Fall 12-17-2022

Roles of Individual Protein in de novo Polycomb Protein Recruitment

Piao Ye
pye@smu.edu

Roles of Individual Protein in *de novo* Polycomb Protein Recruitment

Approved by:

_____

Dr. Richard Jones
Professor of Biology

_____

Dr. Adam Norris
Professor of Biology

_____

Dr. Robert Harrod
Professor of Biology

Roles of Individual Protein in *de novo* Polycomb Protein Recruitment

A Thesis Presented to the Graduate Faculty of the

Dedman College

Southern Methodist University

in

Partial Fulfillment of the Requirements

for the degree of

Master of Science

with a

Major in Biology

by

Piao Ye

B.S., Biotechnology, South China Agricultural University

December 17, 2022

ACKNOWLEDGMENTS

I want to thank Dr. Jones to give me the opportunity to execute my experiment under his guidance and Judith Benes teaching me everything about Drosophila. I'm very grateful for everything I learned in SMU, it played a very important role for my career in molecular biology.

Ye, Piao B.S., Biotechnology, South China Agricultural University

Roles of Individual Protein in *de novo* Polycomb Protein Recruitment

Advisor: Dr Richard Jones

Master of Science degree conferred December 17, 2022

Dissertation completed October 26, 2022

Polycomb Group (PcG) proteins are evolutionarily conserved epigenetic transcriptional regulators that maintain the transcriptional repression of silenced genes by maintaining heritable chromatin states. PcG proteins first discovered as repressors of Hox genes in Drosophila, later were shown to regulate a wide range of genes. In mammals, PcG proteins are involved in maintaining pluripotent state of stem cells and controlling cell differentiation. Misexpression of PcG protein leads to cancers like lymphoma and melanoma. PcG proteins maintain rather than initiate transcriptional repression, once PcG – mediated repression is established it can be maintained through an unlimited number of cycles. Most studies of PcG proteins are in vitro or focus on maintenance phase of repression. Little is known about the molecular mechanisms by which PcG proteins are initially recruited to target genes. The challenge of obtaining a homogenous population of cells in a certain developmental stage in which a target gene is uniformly repressed by PcG proteins, creates a major difficulty in studying recruitment of PcG proteins *in vivo*. To solve this problem, our lab previously generated a genetic system in which giant (gt), a PcG target gene, is ubiquitously repressed. In embryos produced by *bcd osk tsl* homozygous mother, maternal Hunchback (Hb) is ubiquitously expressed due to lack of *osk*. There is no zygotic Hb due to lack of bcd and *tsl*. gt remained repressed after maternal Hb is completely degraded at nuclear cycle

14, at the same time PcG proteins take over repression. Time course chromatin immunoprecipitation (ChIP) assay has previously been done on *bcd osk tsl* system to determine proteins distribution when PcG proteins take over repression. To study contributions of various proteins involved in recruitment, we knocked down each protein by RNAi in *bcd osk tsl* background followed by ChIP assays on embryos of different stages. The goal of my research is to define the roles of individual PcG proteins (as complexes) as well as participated transcription factors in facilitating *de novo* establishment of PcG silencing.

Table of contents

Introduction

PcG proteins are conserved epigenetic regulators. PcG proteins maintain target genes transcriptional state through numerous cycles by altering chromatin structure. PcG proteins function as protein complexes. The most studied PcG protein complexes are Pho-RC, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). PRC2 is mostly conserved in both Drosophila and mammals while PRC1 subunits have many variant homologs in mammals (Schwartz and Pirrotta 2013).

Polycomb Proteins Complexes

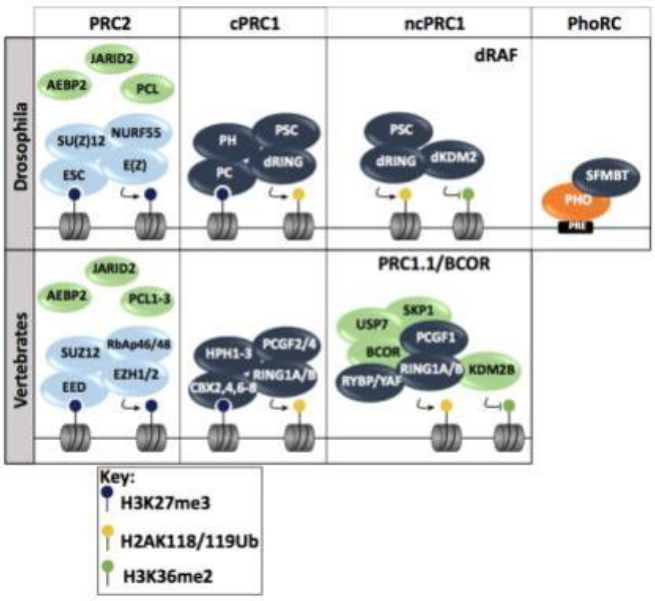

Figure 1 Graph showing components of PcG proteins (Schwartz and Pirrotta 2013)

