immunoprecipitated by anti-E(Z). Protein-A represents beads incubated with extracts alone in the absence of primary antibody, and E(Z) represents Protein-A bound to E(Z) incubated with embryo extracts.

C


Parental
WT
ZnF Δ
P414A
W415A
α- HA
α- tubulin

D

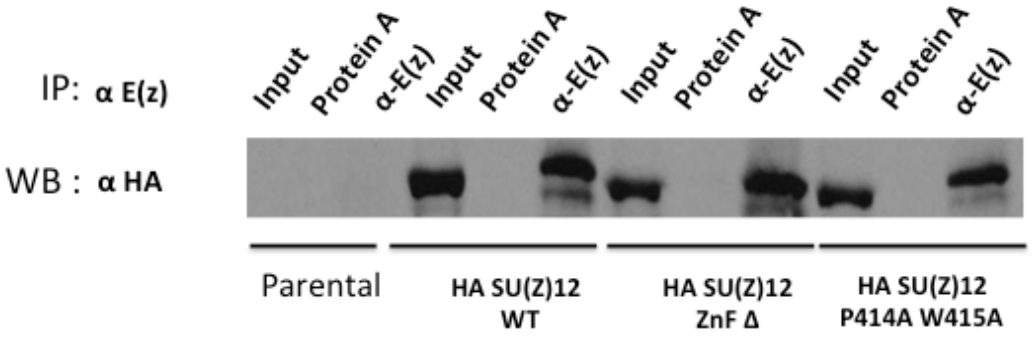
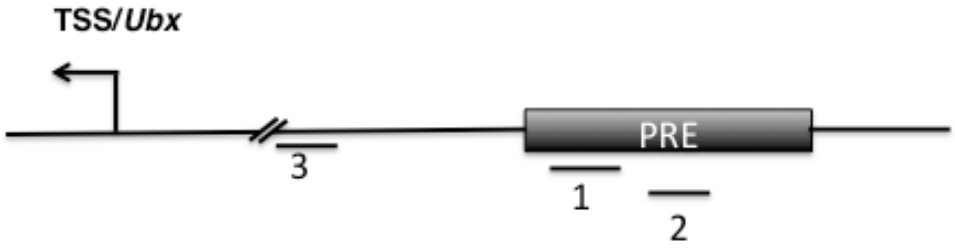

IP: α E(z)
WB : α HA
Input
Protein A
α-E(z)
Parental
HA SU(Z)12 WT
HA SU(Z)12 ZnF Δ
HA SU(Z)12 P414A W415A

Figure 3 A: Schematic representation of the major PRE of *Ubx* located approximately 25kb upstream of the transcription start site. 1 and 2 represent fragments amplified by qPCR for detection in ChIP assays as described previously (Wang et al., 2010). Fragment 3 [within the b1 region described in Wang et al., 2004] represents a region outside of the PRE used as a negative control in ChIP assays (Wang et al., 2004b).

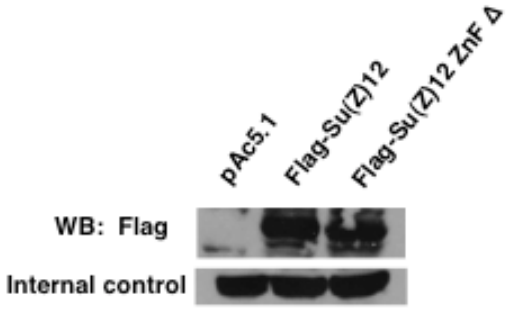
Figure 3B : Expression of Flag-SU(Z)12 and Flag- SU(Z)12 ZnFΔ in *Drosophila* S2 cells. Anti-Flag westerns were performed on S2 cell lysates to detect of accumulation of Flag-tagged wild-type and mutated SU(Z)12. An unidentified protein that cross reacts with the anti-Flag antibody was used as a convenient internal loading control to verify equivalent loading. Lysates from cells transfected with pAc.5 empty vector alone was used as a negative control.

Figure 3C: Transiently transfected Flag-SU(Z)12 and Flag- SU(Z)12 ZnF Δ assemble with endogenous E(Z) in fly S2 cells. Anti-Flag M2 beads were used to immunoprecipitate proteins associated with Flag tagged SU(Z)12 wild type and ZnF mutant. Beads alone IP was performed to control for non-specific interactions. Another negative control was IP from cells transfected with pAc5.1 empty vector. Input lane represent 1/6th of the lysates used for immunoprecipitation. Anti-E(Z) western blots were performed to detect the association of endogenous E(Z) with transfected forms of SU(Z)12. Bands corresponding to E(Z)are indicated and the low molecular weights are presumably degradation products of E(Z).

A


TSS/*Ubx*
PRE
3
1
2

B


pAc5.1
Flag-Su(Z)12
Flag-Su(Z)12 ZnF Δ
WB: Flag
Internal control

C

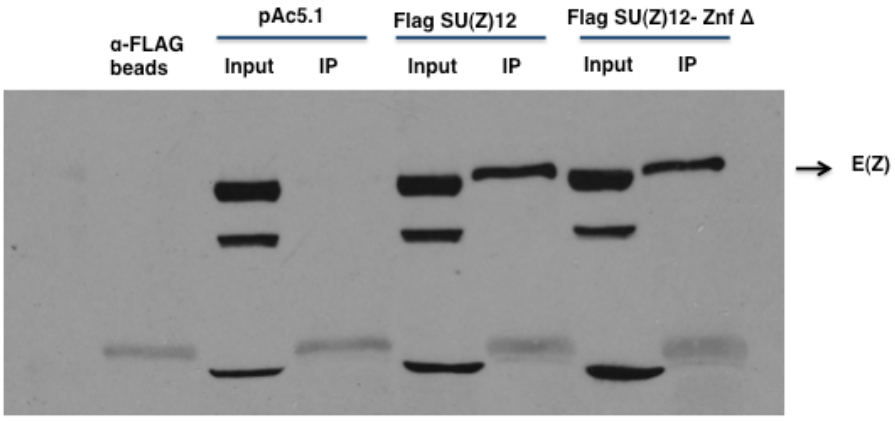
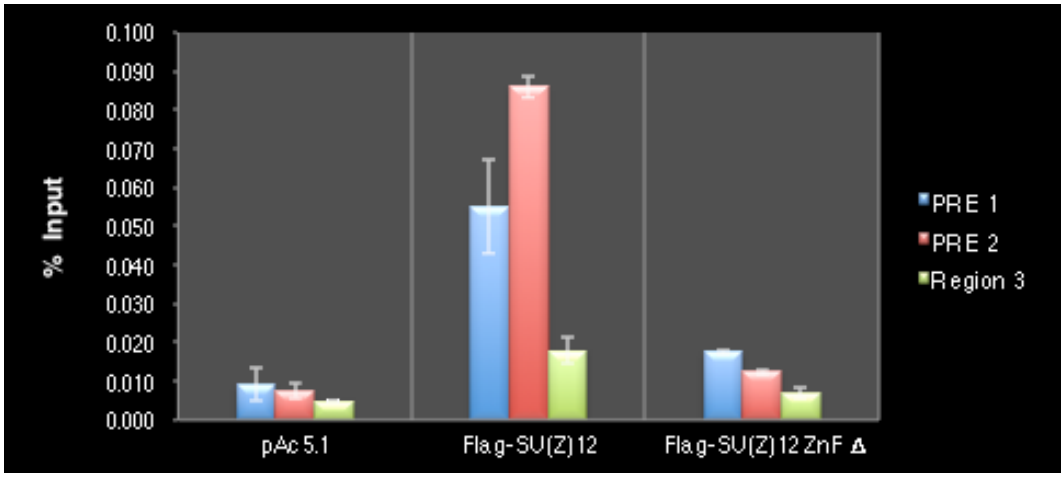

α-FLAG beads
pAc5.1
Flag SU(Z)12
Flag SU(Z)12- Znf Δ
Input
IP
Input
IP
Input
IP
E(Z)

Figure 3D: ChIP assays to detect binding of wild-type or the zinc finger deleted version of the zinc finger to *Ubx* PRE in fly S2 cells. Regions in the major PRE of *Ubx* [PRE1 and PRE2] known to accumulate PRC2 were analyzed (Wang et al., 2004b; 2010). Region 3, which does not bind PRC2 was used as negative control for ChIP. Lysates from vector alone [pAc5.1] transfection were analyzed in parallel. Data represented is ± SD from duplicates within an experimental set. The ChIP was performed at least three times from independent transfections to confirm the results.

D


0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
% Input
pAc 5.1
Flag-SU(Z)12
Flag-SU(Z)12 ZnF Δ
PRE 1
PRE 2
Region 3

2.3.4 The dual role of the conserved VEFS domain in PRC2 function.

The highly conserved VEFS domain is critical for binding of SU(Z)12 to E(Z) (Ketel et al., 2005). However, the missense mutation D546A, a charged conserved residue of the SU(Z)12 VEFS domain, fails to methylate H3K27 without any defects in assembly of the complex. This suggests that the VEFS domain is needed for both stable binding to the complex and to stimulate E(Z) mediated histone methyltransferase function. Recently, several missense mutations were identified in conserved residues of the VEFS domain of SUZ12 [W591C and N618Y] in T-cell acute leukemia (Ntziachristos et al., 2012) and myelodysplastic or myeloproliferative neoplasms [E610G] (Score et al., 2012). The exact mechanism by which these mutations disrupt PRC2 function is unknown.

To gain insight into the contributions of these conserved residues, mutant complexes bearing corresponding mutations in *Drosophila* SU(Z)12 [W555C, E574A, N582Y] were generated, affinity-purified and tested for function *in vitro* after expression and assembly in Sf-9 insect cells [Fig. 4A]. The N582 and E574 residue are highly conserved from plants to mammals whereas the W555 residue is conserved between humans, mice and flies but not in plants. We found that the N582Y mutant failed to assemble into PRC2. Instead we observed an E(Z)-ESC dimer very similar to the defect in assembly observed for SU(Z)12 lacking the VEFS domain [Fig. 4B] (Ketel et al., 2005). The N582Y mutation disrupts SU(Z)12 interaction with E(Z). One possible explanation is that the conserved

Asparagine (N) residue, with its highly polar side chain, resides on the surface of SU(Z)12 in position to interact with key residues in E(Z). Substituting the N582 residue with the hydrophobic Tyrosine (Y) can bury this interaction surface, resulting in an inability to bind E(Z). We also assessed if the inability of N582Y mutant to assemble into a fully functional PRC2 complex is the result of structural changes that lead to degradation of the mutant protein. Western blot analysis performed on Sf-9 cell co-infection lysates confirmed that the N582Y mutant protein is not degraded. Rather, it is expressed at levels comparable to the wild-type protein [Fig. 4C]. Not surprisingly, the N582Y mutated protein fails to methylate polynucleosomes [Fig. 4D]. On the other hand, W555C and E574A [similar change for E610G] mutated proteins retained full assembly [Fig. 4B]. However, the E574A mutant showed partial reduction in enzyme function, while the W555C mutant showed a significant reduction in its ability to methylate histone substrates [Fig. 4D]. We also extended our mutational analysis to another conserved residue flanking E574, K575. Similar to E574A and W555C, K575A retained assembly of the mutant PRC2 complex [Fig. 4E]. However, we observed only slight reduction in the catalytic activity of the K575A mutated protein [Fig. 4F].

Based on these results, one can speculate that the VEFS domain of SU(Z)12 serves a dual role in the PRC2 complex by providing an interface to stably bind E(Z) as well as to allosterically boost its enzyme function. Previous studies reported missense mutations in the conserved charged residues localized

in the N-terminal end of the VEFS domain that preserve full assembly but reduce catalytic function, and a mutation in the C-terminal portion of VEFS that has no effect on assembly or enzyme function (Ketel et al., 2005). Combining our previously reported data (Ketel et al., 2005) with this study, we can functionally demarcate the VEFS into two subdomains, Subdomain-C [C terminal] that is critical for binding to E(Z), and Subdomain-N [N-terminal], critical for enhancing E(Z)-mediated enzyme function [Fig. 4A]. Disrupting either of these functions can have major implications in leukemic disease progression as a result of PRC2 loss of function.

Figure 4A. Shadowed region below represents residues conserved between flies and mammals within the VEFS domain. Point mutations used to study the function of the VEFS domain are depicted and their locations within the domain highlighted in the black box. Residues highlighted by red boxes represent previously analyzed conserved residues (Ketel et al., 2005). Functional demarcation of the two subdomains of the VEFS domain is also indicated.

A

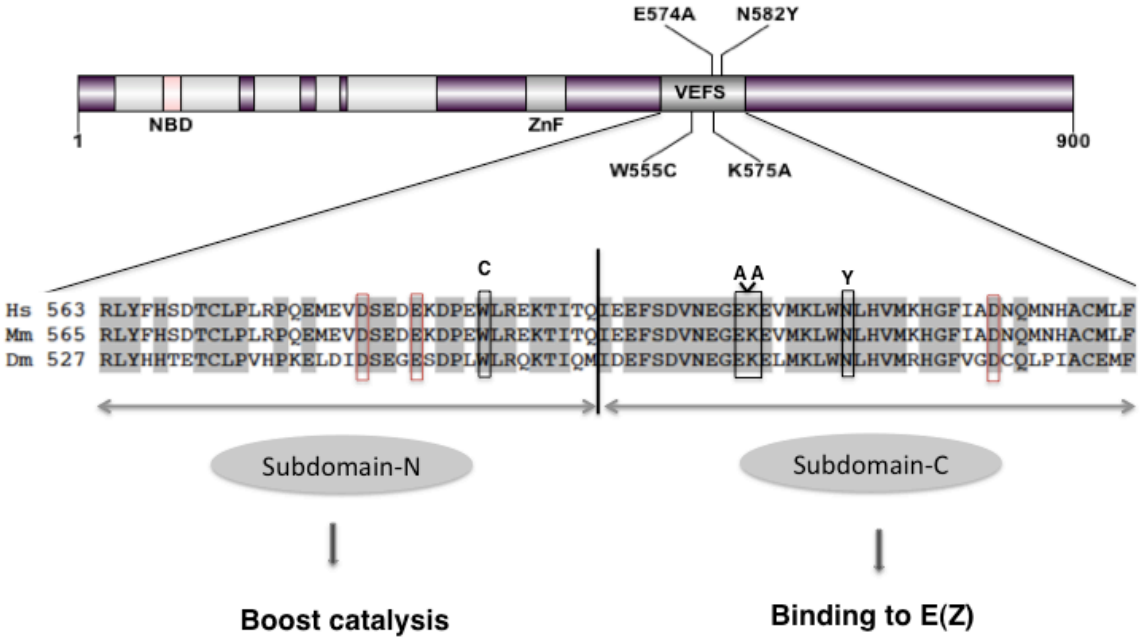
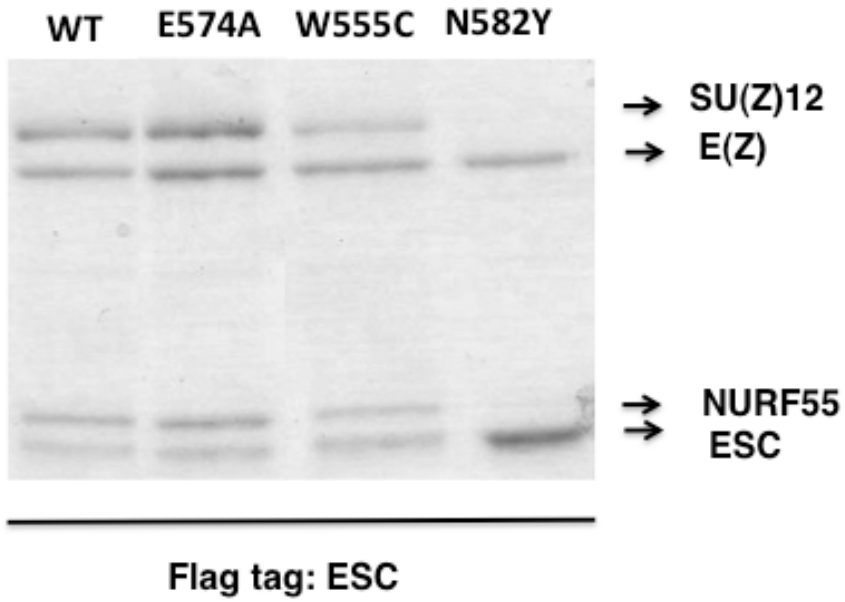

E574A
N582Y
VEFS
NBD
ZnF
1
900
W555C
K575A
C
A A
Y
Hs 563 RLYFHSDTCLPLRPQEMEVDSEDEKDPEWLREKTITQIEEFSDVNEGEKEVMKLWNLHVMKHGFIADNQMNHACMLF
Mm 565 RLYFHSDTCLPLRPQEMEVDSEDEKDPEWLREKTITQIEEFSDVNEGEKEVMKLWNLHVMKHGFIADNQMNHACMLF
Dm 527 RLYHHTETCLPVHPKELDIDSEGESDPLWLRQKTIQMIDEFSDVNEGEKELMKLWNLHVMRHGFVGDCQLPIACEMF
Subdomain-N
Subdomain-C
Boost catalysis
Binding to E(Z)

Figure 4B: Assembly of PRC2 bearing substitutions in conserved residues of the VEFS domain. Flag-tagged ESC was used to purify wild-type and mutant PRC2 complexes with altered conserved residues of the VEFS domain using a baculovirus overexpression system. Sub-unit compositions of the VEFS mutant complexes were analyzed by 8%-SDS PAGE and stained using Coomassie blue for visualization.

Figure 4C: Anti- SU(Z)12 western blots showing the stable accumulation and expression of wild-type and VEFS missense mutants in the Sf-9 co-infection lysate. Anti-tubulin western blot was performed on the same blot to control for lane loading.

B


WT
E574A
W555C
N582Y
SU(Z)12
E(Z)
NURF55
ESC
Flag tag: ESC

C

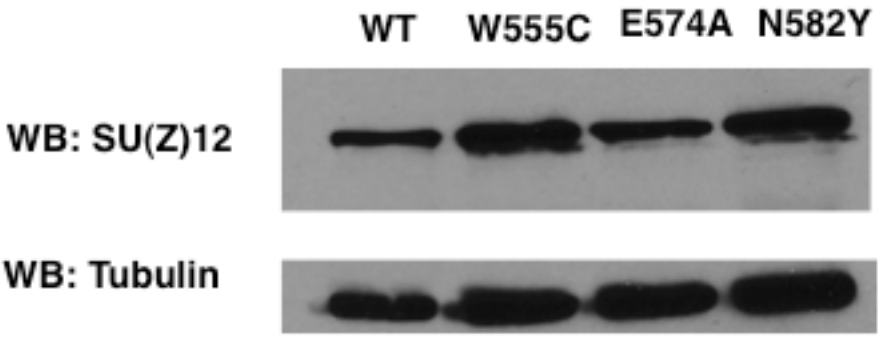
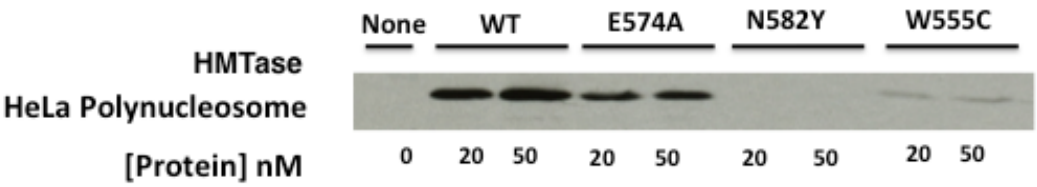

WT
W555C
E574A
N582Y
WB: SU(Z)12
WB: Tubulin

Figure 4D: Catalytic function of PRC2 with mutations in the conserved VEFS domain. Histone methytransferase assays showing the catalytic activity of VEFS mutants on HeLa polynucleosome substrates.

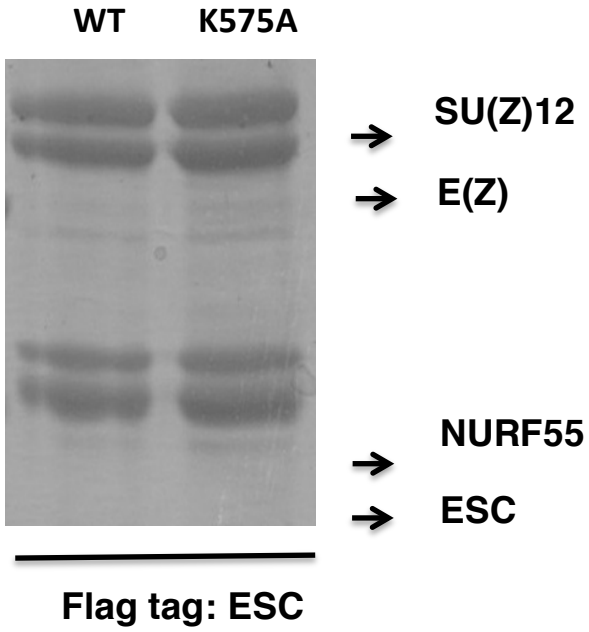
Figure 4E: Assembly and stability of PRC2 complex with the K575A mutation. Flag- tagged ESC was used to affinity-purify wild type and mutant PRC2 complexes.

Figure 4F: Catalytic function of PRC2 with mutations in the conserved K575A residue of VEFS domain. HeLa polynucleosome substrates were used in *in vitro* methyltransferase assays using ^{3}H labelled S-adenosyl methionine as a co-factor.

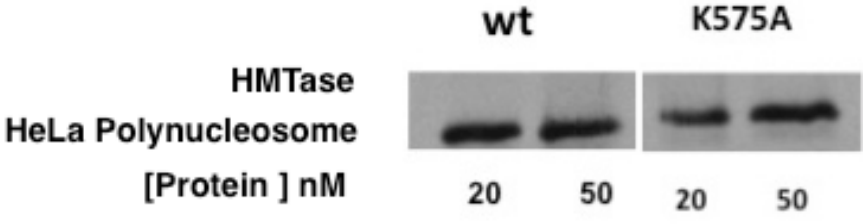
D


None
WT
E574A
N582Y
W555C
HMTase
HeLa Polynucleosome
[Protein] nM
0
20
50
20
50
20
50
20
50

E


WT
K575A
SU(Z)12
E(Z)
NURF55
ESC
Flag tag: ESC

F


wt
K575A
HMTase
HeLa Polynucleosome
[Protein] nM
20
50
20
50

2.3.5 N-terminal conserved domain of SU(Z)12 has E(Z) binding determinants.

The N-terminal domain of SU(Z)12 is a region with scattered short stretches of highly conserved blocks interspersed with less conserved regions in both flies and mammals. The N-terminal domain of SU(Z)12 harbors NBE required for the stable association of NURF55 to SU(Z)12 (Furuyama et al., 2006; Schmitges et al., 2011). Specifically, the region between residues 94-143 in SU(Z)12 is critical for the binding of NURF55 (Schmitges et al., 2011). To understand the contribution of this domain in detail, we made four progressive deletions of the N-terminal domain and a clustered triple mutant in charged successive residues [Fig 5A]. All four in-frame truncations of SU(Z)12 assembled into the PRC2 complex with altered stoichiometry compared to the wild-type PRC2 complex purified in parallel [Fig 5B]. We asked if the weak stoichiometry of SU(Z)12 truncations in the mutant complexes was due to instability of these proteins in Sf-9 cells. Surprisingly, we did not observe any significant reduction in the expression and stability of the truncated SU(Z)12 proteins in Sf-9 cells [Fig 5D]. This suggests that the N-terminal region of SU(Z)12 has E(Z) binding determinants outside of the VEFS domain.