Pho-RC

Pho-RC is the only complex that includes DNA binding protein but no chromatin alteration activity, therefore likely Pho-RC plays the important role of recruiting the rest of PcG proteins and take over repression. Pho-RC has subunits Pho and Sfmbt (Brown et al 1998, Brown et al 2003, Grimm et al 2009). Pho specifically binds to PRE (Polycomb response element) and recruit other PcG proteins. In Drosophila, PcG proteins are recruited to PRE, a specific cis-regulatory DNA elements (Blackledge, Rose and Klose 2015Pho binding sites are found in many PREs although Pho mutants only show mild homeotic phenotype compared to other PcG mutants (Brown et al 2003). Pho is needed but not sufficient for establishing PcG proteins binding on target genes (Wang et al 2004, Poux et al 2001). Pho is the only DNA binding protein in core complexes, it's considered the base of PcG proteins recruitment, however there are evidence show this is not always the case. Sfmbt has a SPM domain and four MBT domains which link it to a PcG protein Scm (Usui 2000). In vitro, Sfmbt and Scm interact with each other through their SPM domain and their interaction is sufficient to recruit Ph (Grimm et al 2009). Sfmbt may also involve in assisting PRC1 and/or PRC2 binding to target gene (Frey et al 2016, Wang et al 2010). Pho-RC, which contains Pho and Sfmbt, is considered the base of PcG proteins recruitment, but it may not always be the case.

PRC2

Polycomb repressive complex 2 is one of the most extensively studied complex. It contains subunits E(z), Su(z)12, Esc and P55 and it's mainly known for adding methylation to histone as repressive mark, followed by spreading methylation to nearby region.

All PRC2 subunits express maternally and lacking either maternal E(z) or Esc results in embryonic lethality (Struhl 1981, Jones and Gelbart 1990, Simon et al 1995). Su(z)12 interacts with E(z) through its VEFS-box domain (O'meara and Simon 2012). Nurf55/P55, also a chromatin assembly factor (Caf1) in Drosophila, has been shown to interact with Su(z)12 and together stabilize PRC2 binding on target gene (Martinez-Balbas et al 1998, Schmitges et al 2011). SET domain of E(z) has catalytic activity of mono-, di-, tri-methylate lysine 27 at histone 3, however E(z) cannot mediate H3K27me3 by itself. In reconstituted nucleosome assay, E(z) needs to be assembled into a complex with Su(z)12 and P55 to bind to nucleosomes and the complex needs Esc to mediate H3K27me3 (Nekrasov et al 2005). Esc has WD40 repeat which preferentially bind to H3K27me3 (Margueron et al 2009). Its N-terminal allows E(z) to interact with H3 and eventually establish H3K27me3 (Tie et al 2007).

Pcl is a substochiometric subunit of PRC2. Pcl germline mosaic stained with Abd-B antibody shows tissue specific phenotype which is a weaker phenotype than other PcG proteins (Soto et al 1995). E(z) can be co-purified with Pcl but Pcl is unable to be co-purified with E(z) (Nekrasov et al 2007). This is consistent with what previously has been shown that E(z) is dramatically decreased when Pcl is knocked down in wing discs while Pcl can still be present on PRE without E(z) (Urmi et al 2008). Pcl enhances PRC2 HMT activity both in vitro and in vivo (Nekrasov et

al 2007). Canonical PRC2 has been extensively studied for its function in mediating H3K27me3, however accessory protein of PRC2 seem to also play a role in mediating tri-methylating chromatin.