To test this possibility, we took two representative deletions and analyzed their ability to directly bind E(Z) in a pairwise binding assay. Flag-tagged E(Z) was used to co-purify SU(Z)12 [122-900] and SU(Z)12 [220-900] using Sf-9 expression system. We observed weak stoichiometric assembly of the SU(Z)12 N-terminal truncations compared to wild-type SU(Z)12. This experiment shows

that together with the VEFS domain, the N-terminus of SU(Z)12 is required for stabilizing effective binding of E(Z) and SU(Z)12 [Fig 5E]. We also observed that the levels of NURF55 were only marginally reduced in all the truncations of the SU(Z)12 analyzed [Fig 5B]. This observation is in partial agreement with recent studies that showed that the N-terminal domain harbors a NURF55 binding element (Schmitges et al., 2011). However, our studies also suggest the presence of NURF55 binding determinants outside of the NBE in SU(Z)12. Thus, one can speculate that there is a NURF55 binding site in E(Z) required for NURF55 assembly into PRC2 that is yet to be mapped. In a separate test to determine the contribution of the N-terminal conserved domain for PRC2 enzyme function, we observed a moderate reduction in the ability to methylate histones [Fig 5C]. We provide evidence that this reduction in enzyme function is due to the defective binding to E(Z) as opposed to low levels of NURF55 in the complex since mutant complexes show lower activity here than wild-type PRC2 complexes lacking NURF55 [Fig 5C].

We also analyzed a cluster of charged residues in this N-terminal domain [K174A, R175A, K176A]. We did not see any significant defects in binding to E(Z) or enzyme function, although some minor defects in binding to NURF55 were observed [Fig 5F and 5G]. How the defects in assembly and the slight reduction of enzyme function translates *in vivo* require further analysis.

Figure 5A. Illustration of the clustered triple mutant and the four progressive deletions of the SU(Z)12 N-terminal domain used for *in vitro* analysis in this study.

A

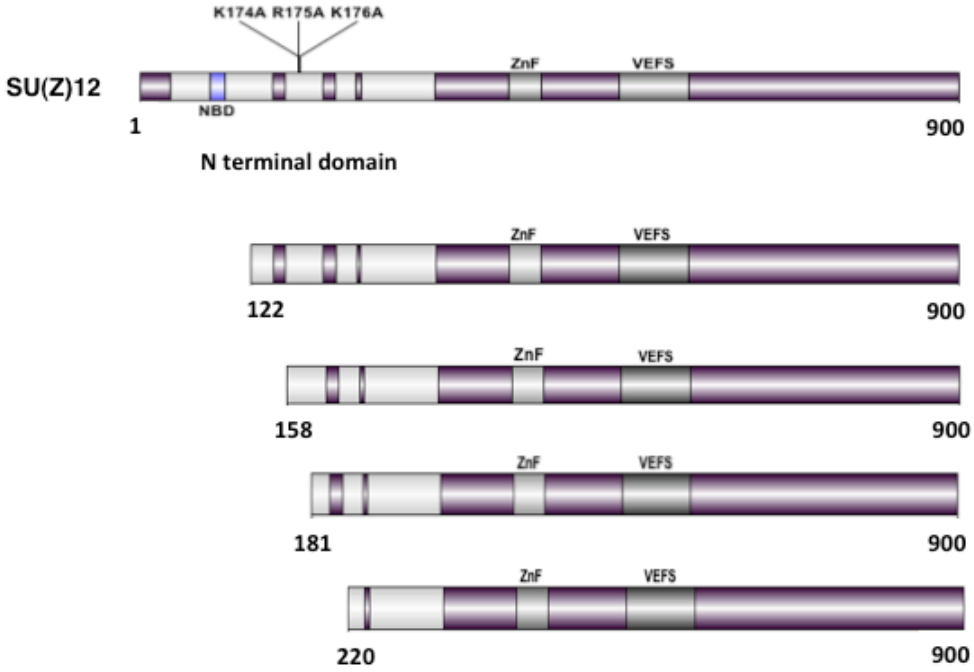
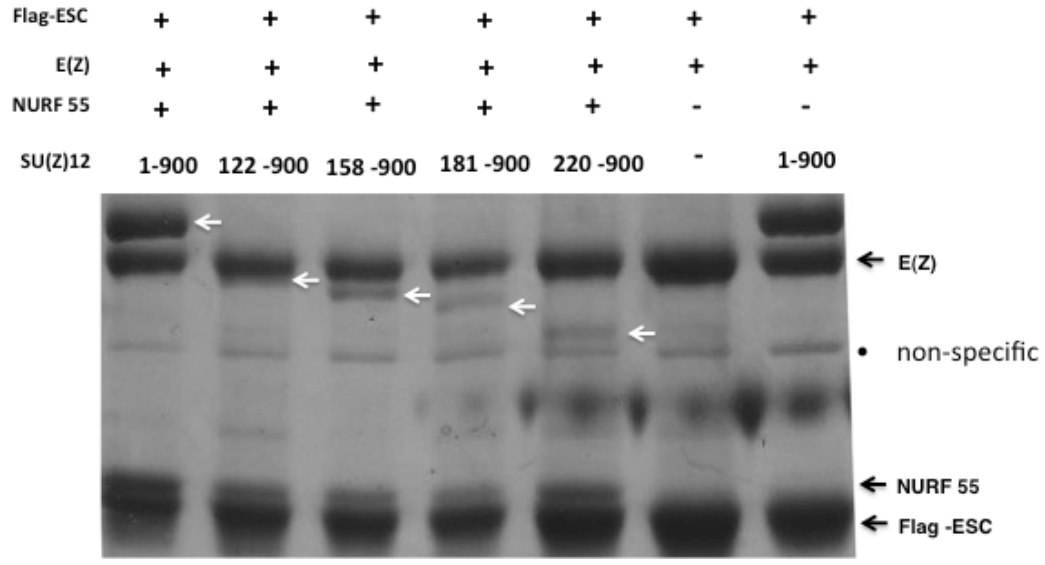

K174A R175A K176A
SU(Z)12
NBD
ZnF
VEFS
1
900
N terminal domain
122
158
181
220

Figure 5B. Effect of N-terminal deletions in SU(Z)12 on the stability of the PRC2 complex. Arrows depict SU(Z)12 truncations in the purified PRC2 complex. Flag-tagged ESC was used to purify reconstituted mutant and wild-type complexes. The dimeric PRC2 complex Flag-ESC/E(Z) was purified as a negative control for *in vitro* enzyme assays. The trimeric complex lacking NURF55 was also purified in parallel as a negative control for NURF55 binding.

Figure 5C: HMTase assays showing the catalytic activity of wildtype, trimeric and dimeric PRC2 alongside PRC2 with progressive deletions in the N-terminal domain.

B


Flag-ESC + + + + + + +
E(Z) + + + + + + +
NURF 55 + + + + + - -
SU(Z)12 1-900 122 -900 158 -900 181 -900 220 -900 - 1-900
E(Z)
non-specific
NURF 55
Flag -ESC

C

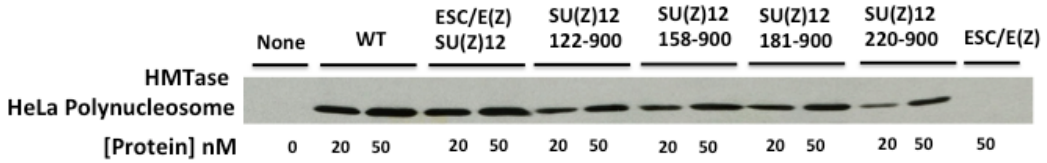
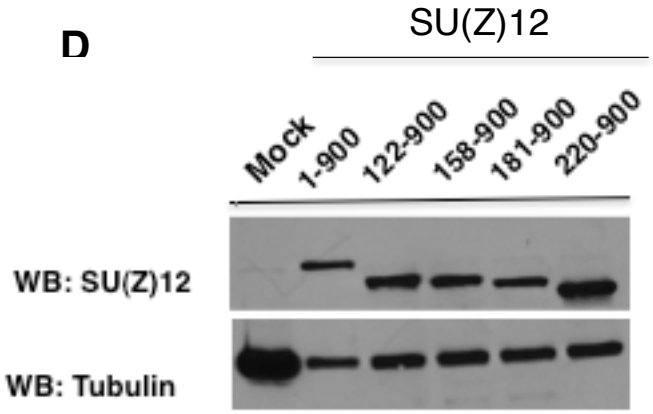

None
WT
ESC/E(Z) SU(Z)12
SU(Z)12 122-900
SU(Z)12 158-900
SU(Z)12 181-900
SU(Z)12 220-900
ESC/E(Z)
HMTase
HeLa Polynucleosome
[Protein] nM
0 20 50 20 50 20 50 20 50 20 50 20 50 50

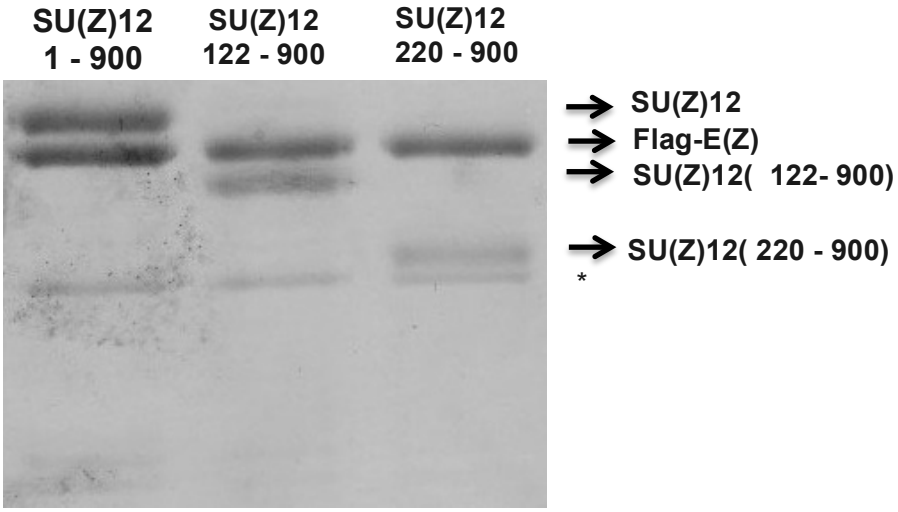
Figure 5D. Tests for the stablity of SU(Z)12 proteins with progressive deletions in the conserved N-terminal domain. Sf-9 co-infection lysates were analyzed for accumulation and stable expression of SU(Z)12 wild-type [1-900] and truncated mutants. Anti-SU(Z)12 western blots were performed to detect the expression of mutant proteins. Anti-tubulin western blots were performed on the same blot to control for even loading.

Figure 5E: Tests to determine direct binding of SU(Z)12 bearing N-terminal truncations to E(Z). Flag-tagged E(Z) was used to purify recombinant dimers from co-infection lysates of Sf-9 cells. The dimers were analyzed by 8% SDS-PAGE and expression detected by Coomassie blue staining.

D



E

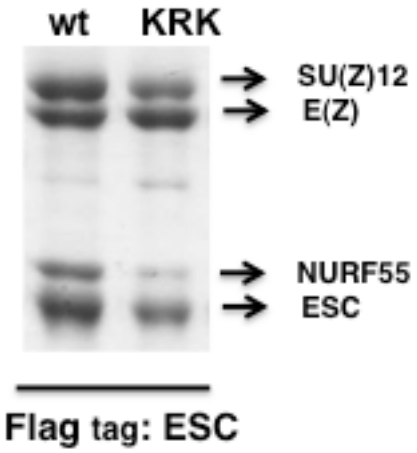


Tag: Flag -E(Z)

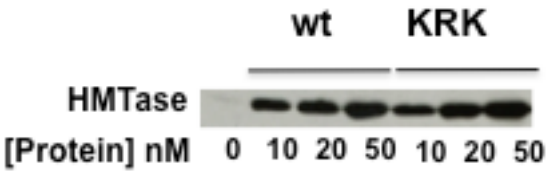
Figure 5F. Effect of a clustered point mutation in the conserved charged residues in the N-terminal domain of SU(Z)12 on the stability of the PRC2 complex. Flag-tagged ESC was used to purify reconstituted mutant and wildtype complexes.

Figure 5G: HMTase assay showing the catalytic activity of wildtype and triple point mutants on HeLa polynucleosome substrates.

F


wt
KRK
SU(Z)12
E(Z)
NURF55
ESC
Flag tag: ESC

G


wt
KRK
HMTase
[Protein] nM
0 10 20 50 10 20 50

2.3.6 N-terminal domain of SU(Z)12 is required for normal development *in vivo*

We examined if the moderate defects in enzyme function and altered stoichiometry of SU(Z)12 in mutant complexes manifest into significant PRC2 loss of function *in vivo*. Is the combination of the zinc finger and VEFS domains of SU(Z)12 sufficient for a fully functional PRC2? To address this, we tested two representative N-terminal mutants of SU(Z)12 for ability to rescue SU(Z)12 null lethality. We used PhiC31 site-directed transgenesis to generate fly lines with N-terminal deletions [122 -900] and [220-900] inserted in the 2nd chromosome [Fig. 6A]. When tested for the ability to rescue the larval lethal phenotype *Su(z)12^{3}/Su(z)12^{4}*, both mutants failed. However, it is yet to be determined if the failure to rescue is the result of failure of the transgenic proteins to accumulate or assemble into PRC2 *in vivo*. These initial observations suggest that SU(Z)12 with the zinc finger and VEFS, though sufficient for H3K27 methylation *in vitro* is not enough for function *in vivo*. This observation is also consistent with the hypomorphic nature of the SU(Z)12^{2} (G274D) allele and the mild defects in assembly observed when G274D mutant complexes were generated *in vitro* (Ketel et al., 2005). It is likely that the N-terminal domain of SU(Z)12 binds to other modulatory subunits that contribute to PRC2 function. In mammals, JARID2 directly interacts with the N-terminal domain of SUZ12 (Peng et al., 2009). It is yet to be determined if this interaction is conserved in *Drosophila.* Considering that the deletion of the N-terminal domain did not completely disrupt the ability of

NURF55 to accumulate in the complex, the failure to rescue *in vivo* could only be partially attributed to loss of NURF55 binding.

Figure 6: Schematic representation of the hybrid SU(Z)12 genomic promoter-HA-SU(Z)12 cDNA constructs used to generate transgenic fly lines with in-frame truncations in the N-terminal conserved domain. Transgenic lines with HA-SU(Z)12 [122-900] and HA-SU(Z)12 [220-900] were generated using PhiC31 site-directed transgenesis.

Table 3: Rescue of SU(Z)12 lethality by expression of transgenic SU(Z)12 by PhiC31 site-directed transgenesis. HA-tagged wild-type and mutant SU(Z)12 with N-terminal truncations were inserted in the same site on the second chromosome by PhiC31 site-directed transgenesis. (-) indicates the failure to rescue. The expected ratio of full rescue is 0.125. VK37 parental lines were analysed in parallel as a negative control. Rescue tests were performed in duplicate to confirm the failure to rescue.

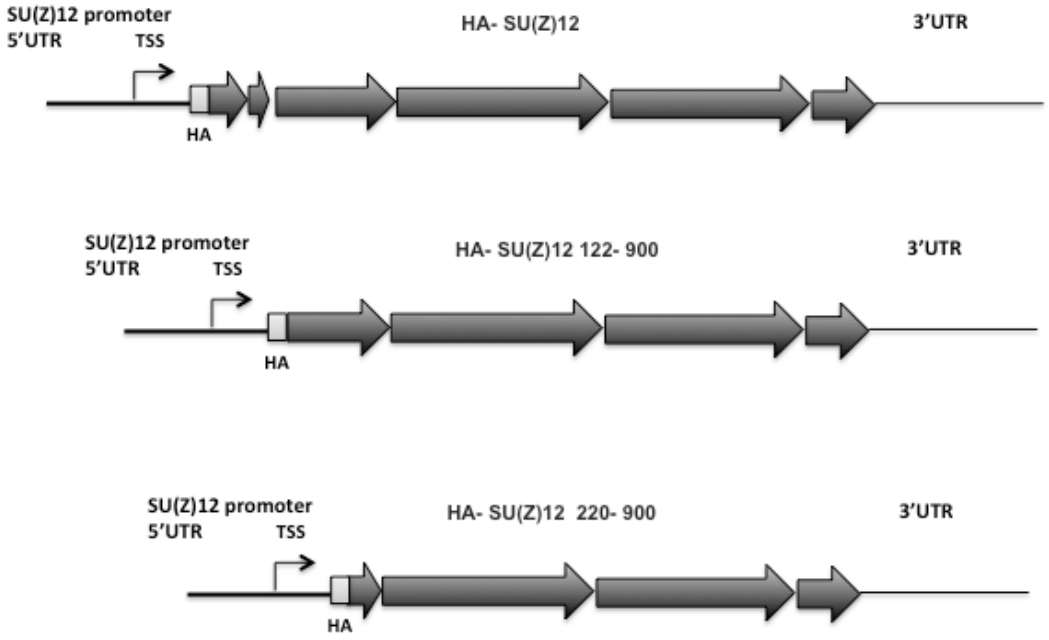


Table 3

Line	Rescued Adults	Total Adults scored	Observed ratio
HA-SU(Z)12	121	928	.113
HA- SU(Z)12 122-900	0	769	-
HA-SU(Z)12 220-900	0	875	-
Parental (no transgene)	0	879	-

2.4 Discussion and future directions

SU(Z)12 is an indispensable non-catalytic subunit for PRC2 function *in vitro* (Müller et al., 2002; Ketel et al., 2005; Nekrasov et al., 2005) and *in vivo* (Pasini et al., 2004). However, the molecular basis of SU(Z)12 contribution to PRC2 function is poorly understood. Genome-wide studies in mammalian ES cells and flies implicate SU(Z)12 co-localization with binding partners of PRC2 including JARID2, PCL [PHF1, MTF2, PHF19] and JING [AEBP2]. These proteins have critical roles in targeting PRC2 to genomic loci. In mammals, non-coding RNA can also target PRC2, and evidence points towards SUZ12 as a subunit that mediates interactions with non-coding RNA (Kanhere et al., 2010). Current knowledge about SU(Z)12 domains is very limited. Previous reports identified the highly conserved VEFS domain to be critical for binding E(Z) and the formation of a fully functional PRC2 complex , as well as modulating enzyme function (Ketel et al., 2005). Although the VEFS domain is the most characterized region of SU(Z)12, there are two other conserved domains whose function is relatively less characterized or unknown. Missense mutations in the highly conserved zinc finger domain maintained the property of a fully functional PRC2 *in vitro* (Ketel et al., 2005). Another implicated role of SU(Z)12 is to aid PRC2 binding to nucleosomes via interactions with NURF55 (Nekrasov et al., 2005). NURF55 binding epitopes were recently mapped to the N-terminal domain of SU(Z)12 (Nowak et al., 2011; Schmitges et al., 2011). In *Drosophila*, there are no known strong loss of function missense alleles of SU(Z)12 in the conserved

domains with the exception of *Su(z)12*^{2}, a G to a D change in residue 274 [G274D] in the N-terminal domain. G274D is a partial loss of function allele with flies arresting as pharate adults (Birve et al., 2001). In this study, we took a systematic approach to dissect the function of the conserved domains in SU(Z)12.

2.4.1 Role of the conserved C2H2 zinc finger domain of SU(Z)12.

This study reveals a functional requirement for the zinc finger domain of SU(Z)12. Our use of *in vitro* assays first showed that the loss of zinger finger domain does not alter the intrinsic E(Z) mediated catalytic function of PRC2 [Fig. 1E]. Although zinc fingers are known nucleic acid and protein-interacting domains, in SU(Z)12 the zinc finger does not have DNA binding properties (Birve et al., 2001) leaving RNA or protein-interactions as possible functions. Mutations that disrupt the zinc finger domain retained complete assembly of the PRC2 complex *in vitro* [Fig. 1B and Fig. 1D] suggesting that the zinc finger is neither required for SU(Z)12 stability nor the stability of assembled PRC2. Similar observations were made in fly embryos *in vivo*. As a first step toward studying the function of SU(Z)12 zinc finger domain *in vivo*, we defined a genomic region that is sufficient to rescue the SU(Z)12 null phenotype. We then exploited this information to develop a cDNA rescue construct. We show that SU(Z)12 bearing a deletion or mutations that disrupt the zinc finger do not rescue SU(Z)12 nulls. Thus, despite dispensability of this zinc finger for PRC2 enzyme activity *in vitro*,

an *in vivo* requirement was established. This shifted the focus towards a potential role for the zinc finger domain in targeting PRC2 to its chromatin targets. In fly S2 cells, lack of the zinc finger resulted in the failure to efficiently bind the major PRE upstream of the *Drosophila Hox* gene *Ubx*. This observation confirmed the requirement of the zinc finger domain for *in vivo* PRC2 function, and it suggests a role in navigating PRC2 to PREs in flies. Future tests should determine if PCL, JING or even JARID2 binding is disrupted in the cells with PRC2 lacking the SU(Z)12 zinc finger. This could be assessed by Co-IPs on embryo extracts from transgenic animals expressing the HA-tagged SU(Z)12 mutants. Alternatively, we can test for these interactions in S2 cells expressing SU(Z)12 lacking its zinc finger. In mammals, the long non-coding RNA, HOTAIR can recruit PRC2 to target loci (Tsai et al., 2010; Chu et al., 2011). However, PRC2-interacting non-coding RNAs are poorly characterized in *Drosophila.* To begin addressing this, one could use biotinylated RNA pulldown assays (Tsai et al., 2010) to determine if fly PRC2 can bind HOTAIR non-coding RNA similar to mammalian PRC2.

2.4.2 Contributions of the VEFS domain to SU(Z)12 function.

As described above, the VEFS domain of SU(Z)12 can bind E(Z) as well as stimulate PRC2 catalytic function. A recent report also suggests that a C-terminal region of SU(Z)12 inclusive of the VEFS domain can sense the active H3K4me3 chromatin mark thereby inhibiting PRC2 enzyme activity (Schmitges et al., 2011; O'Meara and Simon, 2012). The VEFS domain is also gaining a lot of

attention from clinical studies reporting missense mutations in the conserved residues in this domain in T-ALL and myeloid malignancies (Ntziachristos et al., 2012; Score et al., 2012). These mutations create a loss-of-function phenotype and ascribe a tumor suppressor function to SU(Z)12. Previously, we analyzed a series of missense mutations in certain charged residues of the VEFS domain that to varying degrees disrupted the catalytic function of PRC2 (Ketel et al., 2005). Here, we extended this analysis of the VEFs domain by generating mutations in fly PRC2 corresponding to changes seen in T-ALL and Myeloid malignancies namely E574A [E610G], W555C [W591C] and N582Y [N618Y]. Interestingly, we observed a pattern in the behavior of these mutants that, based on previous work and the current study, suggest functional sub-divisions of the VEFS domain [Fig 4A]. Mutations in the N-terminal region of the VEFS domain, [Subdomain-N], had specific impact in disrupting E(Z) mediated PRC2 catalytic function without perturbing PRC2 assembly. In contrast, several mutations in conserved residues in the C-terminal domain of VEFS, [Subdomain-C], selectively impaired the HMTase function. However, consistent with the overall requirement for the VEFS domain to stably bind E(Z) (Ketel et al., 2005), a single point mutation of the conserved residue N582 abolished binding of SU(Z)12 to E(Z). Taken together, we propose that the VEFS domain has two sub-regions with different contributions to the PRC2 holoenzyme [Fig 4A]. This may have clinical significance since it suggests that different VEFS mutations that reduce H3K27me3 levels in cancer cells may accomplish this via two different

mechanisms. While the W555C [W591C] mutation in T-ALL imparts significant reduction in HMTase activity of a fully assembled complex, the N582Y [N618Y] missense mutation disrupts the binding of SU(Z)12 to E(Z) and thereby perturbs PRC2 complex assembly. These data underscore the importance of revealing the mechanistic consequences of disease promoting mutations to help translate clinical observations into therapeutic approaches. Future work should explore the function of the two VEFS domains *in vivo*. The genetic rescue assay developed here to assess zinc finger functional requirement *in vivo* will be further deployed to gain more insight into its mechanistic contributions to PRC2.