PRC1

There are two mechanisms, canonical and non-canonical, by which PRC1 is thought regulate gene expression. Canonical PRC1 is known to compact chromatin to maintain repression of target gene. It has been proposed PRC1 and SWI/SNF compete for nucleosome *in vitro* since nucleosomal template is unable to be remodel by SWI/SNF but ability to remodel is not blocked (Shao et al 1999). PRC1 also block RNA polymerase II preinitiation complex assembly (Lehmann et al 2012). Non-canonical PRC1 mediate H2A ubiquitination (King et al 2002), and it's called dRAF in Drosophila. dRAF has E3 ligase activity for ubiquitylating histone H2A. It mono- ubiquitinates H2AK118 in Drosophila and H2AK119 in mammals. It contains dRing/Sce, Psc and dKDM2. dRing is responsible for H2AK118ub in dRAF (Lagarou et al 2008). There is no evidence dRAF is present on *gt*, so non-canonical PRC1 is not a focus in this thesis.

Canonical PRC1 has no known enzymatic activity and contains four core subunits, Pc, Ph, dRing/Sce, Psc. Chromodomain of Pc can bind to H3K27me3, and its C-terminus is able to bind to nucleosome cores (Breiling et al 1999, Min et al 2003). dRing is a Ring finger protein and it doesn't have enzymatic activity in canonical PRC1 (Fristch et al 2003, Lagarou et al 2008). dRing itself can't compact nucleosome in vitro (King et al 2002) but it is needed for PcG mediated repression (Fristch et al 2003). Ph itself is sufficient to compact nucleosome *in vitro*, however Ph is not required for PRC1 to compact chromatin *in vivo* (King et al 2002, Francis et al 2004). Ph

contains a SPM domain which is conserved with Sfmbt and Scm, a substoichiometric subunit of PRC1. It has been proposed Sfmbt interacts with Scm, Scm interacts with Ph all through SPM domain and results in recruitment of PRC1. PRC2 is not mentioned in this model (Frey et al 2016). It has also been shown both *in vitro* and *in vivo*, knocking down Scm results in reduced level of PRC1 and PRC2 (Wang et al 2010). Subunit Psc is related to Su(z)2, which is not included in PcG. psc and su(z)2 are adjacent in Drosophila genome (ModENCODE). Even though Su(z)2 is not a PcG protein, it shows synergetic interaction with Scm and mutating both psc and su(z)2 enhances Psc mutant phenotype (Soto et al 1995). Psc is also sufficient to compact chromatin by itself *in vitro*. C- terminal region of Psc is responsible for inhibition of chromatin remodeling and transcription (King et al 2005). Psc and Su(z)2 are partially redundant in embryo and can fully substitute for each other in larvae (Beuchle et al 2001, Soto et al 1995). Canonical PRC1 recognizes repressive covalent histone modification like H3K27me3, compact chromatin and maintain repressive state of target gene, however individual role of each PRC1 component is not clear.

Mammalian PcG proteins complexes

Mammalian PcG proteins involve in embryonic development as well as cancers. PcG proteins play a role in controlling cell differentiation and maintaining embryonic stem cells pluripotency in mammal (Boyer et al 2006, Schwartz and Pirrotta 2013). PcG proteins are also responsible for maintaining x chromosome inactivation in females (Tie et al 2003). In various types of cancers, PcG proteins have been found mis-regulated and that results in abnormal silencing pattern in genes, especially tumor suppressor genes. (Kogo et al 2011, Bracken and Helin 2009).

Some subunits in mammalian PRC1 and PRC2 are structurally and functionally conserved with Drosophila PcG proteins. However, Ying-Yang-1 (YY1) which is the homolog of Pho in Drosophila, may not act the same as Pho in Drosophila.

Pho

Pho's mammalian homolog is YY1, which has a zinc finger domain. Pho, as a subunit of PhoRC complex recruits PcG proteins by binding to PRE, however there is no known PRE in mammals. There is evidence that CpG island in mammals may play a similar role as PREs in Drosophila (Bauer et al 2016). However, YY1's zinc finger domain is not necessary for PcG repression, REPO domain in YY1 is, even though mechanism is unknown (Wilkinson et al 2006).