2.4.3 Role of the conserved N-terminal domain of SU(Z)12 and implications in PRC2 function.

The N-terminal domain of SU(Z)12 is a region with short stretches of highly conserved blocks interspersed with less conserved stretches. This region harbors the recently mapped, highly conserved NURF55 binding epitope [NBE] (Nowak et al., 2011; Schmitges et al., 2011). Also of significance is the observation that recombinant mammalian JARID2 can directly interact with the N-terminal region of SUZ12 (Peng et al., 2009). The only known missense allele in a conserved domain of SU(Z)12 resides in the N-terminal domain of SU(Z)12 [G274D]. We previously showed that recombinant complexes bearing the G274D mutation display subtle defects in the assembly of the complex and HMTase function (Ketel et al., 2005). In this study, we performed a more detailed

analysis of this N-terminal domain. When four progressive deletions of this domain were tested for assembly and enzyme function, we found a modest defect in the assembly of the SU(Z)12 subunit into PRC2 [Fig. 5B]. This data is suggest E(Z) binding determinants exists in the N-terminal domain of SU(Z)12 outside of the VEFS domain. Thus, the modest reduction in the HMTase function of SU(Z)12 can be attributed to defective assembly of the complex [Fig. 5C].

A recent report suggested that the zinc finger plus the VEFS domain constitute the minimal region required for SU(Z)12 mediated function within PRC2 (Schmitges et al., 2011) suggesting that the N-terminal domain is not critical. However, our analysis found that truncations in the N-terminal domain failed to rescue the larval lethality of SU(Z)12 nulls. Although, PRC2 can still methylate histones without this domain present, this result suggests a significant functional contribution *in vivo*. In the same study, the authors mapped the region in SU(Z)12 required for the stable interaction with NURF55. The authors reported that the region between 73-143 was required. We tested the contributions of this domain to assembly of the full PRC2 complex. Our data [Fig. 5B] suggests the existence of previously unmapped NURF55 binding regions in SU(Z)12 outside of the NBE and possible binding sites for NURF55 in E(Z). The data on the exact locations of NURF55 binding determinants in PRC2 complex is conflicting. Data from our group showed that NURF55 does not bind to E(Z) in pairwise binding tests (Ketel et al., 2005). However there are other reports of NURF55 directly binding to E(Z) (Tie et al., 2001; Nekrasov et al., 2005). This conundrum remains

unsolved based on two different observations made in this study. First, the VEFS mutant N582Y disrupts the binding of SU(Z)12 to E(Z) [Fig 4B]. However, we still observed low levels of NURF55 in this complex. Second, the progressive N-terminal deletions of SU(Z)12 tested in reconstituted PRC2 [Fig 5B] retained NURF55, albeit at reduced levels. The uncertainity in the exact location of NURF55 binding sites can be attributed to differences in conditions used to purify the recombinant complexes combined with the weak nature of its interaction with E(Z). SU(Z)12 can directly bind NURF55 (data not shown), however, in the context of a fully functional complex, E(Z) may be required to stabilize this interaction. More binding tests using different salt and buffer conditions may shed further light on these interactions. Ultimately, the derivation of a three-dimensional structure for the intact PRC2 complex should reveal much about these unresolved subunit-subunit contacts.

Chapter 3

Phosphorylation of EZH2 by CDKs: Modulation of gene silencing by PRC2.

This chapter is part of a published work (Chen et al., 2010) with contents partially modified or unmodified to highlight principal findings and my individual contributions. Sections on methodology, figure legends and portions of the results section are presented as published with permission from the publisher to reproduce the contents [attached in the previous page]. The work described in this chapter is the outcome of a collaboration with Prof. Haojie Huang, Mayo Clinic, Rochester MN (corresponding author) and Dr. Shuai Chen (first author). I performed the experiments in Supplementary figure 4 (a) and 4 (b) in the original publication [Fig 3A and Fig 3B in this chapter].

3.1 Introduction

Polycomb group [PcG] gene silencing regulates genes that control cell proliferation, cell fate decisions and development. Mammalian PRC2 consists of three PcG proteins EZH2, SUZ12 and EED and the histone binding protein RbAP48/46 (Cao et al., 2002; Kuzmichev et al., 2002). Similar to fly PRC2, the catalytic function of mammalian PRC2 is to methylate H3K27 provided by EZH2 via its SET domain (Cao et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). Unlike its fly counterparts, mammalian PRC2 subunits EED and EZH2 have four and two isoforms respectively, contributing to different flavors of PRC2 (Kirmizis

et al., 2004; Simon and Kingston, 2009). The last decade has seen considerable progress in determining the composition of PcG complexes and their roles in several biological processes including cancer progression and stem cell renewal and differentiation (Sauvageau and Sauvageau, 2010). However, our understanding of the regulatory inputs that modulate the activity of the core PcG components is minimal. Levels of SU(Z)12, EED and EZH2 are regulated by the pRB/E2F cell cycle regulatory pathway (Bracken et al., 2003; Cao and Zhang, 2004a). Deregulation of the pRB pathway is implicated in uncontrolled cell proliferation and, not surprisingly, levels of the core PRC2 components are up regulated in several metastatic forms of cancer (Varambally et al., 2002; Bracken et al., 2003). High levels of EZH2 is associated with poor prognosis in prostate cancer patients (Varambally et al., 2002), with suggested roles in hyper-silencing tumor suppressor genes like $p16^{INK4A}$, DAB2IP and ARB2 (Chen et al., 2010). In short, abnormally high levels of EZH2 and H3K27me3 are consistent with an oncogenic role of EZH2 in solid tumors, particularly in the most aggressive tumor types. EZH2 is subject to regulation by post-translational modifications like phosphorylation (Cha et al., 2005) and sumoylation. Histone methyltransferase activity of mammalian PRC2 is regulated by the Akt signaling pathway through the phosphorylation of Ser21 in EZH2 (Cha et al., 2005). This phosphorylation event is reported to inhibit the ability of EZH2 to associate with H3 but not SUZ12 or EED. Transcription of the *EZH2* gene is negatively regulated by microRNA miR-101(Varambally et al., 2008; Cao et al., 2010). EZH2 levels are high in

actively dividing cells, while the levels are low in differentiated cells, suggesting the possible involvement of CDKs in regulating EZH2 mediated PRC2 function (Bracken et al., 2003; Chen et al., 2010). The functional partnerships of cyclins and cyclin dependent kinases (CDKs) are critical to drive the cell cycle. Combined with the long standing link of EZH2 to cancer progression (Varambally et al., 2002; Simon and Lange, 2008), EZH2 could be functionally regulated by cyclin dependent kinases. In this study, a role for cyclin dependent kinases in regulating EZH2 and PRC2 enzyme function was revealed. CDK1 and CDK2 levels are high in actively dividing cells where, the demand for EZH2 is high (Zeng et al., 2011). We show that cyclin dependent kinases CDK1 and CDK2 can phosphorylate Threonine (T) residues in the consensus CDK phosphorylation motifs K/R S/T P X K/R (where X is any amino acid) which is associated with subsequent increased deposition of H3K27me3 at target loci. We conclude that the phosphorylation event does not affect the intrinsic enzyme function, stability, or formation of PRC2. Instead, PRC2 function is stimulated through enhanced targeting of PRC2 to genomic sites. This report is one of the first to demonstrate a mechanism that positively regulates the function of EZH2 by cell cycle regulators. This study has clinical significance as there is a lot of interest in developing CDK inhibitors as anti cancer therapeutics, and some that are already in clinical trials (Schwartz, 2005).

3.2 Materials and Methods

3.2.1 *In vitro* HMTase assay.

The HMTase assay was modified from the method published previously (Müller et al., 2002). Immunoprecipitated proteins from transfected 293T cells or proteins expressed and purified from insect Sf9 cells, and HeLa polynucleosomes were added to the HMTase reaction buffer (12 mM Hepes [pH 7.9], 0.24 mM EDTA, 12% glycerol, 4 mM DTT, 2.5 mM MgCl2, 0.5~1 M 3H-SAM, and 60~110 mM KCl), and incubated for 60 min at 30ºC. Reactions were stopped with SDS-sample buffer, resolved by 18% SDS-PAGE, and transferred to Immobilon-P (Millipore), sprayed with EN3HANCE (NEN) and exposed to film. The immunoprecipitated wild-type and mutated EZH2 proteins were analyzed by Western blot analysis with an anti-EZH2 antibody, and the insect cell expressed recombinant PRC2 complexes were visualized by Coomassie blue staining.

3.2.2 Chromatin immunoprecipitation (ChIP) assay.

LNCaP cells were transfected with control siRNA, EZH2 siRNA or EZH2 siRNA plus siRNA-resistant wild-type EZH2 or T350A mutant. At 60 h after transfection, cells were crosslinked with 1% formaldehyde for 10 min, harvested, lysed in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0 and protease inhibitor cocktail (Sigma-Aldrich), and sonicated to produce soluble chromatin with the genomic DNA at an average size of 400-1000 bp. The soluble chromatin was pre-cleared by incubation with sheared salmon sperm DNA. One

portion

of the supernatant was used as a DNA input control, and the remainder was incubated with an anti-H3K27me3 antibody overnight at 4°C. Immunoprecipitated complexes were washed successively with wash buffer (300 mM NaCl, 50 mM Tris pH 8.0, 2.7 mM KCl, 0.05% Tween 20 and 1% deoxycholate) and Tris-EDTA (TE) buffer. The bound protein-DNA immunocomplexes were eluted three times with ChIP elution buffer (1% SDS, 0.1 M NaHCO3) and de-crosslinked at 65°C for 4 h. The DNA was extracted with a PCR purification kit (Invitrogen) and was subjected to real-time PCR amplification using the primers specific for the H3K27me3 region in the promoters of the HOXA9 and DAB2IP genes. The data for the occupancy of H3K27me3 are relative to control siRNA (si-C)-transfected cells and are expressed as a ratio of the cycle threshold for the chromatin immunoprecipitation DNA versus the cycle threshold for the input (1%) samples.

3.2.3 *In-vitro* kinase assay.

Kinase assays were carried out in the presence of [^{32}P] ATP using an *in vitro* kinase buffer system from Cell Signaling Technology. Briefly, recombinant CDK and cyclin complexes were incubated with various substrates at 30°C for 45 min, and the reaction samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

3.3 Results

3.3.1 Identification of consensus CDK phosphorylation motifs in EZH2.

EZH2 possesses the highly conserved SET domain and CXC domains in

its C-terminal region, and EID [EED interacting domain], Domain I and II in its N-terminal region (O'Meara and Simon, 2012). In between Domain II and the CXC domain, EZH2 harbors one perfectly (threonine 350, T350) and two imperfectly matched (threonine 421, T421 and threonine 492, T492) CDK phosphorylation motifs K/R S/T P X K/R, where X denotes any residue [Fig. 1A]. To assess the phosphorylation of EZH2 by CDKs, *in vitro* protein kinase assays were performed on GST- fusions of the amino terminal (amino acids 1-559), and carboxy terminal (560-741) portions of EZH2. As expected, histone H1B, a known CDK1 substrate, was readily phosphorylated in these assays whereas no phosphorylation of the control Glutathione S transferase protein was observed [Fig. 1B]. Of the two EZH2 portions tested, only EZH2 [1-599] gave a signal, suggesting direct phosphorylation by CDK1 [Fig. 1B].

Mutation of T350 to alanine (T350A) resulted in approximately 60% reduction in EZH2- (1-559) phosphorylation mediated by CDK1 [Fig. 1C]. In contrast, approximately 30% or no reduction in EZH2-(1-559) phosphorylation was observed when the T421A and T492A mutants were used as substrates [Fig. 1C]. This suggests that T350 in EZH2 is the major site phosphorylated by the CDK1/cyclin B1 complex *in vitro*. Further analysis showed that the CDK2/cyclin E and CDK2/cyclin A complexes can also phosphorylate EZH2 and that this phosphorylation was largely or completely abolished by the T350A mutation [Fig. 1D]. These data indicate that EZH2 protein can be specifically phosphorylated at the T350 residue by different CDKs *in vitro*. Notably, this

residue is present in a consensus CDK phosphorylation motif that is evolutionarily conserved from fruit flies to humans [Fig. 1A]. The *in vitro* analysis was confirmed *in vivo* by generating a T350 phosphorylation specific antibody. Ectopic expression of CDK1/cyclin B1 and CDK2/cyclin E in LNCaP prostate cancer cells and 293T cells substantially increased phosphorylation levels of both endogenous and Myc-tagged EZH2, whereas the mutant EZH2 T350A did not [data not shown; (Chen et al., 2010)]. Consistent with this observation, co-transfection of CDK1 inhibitor $p21^{WAF1}$ significantly reduced the levels of phosphorylation of the T350 residue [data not shown; (Chen et al., 2010)].

3.3.2 Functional consequence of T350 phosphorylation upon EZH2 mediated H3K27me3.

The functional readout of PRC2 function is H3K27me3 and silencing of target genes genome wide. In an attempt to gain insight into the functional consequence of EZH2 phosphorylation by CDK1 and CDK2, microarray profiling of known EZH2 target genes were performed. LNCaP cells were depleted of endogenous EZH2 [data not shown; (Chen et al., 2010)] using an siRNA approach, and wild-type levels restored with siRNA resistant EZH2 and EZH2 T350A. Cells treated with roscovitine, a known CDK inhibitor were used for comparison. Significant loss of global methylation was observed in cells expressing the T350A mutation as well as roscovitine treated cells compared to cells expressing wild-type levels of EZH2. Together, these results demonstrate

that CDK mediated phosphorylation of EZH2 via T350 is critical to deposit H3K27me3 on PRC2 targets [data not shown; (Chen et al., 2010)]. To understand the modulatory effect of T350 phosphorylation in detail, *HOXA9* and *DAB2IP*, two known PRC2 target genes were analyzed further. To determine whether EZH2 phosphorylation at T350 affects the level of H3K27me3 in the HOXA9 and DAB2IP promoters, endogenous EZH2 was knocked down and levels restored by ectopic expression of siRNA-resistant wild-type or T350A-mutated EZH2 [Fig 2A]. As expected, chromatin immunoprecipitation (ChIP) analysis demonstrated that knockdown of EZH2 decreased the level of H3K27me3 in the promoter regions analyzed [Fig. 2B]. This effect was largely reversed by the restored expression of the wild-type EZH2, but not the T350A mutant [Fig. 2B]. Accordingly, knockdown of endogenous EZH2 resulted in an increase in HOXA9 expression in LNCaP cells [data not shown; (Chen et al., 2010)]. HOXA9 and DAB2IP gene expression were silenced by restored expression of the wild-type EZH2. However, this effect was substantially compromised by the expression of the T350A mutant (data not shown). In agreement with these findings, overexpression of CDK1/cyclin B1 or CDK2/cyclin E resulted in a decrease in HOXA9 expression [data not shown; (Chen et al., 2010)] whereas knockdown of both endogenous CDK1 and CDK2 significantly increased the levels of HOXA9 mRNA [data not shown; (Chen et al., 2010)]. We conclude that T350 phosphorylation of EZH2 by CDKs is important for control of H3K27me3 levels in the HOXA9 promoter and for HOXA9 expression.

Figure 1A: Comparison of the amino-acid sequence of EZH2 homologues near the CDK phosphorylation site (Thr 350 indicated by asterisk).

A

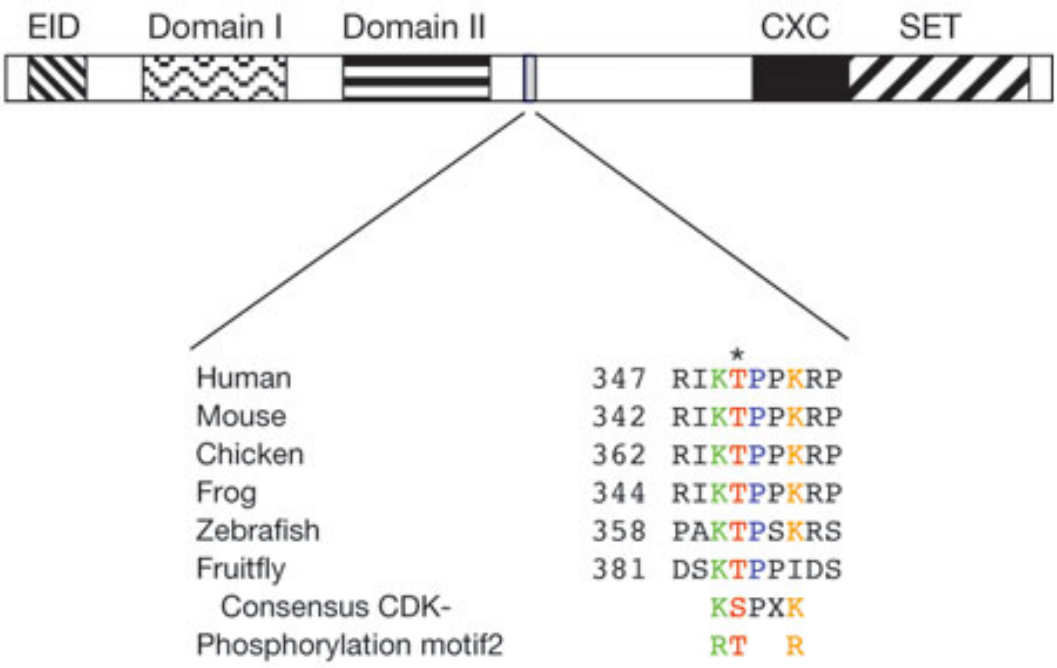

EID
Domain I
Domain II
CXC
SET
Human 347 RIKTPPKRP
Mouse 342 RIKTPPKRP
Chicken 362 RIKTPPKRP
Frog 344 RIKTPPKRP
Zebrafish 358 PAKTPSKRS
Fruitfly 381 DSKTPPIDS
Consensus CDK-Phosphorylation motif2 KSPXK
RT R

Figure 1B

Left: *In vitro* kinase assay. Recombinant CDK1–cyclin B1 protein complex was incubated with [γ-32P]ATP and the indicated substrates. Reaction samples were resolved by SDS–PAGE and visualized by autoradiography. Right: protein substrates revealed by Coomassie blue staining.

B

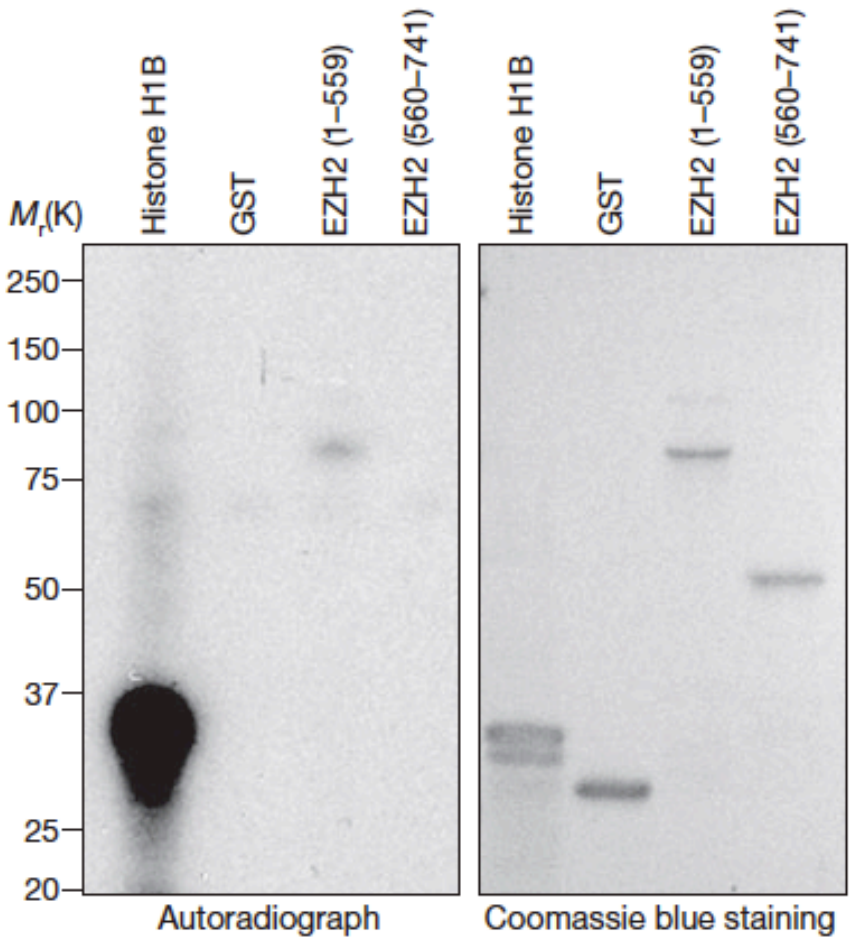

Histone H1B
GST
EZH2 (1–559)
EZH2 (560–741)
Histone H1B
GST
EZH2 (1–559)
EZH2 (560–741)
M_r(K)
250
150
100
75
50
37
25
20
Autoradiograph
Coomassie blue staining

Figure 1C

Left: *In vitro* CDK1 kinase assay using a wildtype

(WT) EZH2 GST-fusion protein fragment (amino-acid residues 1–559), and

T350A, T421A, and T492A mutants of EZH2 (amino-acid residues 1–559), as

substrates. Right: protein substrates indicated by Coomassie blue staining.