PRC2

PRC2, one of the key complexes in PcG proteins, is mostly conserved in organisms. It's the only PcG proteins complex that find in unicellular eukaryotes (Margueron and Reinburg 2011). In mammals, PRC2 has four core subunits, Ezh1/2, Suz12, Eed and RbAp46/48. RbAp46/48 is homolog of Nurf55 in Drosophila. Eed is homolog of Esc and mutating Eed can result in loss of methyltransferase activity (Margueron et al 2009). Ezh1 and Ezh2 are not interchangeable and Ezh2 is a homolog of E(z) in Drosophila. Ezh1 presents in both dividing and differentiated cells while Ezh2 only presents in dividing cells. It's also been shown that Ezh1 has much lower H3K27me3 activity than Ezh2 (Margueron et al 2008, Shen et al 2008) and Ezh1 associates with H3K4me3 (Mousavi et al 2012). PRC2 also associates with its substoichiometric subunits. mammalian homologs of Pcl have PHD fingers which interact with PRC2. PHF19 has a TUDOR domain which specifically binds to H3K36me3 in mammal but not in Drosophila. PHF19 recruits

demethylase KDM5a which can remove methylation on H3K36. H3K36me3 has been shown to prevent PRC2 from methylating H3K27, removing H3K36me3 will facilitate binding of PRC2 to target genes. This mechanism only affects a small number of PcG target genes and it's not clear how it targets this group of genes. (Abed 2012).

PRC1

PRC1 has more variation in mammals compares to Drosophila. In mammals, canonical PRC1 contains subunits Ring 1A/B as homolog of dRing, CBX as homolog of Pc, BMI and PCGF family as homolog of Psc/Su(Z)2, Ph as homolog of Ph. Mammalian homolog variants function in combinatorial fashion thus there are different versions of both canonical and non-canonical PRC1, and they have distinct functions (Gao et al 2012).

One of the most related non-canonical PRC1, also called BCOR, is the homolog complex of dRAF in Drosophila. Ring1B has E3 ligase activity. In some cases, it has been suggested that PRC1-mediated-H2AK118ub is sufficient to recruit PRC2 and establish silencing in both Drosophila and mammals. However, it has also been shown that E3 ligase activity is not essential in mouse ESC (Illingworth et al 2015).

Recruitment of Polycomb Group Proteins

Recruitment of Polycomb Group Proteins in Drosophila

In Drosophila, PcG proteins are recruited to a DNA motif called PRE (Polycomb Respons Element). It's not clear how PcG proteins recognize PRE as deleting core PREs won't completely deplete PcG activity (Kassis and Brown 2013). A hierarchical model is proposed in Drosophila

that PhoRC recruits PRC2, PRC2 establishes H3K27me3, followed by recruitment of PRC1 (Wang et al 2004).

It is demonstrated that PcG proteins have highest affinity binding on PRE. PRE is a regulatory DNA sequence that is enough to maintain PcG protein target genes boundaries and it is not location or gene specific (Brown et al 1998, Muller and Kassis 2006). A PcG protein target gene can have multiple PREs, for example, engrailed has two PREs, gt has two PREs. Reports show PREs are redundant (Devido et al 2008, Ghotbi 2020). PREs can be a few kb apart from each other (Kassis and Brown 2013) and yet they function together in PcG mediated repression. A few reports proposed that cohesin brings two PREs closer to each other, eventually form a loop by recruiting PcG proteins and result in compacting chromatin (Dorsett and Kassis 2014, Cheutin and Cavalli 2014). Cohesin initially discovered as a factor stabilizing sister chromatid, later confirmed a role in facilitating enhancer-promoter communication. Cohesin generally found in transcription start sites and enhancers pausing RNA pol II allowing rapid transcription activation. Increase cohesin binding counteract PcG silencing whereas decrease cohesin enhances PcG silencing (Schwartz et al 2008). PRC1 has been found co-localize with both H3K27me3 and H3K4me3. In imaginal discs, cohesin has been found colocalized with PRC1 on where invected and engrailed are active while H3K27me3 and PRC1 are colocalized on where they are repressed (Schaaf et al 2013). In cultural cells depletion of cohesin can lower PRC1 binding at H3K4me3 and simultaneously increased binding at H3K27me3, so a theory is proposed that cohesin involves in interacting with PRC1 to pause RNA pol II and PcG proteins controls the switch of gene rapid activation (Schaaf et al 2013, Dorsett and Kassis 2014).

Sfmbt and Pho form Pho-RC complex. Sfmbt has the same domain as other two PcG proteins Scm and Ph. Ph is a subunit of canonical PRC1 and Scm is considered as substoichiometric subunit of PRC1. Mutating SPM domain in Scm results in reduced binding of PRC1 and PRC2 also ectopic expression of ubx in wing imaginal discs, it suggests Scm plays a role in recruiting PRC1 and/or PRC2 and helps maintaining repression. Pho-RC complex is co-purified with PRC1 including Scm, crystal of Sfmbt and Scm dimer is obtained, thus show Sfmbt and Scm has physical interaction. There's no direct evidence that Ph interacts with Scm *in vivo*. (Wang et al 2010, Frey et al 2016).

In wing discs, knock down Pho results in absent of PRC2 and PRC1 and knock down PRC2 results in absent of PRC1. Based on results of knocking down Pho and PRC2 in wing discs, a classic hierarchical model is proposed that Pho binds to PRE with Sfmbt, then recruits PRC2. PRC1 recognizes and binds to PRC2 mediated H3K27me3 which leads to PcG mediated silencing established (Wang et al 2004).

Recruitment of Polycomb Group Proteins in Mammal

Hiearchical model has been used to explain recruitment in mammal (blackledge, Rose and Klose 2015), but PRC2 and PRC1 doesn't always colocalize with each other. PRC1 has many subunits variants that don't have the same responsibility (Gao et al 2012). That suggest mechanisms recruiting PRC1 and PRC2 independently exist.

Do date, no sequences have been found in mammals that function in the way PRE does in Drosophila. However, PcG proteins especially PRC2 enriched in CpG island, and artificial DNA

sequence enriched with CG is sufficient to recruit PRC2, thus suggest CpG island plays a role in recruiting PRC2 (Mendenhall et al 2010, Riising et al 2014). Kdm2b has zinc finger domain CxxC specifically recognizes un-methylated CpG dinucleotides, Kdm2b is a subunit of BCOR and knocking down Kdm2b results in upregulating of several target genes, suggest Kdm2b may involve in recruiting PRC1 to CpG island through H2AK119ub. Lacking Kdm2b impairs H2AK119ub as well as decreases level of PRC2 binding and H3K27me3 on certain target genes but both H2AK119ub and H3K27me3 are not eliminated suggest there are other mechanisms involved (Blackledge et al 2014, Farcas et al 2015).

It has been suggested lnc RNA can recruit PcG proteins to specific loci, the most studied case is the relationship between PcG proteins and xist. The chromatin remodeling protein ATRX induces conformational changes within the Xist RNA and recruit PRC2 (Sarma et al 2014). It has been proposed either A- repeats of Xist RNA or a 4- kb region downstream of Xist exon 1 (XN region) mediates recruitment of PRC2. Recently a study not only show that XN region is the one that recruits PRC2, also show that the recruitment is initiated by PRC1 mediated H2AK119ub (Almeida et al 2017). HOTAIR lnc RNA transcribed from the HOXC locus on human chromosome 12. It's been reported that increased HOTAIR expression leads to increase binding of PRC2 and H3K27me3 signal and PRC2 needs HOTAIR in cancer progression (Gupta et al 2010). On the other hand, it has also been proposed that PRC2 binds to nascent RNA and PRC2 activity is inhibited (Kaneko et al 2014).

Regulation of giant (*gt*)

gt is a zygotic gap gene that regulates segmentation in Drosophila development. E(z), a subunit of PRC2, is needed to maintain gt expression pattern in early embryogenesis, thus, identified gt as PcG proteins target gene (Pelegri and Lehmann 1994).

gt is first detected in nuclear cycle 12. *gt* initially express in two broad stripes, one anterior activated by bicoid (bcd), one posterior activated by maternal caudal (cad) (Kraut and Levine 1991, Rivera-Pomar et al 1995). During nuclear cycle 14, posterior stripe becomes narrower and shift to anteriorly, the anterior domain splits into two bands, expression in the most ventral part of anterior stripe is lost simultaneously. By the end of nuclear cycle 14, a third anterior stripe appears (Kraut and Levine 1991). Four enhancers that are responsible for controlling *gt* expression have been mapped. gt (-1) enhancer controls both anterior and posterior stripes. gt (-3) enhancer produces the posterior stripes while gt (-10) produces two anterior stripes. the stripe that is closest to anterior pole of the embryo is controlled by gt (-6) enhancer (Schroeder 2004).