C

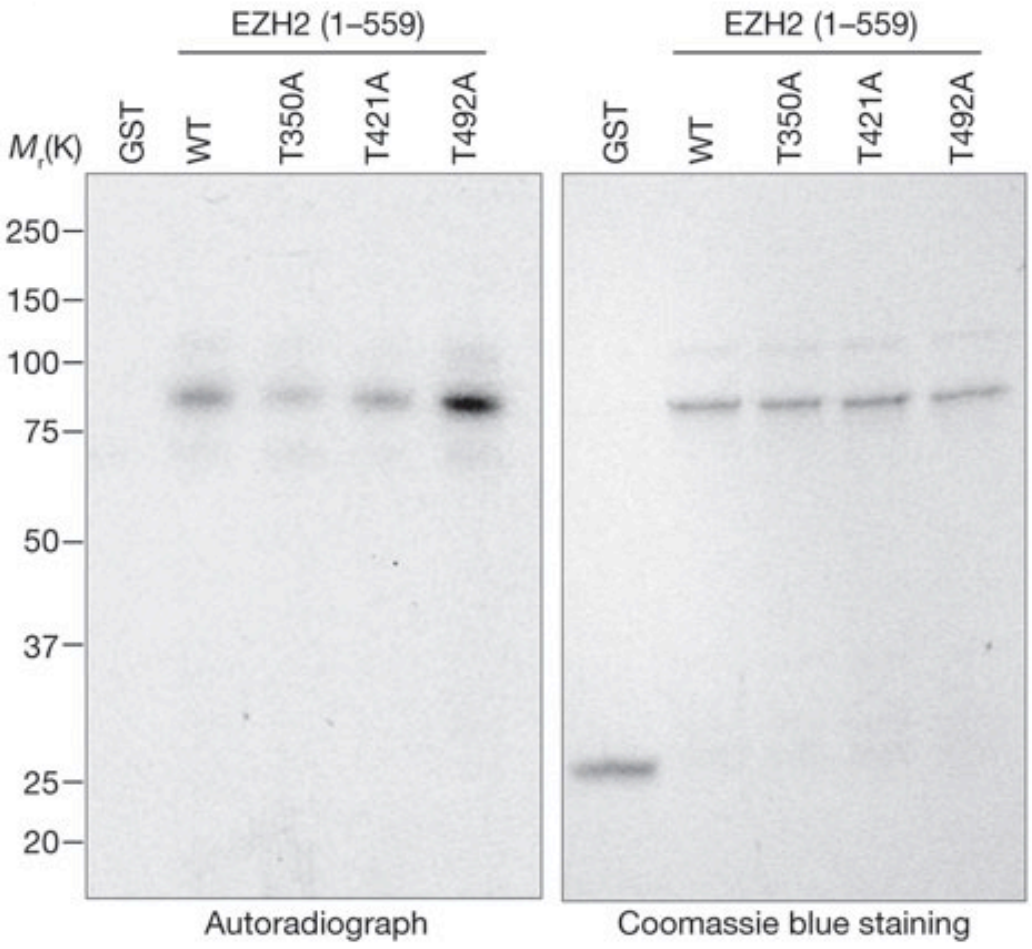

EZH2 (1–559)
EZH2 (1–559)
M_r(K)
GST
WT
T350A
T421A
T492A
GST
WT
T350A
T421A
T492A
250
150
100
75
50
37
25
20
Autoradiograph
Coomassie blue staining

Figure 1D

Top: *In vitro* CDK2 and CDK6 kinase assays using GST, a wild-type EZH2 GST fusion protein segment (residues 1–559) and a EZH2 (residues 1–599) T350A mutant, as substrates. Bottom: protein substrates indicated by Coomassie blue staining.

D

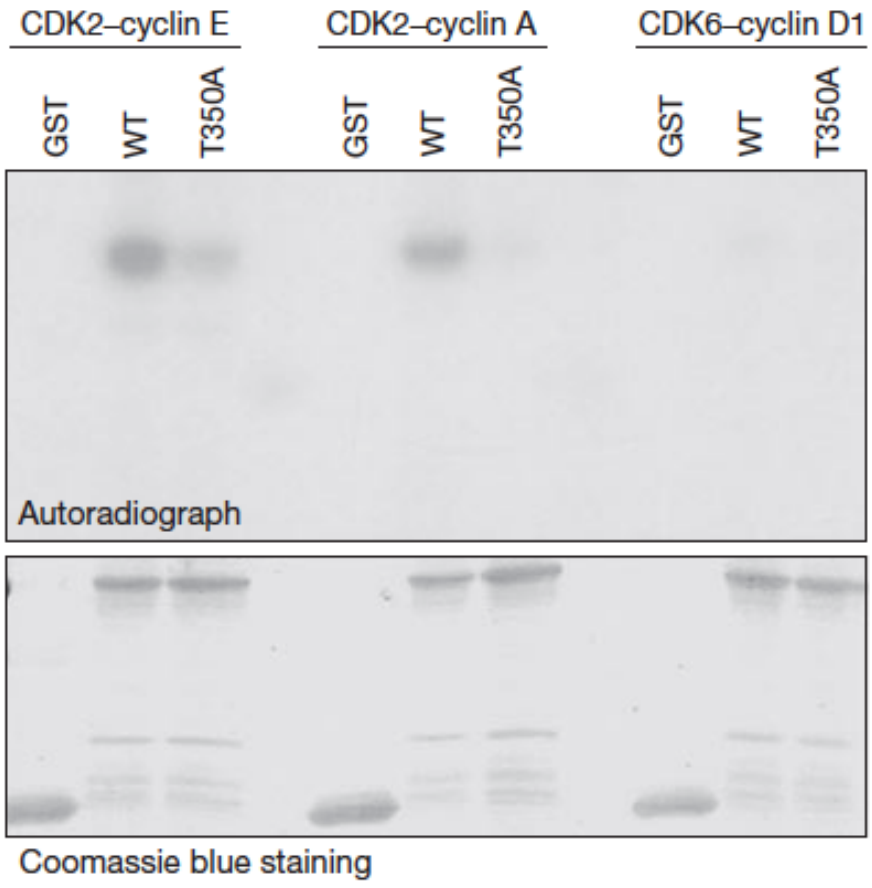
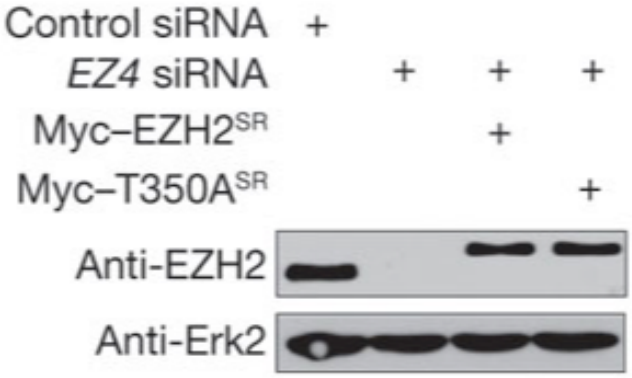

CDK2–cyclin E
CDK2–cyclin A
CDK6–cyclin D1
GST
WT
T350A
GST
WT
T350A
GST
WT
T350A
Autoradiograph
Coomassie blue staining

Figure 2: The effect of Thr 350 phosphorylation on H3K27me3 levels at EZH2-target-gene promoters.

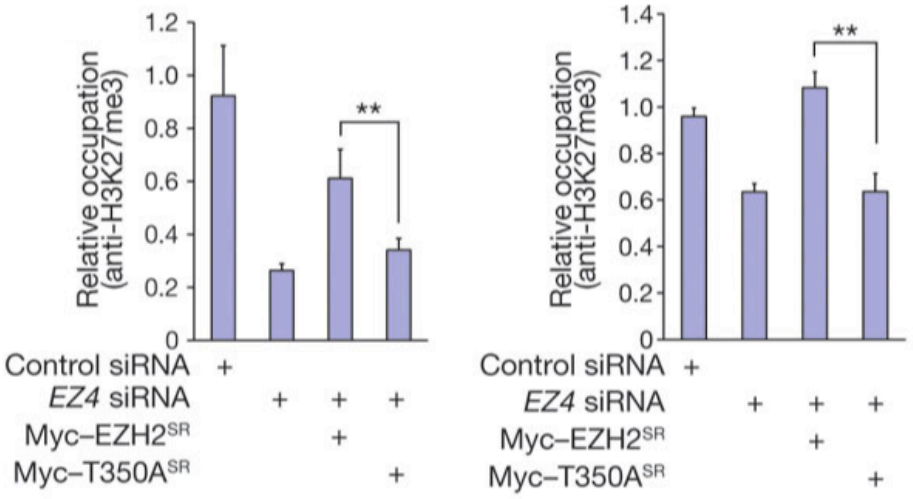
A) LNCaP cells were transfected with control or *EZH2*-specific siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2T350A (or empty control plasmids), as indicated. At 60 h after transfection, expression of EZH2 was examined by western blot.

B) H3K27me3 levels in promoters of the EZH2-target-genes, *HOXA9* (left) and *DAB2IP* (right), were assessed by ChIP assays using anti-H3K27me3 antibodies. Data are means ± s.d. from three individual experiments ($n = 3$). Asterisks indicate $P < 0.01$.

A


Control siRNA +
EZ4 siRNA + + +
Myc–EZH2SR +
Myc–T350ASR +
Anti-EZH2
Anti-Erk2

B


Relative occupation (anti-H3K27me3)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Control siRNA +
EZ4 siRNA + + +
Myc–EZH2SR +
Myc–T350ASR +
Relative occupation (anti-H3K27me3)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Control siRNA +
EZ4 siRNA + + +
Myc–EZH2SR +
Myc–T350ASR +

3.3.3 Phosphorylation of EZH2 by CDK1 and CDK2 does not affect the intrinsic histone methyltransferase activity of PRC2.

The reduced level of H3K27me3 at genomic sites in cells expressing phosphorylation-defective EZH2 can be explained in one of the two ways; it could result from 1) EZH2 losing its enzymatic ability to transfer methyl groups to histone substrates or 2) failure of PRC2 bearing T350A mutant EZH2 to target properly to chromatin sites. To distinguish these two possibilities, we generated, purified and tested a recombinant mammalian PRC2 containing mouse ENX [EZH2], human SUZ12, human EED and human RbAp48/46 [expression constructs provided by Prof. Yi Zhang, University of North Carolina, Chapel Hill] using a baculovirus expression system. Mutant PRC2 bearing mutations in T345A [the residue corresponding to T350 in humans], and in-frame deletions of the SET domain [negative control] were generated in parallel. The T345A PRC2 complex retained the ability to assemble *in vitro* [Fig 3A, top], suggesting that phosphorylation of EZH2 on T345 does not alter subunit interactions with PRC2 [Fig 3A]. As expected, when tested for the ability to methylate HeLa polynucleosome substrates, wild-type PRC2 showed robust methylation and PRC2 lacking SET domain failed to methylate H3 tails. Surprisingly, the T345A mutant showed robust levels of methylation comparable to wild-type PRC2 *in vitro* [Fig 3A, bottom].

Similar observations were made on wild-type and T350A PRC2 complexes using the prostate cancer cell line, LNCaP. Myc-tagged wild-type or T350A-

mutated EZH2 were transfected into LNCaP cells and the resulting PRC2 complexes containing wild-type or mutated EZH2 proteins were recovered by immunoprecipitation using anti-myc antibody [Fig 3B, top]. These complexes were then subjected to *in vitro* HMTase assays [Fig 3B, bottom]. We observed comparable levels of HMTase with the wild-type and T350A mutant versions of PRC2. Taken together, these data suggest that T350 phosphorylation of EZH2 by CDKs does not affect the intrinsic HMTase activity, stability, or formation of the PRC2 complex.

3.3.4 Phosphorylation of T350A enhances targeting of PRC2 to chromatin.

To gain insight into the mechanism of PRC2 modulation by CDK-mediated phosphorylation of EZH2, we next tested if PRC2 lacking the T350 residue affects PRC2 targeting to chromatin sites. Chromatin IP on promoters of *HOXA9* and *DAB2IP*, known PRC2 targets, were performed to detect the localization of PRC2 in cells expressing wild-type and T350A EZH2 mutant. We observed that binding of PRC2 to promoters of HOXA9 and DAB2IP was significantly lower in cells expressing theT350A mutant form of PRC2 [Fig 4A and 4B]. We conclude that the reduction of H3K27me3 levels in cells expressing EZH2 T350A [Fig 2] is likely due to a defect in genomic targeting of PRC2.

Figure 3A:

Effects of T345 mutation on *in vitro* HMTase activity and protein assembly of recombinant PRC2.

Baculovirus vectors for wild-type or a phosphorylation-resistant mutant of mouse Ezh2 (Enx-1), in which the CDK site T345 (Fig.1A) was mutated to alanine, were co-infected with the other three PRC2 components (Flag-EED, SUZ12, RbAp48) into insect Sf9 cells. PRC2 complexes containing different forms of Enx-1 were immunopurified by anti-Flag antibody (top panel) and then subjected to in vitro HMTase assays using HeLa polynucleosomes as substrate (bottom panel).