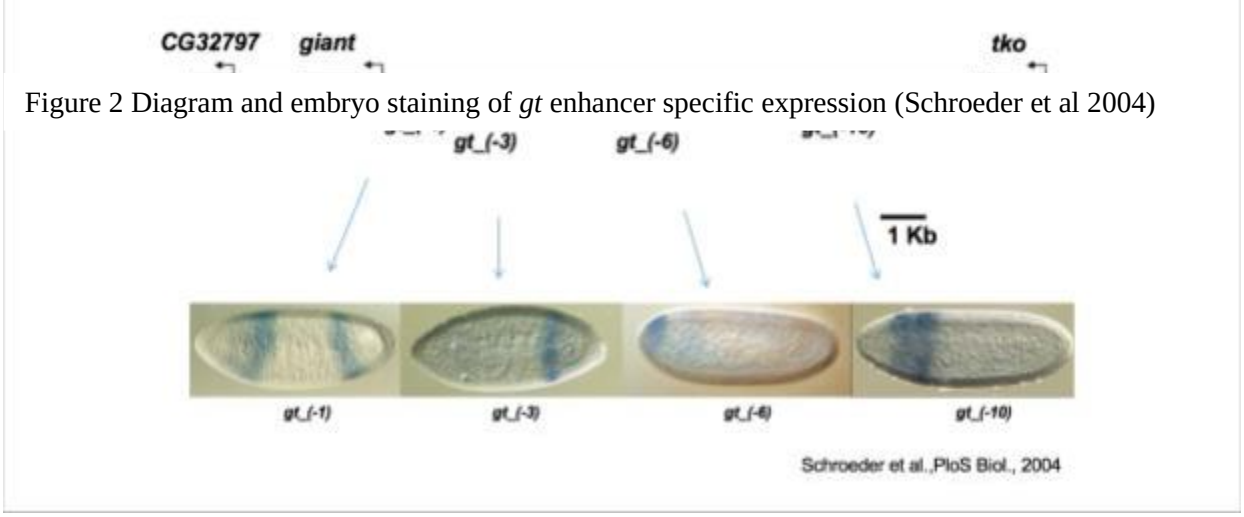

Figure 2 Diagram and embryo staining of *gt* enhancer specific expression (Schroeder et al 2004)

Bcd and Cad are *gt* activators, Bcd activates anterior stripe and Cad activates posterior stripes at nuclear cycle12. *gt* also regulates itself but it seems to play a minor role in

setting its expression boundaries. Maternal Hb represses *gt* expression, when nanos (nos) is mutated, *gt* is ubiquitously repressed by uniformly expressed Hb in embryo (Pelegri and Lehmann 1994). Knirpes (Kni) is repressor of anterior stripe of *gt* expression while tailless (tll) represses the posterior stripe. Krupple (Kr) is repressor of both anterior and posterior stripe of *gt* (Stanojevic et al 1991). High concentration of Hb blocks expression of kni, kr and *gt*, however lower concentration of Hb activates kr and even lower concentration of it will activate kni (Struhl 1992).

bcd osk tsl **genetic system**

Due to the difficulties of *in vivo* study for PcG proteins, most studies are done *in vitro*. Most of the *in vivo* studies use imaginal discs in which is the maintenance phase of PcG mediated repression. A system in where PcG target gene is ubiquitously repressed is needed to study initiation of PcG silencing. Previous graduate student generated *bcd osk tsl* genetic system to study of *de novo* PcG protein recruitment. Hb express both maternally and zygotically. In wildtype, maternal hb mRNA is ubiquitously distribute in early embryos, but Hb protein form a gradient from anterior to posterior due to translation inhibited by nos in the posterior end. Zygotic Hb is activated by bcd and *tsl*. In *bcd osk tsl* system, bcd, *osk* and *tsl* are mutated on the third chromosome. nanos is unable to localize Hb in the posterior end due to mutated *osk*, thus maternal Hb translation is not inhibited and express uniformly in embryo. Maternal Hb express ubiquitously result in *gt* expression is repressed ubiquitously in embryo. Lacking *bcd* and *tsl* results in no activation of zygotic Hb. Previous results have shown *gt* remain repressed after maternal Hb is fully degraded in nuclear cycle 14 and no zygotic Hb present to maintain *gt* repression.

Temperature sensitive E(z) allele is used to confirm *gt* maintain repressed after nuclear cycle 13 is due to PcG proteins.