A

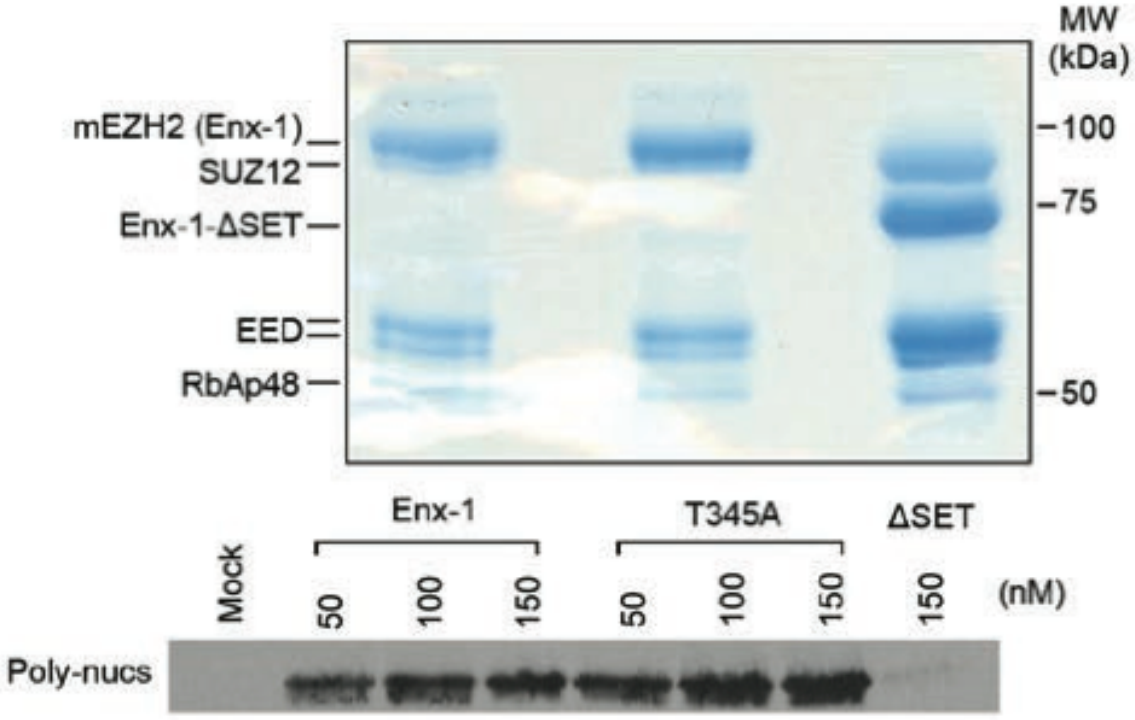

MW
(kDa)
mEZH2 (Enx-1)
SUZ12
Enx-1-ΔSET
EED
RbAp48
100
75
50
Enx-1
T345A
ΔSET
Mock
50
100
150
50
100
150
150
(nM)
Poly-nucs

Figure 3B:

Effects of EZH2 T350 phosphorylation on HMTase activity and assembly of PRC2 isolated from LNCaP cells.

Myc-tagged EZH2, T350A, or ΔSET mutants were overexpressed in LNCaP cells, and immunoprecipitated by anti-myc antibodies. The recovery of these proteins and SUZ12 and EED binding to EZH2 were evaluated by western blots (top panel). The methyltransferase activities of these PRC2 complexes were then tested by *in vitro* HMTase assay using HeLa polynucleosomes (poly-nucs) as substrates (bottom panel). The asterisk (*) indicates nonspecific bands.

B

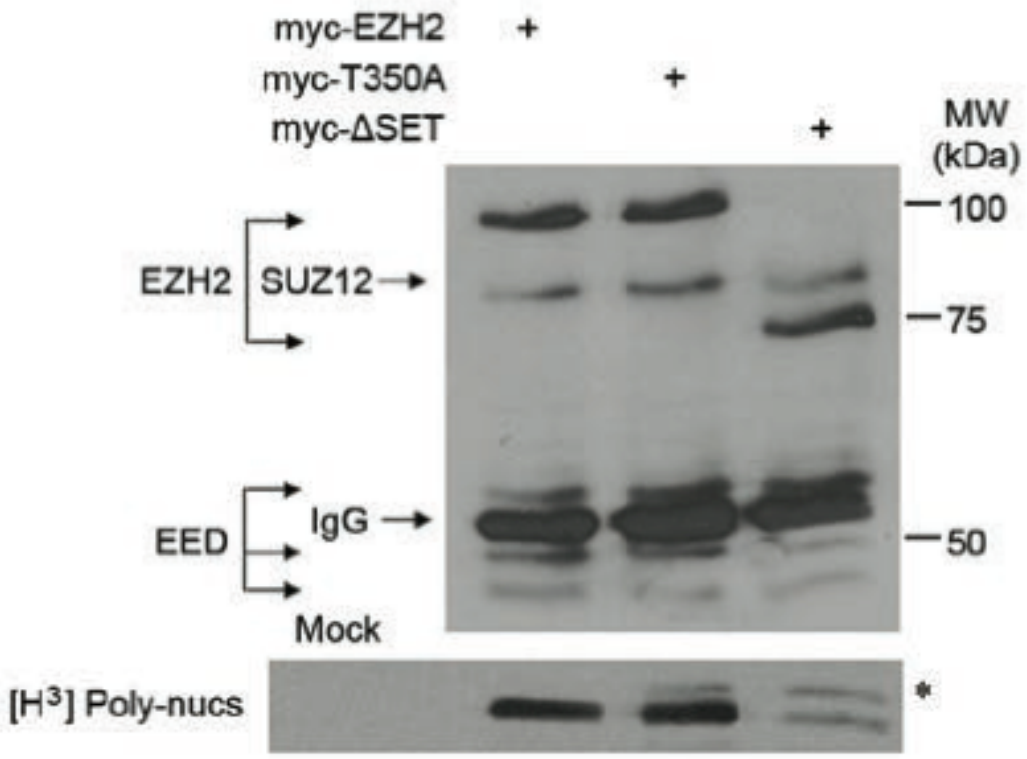
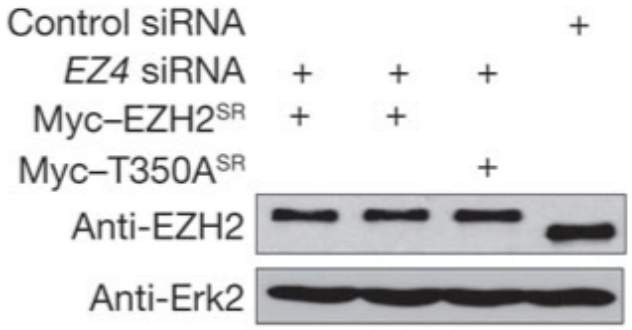

myc-EZH2 +
myc-T350A +
myc-ΔSET +
MW (kDa)
100
75
50
EZH2
SUZ12
EED
IgG
Mock
[H3] Poly-nucs
*

Figure 4: The effect of Thr 350 phosphorylation on EZH2 recruitment to EZH2-target-gene promoters.

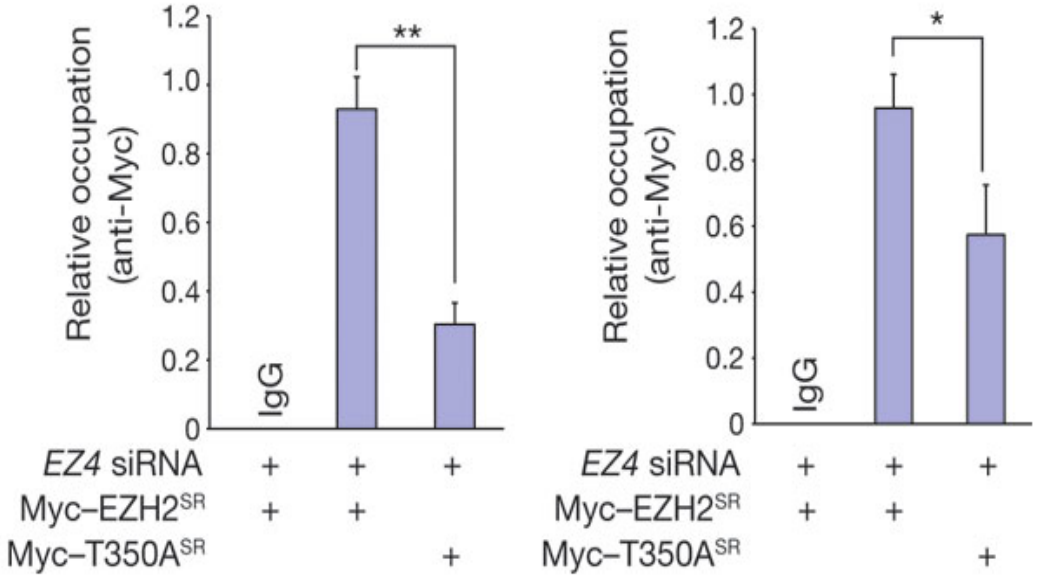
A) LNCaP cells were transfected with control or *EZH2*-specific. siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc tagged, siRNA-resistant EZH2 T350A as indicated. At 60 h after transfection, expression of endogenous and restored EZH2 was examined by western blot.

B) The binding of Myc–EZH2 and Myc–EZH2 T350A to *HOXA9* (left) and *DAB2IP* (right) promoters was examined by ChIP assays with anti-Myc antibody. Data are means ± s.d. from three individual experiments ($n = 3$). Asterisk indicates $P < 0.05$, double asterisks indicate $P < 0.01$. IgG; immunoglobulin G used as control antibody in ChIP assay.

A


Control siRNA +
EZ4 siRNA + + +
Myc–EZH2SR + +
Myc–T350ASR +
Anti-EZH2
Anti-Erk2

B


Relative occupation (anti-Myc)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
IgG
EZ4 siRNA + + +
Myc–EZH2SR + +
Myc–T350ASR +
Relative occupation (anti-Myc)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
IgG
EZ4 siRNA + + +
Myc–EZH2SR + +
Myc–T350ASR +

3.4 Discussion

This study demonstrates a role for CDKs in the regulation of PcG silencing. We find that EZH2 is phosphorylated at an evolutionarily conserved CDK phosphorylation motif that in turn modulates H3K27me3 deposition. The high degree of conservation of this motif suggests the existence of similar mechanisms in other organisms. We show that the functional consequence of EZH2 phosphorylation by CDKs is to enhance H3K27me3 levels at target gene promoters. Enhanced methylation however is not achieved by direct regulation to the intrinsic enzyme function of PRC2. Instead, EZH2 phosphorylation stimulates recruitment of PRC2 to promoters of target genes. Consistent with our studies, another report showed that mouse ENX gets phosphorylated at the T345 residue in the same consensus CDK phosphorylation motif and a mutation in this residue disrupts binding to HOTAIR, a non-coding RNA with a well-characterized role in recruiting PRC2 to genomic sites (Kaneko et al., 2010). However, two other studies report CDK phosphorylation at EZH2 residues T492, as well as T345, with different mechanistic outputs (Wei et al., 2011; Wu and Zhang, 2011). Wei et.al, 2011 showed that phosphorylation of EZH2 T492 reduces PRC2 enzyme function as a result of disrupting its association with the non-catalytic subunits. On the other hand, Wu et.al., 2011 showed that phosphorylation of mouse EZH2 at T345 and T487 targets EZH2 for ubiquitin-mediated degradation. Given that EZH2 is required to silence differentiation specific genes, our results tend to support a model wherein induction of CDK inhibitors, such as p16INK4A,

p21WAF1, leads to CDK inactivation and de-repression of cell differentiation regulators due to decreased phosphorylation and function of EZH2. In addition to its normal role in repression of cell differentiation, EZH2 is also important for oncogenesis by driving cancer cell proliferation and migration. We also provided evidence that T350 phosphorylation is essential for these functions of EZH2 in prostate cancer cells (Chen et al., 2010). Because CDK activity is often elevated in human cancers, our data suggest that aberrant activation of CDKs may contribute to the aggressive phenotype of tumors by phosphorylating and potentiating the oncogenic and gene-silencing functions of EZH2. Considering CDK inhibitors are in clinical trials as potential cancer therapeutics, it is critical to understand the precise mechanisms and pathways by which CDKs control PRC2 function.

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