A. Specific Aims:

PcG proteins are conserved epigenetic regulators and their misregulation can lead to various kinds of cancer, however how PcG proteins initially recruited target genes is not clear, nor what role each protein plays in recruitment. It is beneficial in drug development to better understand mechanisms of PcG protein recruitment. In Drosophila PcG proteins are recruited to a DNA motif, called polycomb response elements (PRE). Many/Numerous related studies are done in cultural cells or in imaginal discs in which is the maintenance phase of PcG proteins mediated repression. There is no known study about how PcG – mediated silencing is established because a system where a PcG proteins target gene is ubiquitously repressed by PcG proteins is needed to study initiation of PcG – mediated repression in vivo. To solve this problem, our lab previously generated a *bcd osk tsl* genetic system in which PcG target gene *gt* is ubiquitously repressed in early embryos. Previous work in the lab have discriminated the temporal order and distribution of PcG protein complexes binding to *gt* in blastoderm stage embryos, the goal of my research is to determine the contribution of individual proteins or complexes to PcG recruitment.

Specific Aim 1: Determine contributions of PRE binding proteins in *de novo* recruitment of PcG proteins

Pho-RC

Pho-RC contains subunits Pho and Sfmbt. Contributions of Pho and Sfmbt will be determined separately. Pho is the only PRE as well as DNA binding protein in PcG core complexes and has been shown to play an important role in recruiting PcG proteins, however it's not known if Pho can bind to PRE independent of Sfmbt. Sfmbt might be assisting PRC1 and/or PRC2 binding to *gt* by interacting with Scm.

Pho and Sfmbt may not need to be in the same complex to stably bind to target gene and subsequently recruit other PcG proteins. I will test the hypothesis that knockdown of either Pho or Sfmbt will result in PRC2 and PRC1 not be recruited to target gene *gt*.

Experimental Design: To test this hypothesis a UAS-Galr4 system paired with RNAi will be used knock down Pho and Sfmbt individually in *bcd osk tsl* embryo. ChIP will be done using embryos from developmental stage NC13-14b to examine PcG proteins binding across various developmental stages.

Specific Aim 2: Determine contributions of transcription factors in *de novo* recruitment of PcG proteins

Hunchback (Hb)

gt is transcriptionally activated ubiquitously by knocking down Hb in *bcd osk tsl* background, a repressor of Gt. PcG proteins only takes over repression but not initiating it. Transcriptionally active *gt* may inhibit PcG proteins recruitment of target genes.

I will test the hypothesis that transcriptionally active *gt* will inhibit PcG protein binding on target genes

Experimental Design: To test this hypothesis a UAS-Galr4 system paired with RNAi will be used knock down Hb in *bcd osk tsl* embryo. ChIP will be done using embryos from developmental stage NC10-14b to examine PcG proteins binding.

Caudal (Cad)

Cad is present on *gt* in *bcd osk tsl* embryos as an activator of *gt*. Without Cad in *bcd osk tsl* background, *gt* won't be activated as Cad is the only activator in the genetic system. It's possible after knocking down Cad, PcG proteins will be recruited sooner due to the removal of activator.

Hypothesis: I will test the hypothesis that by silencing/knockdown of Cad, PcG proteins will be recruited to target genes earlier in development.

Experimental Design: To test this hypothesis a UAS-Galr4 system paired with RNAi will be used knock down Cad in *bcd osk tsl* embryo. ChIP will be done using embryos from NC10-14b to examine PcG proteins binding.

B. Methods:

Fly stocks

All RNAi transgenic stocks as wells as Gal4 driver stock has been made into *bcd osk tsl* genetic background for the following ChIP experiments.

Fly stocks for constructing *bcd osk tsl* genetic background

We received a triple mutant stock (*bcd*6 *osk*6 tslPZREV32) from Leslie Stevens (Stevens et al. 2003), which cannot homozygous due to accumulation of one or more recessive lethal mutations on the third chromosome. The Bloomington stock BL-3252 (*bcd*7 *osk*6), was crossed to a tsl stock, BL-3289 (tsl4) stock to make an alternative triple mutant stock. That stock also had a recessive lethal mutation. To make a viable trans-heterozygous stock, these two stocks were crossed to each other when needed. We now have two balanced *bcd osk tsl* stocks that are crossed to produce *bcd osk tsl* females.For brevity, these will be referred to as *bcd osk tsl* embryos.

Constructing RNAi transgenic fly stocks

TRiP project from Harvard medical school developed VALIUM 20 and VALIUM 22 vectors that allow RNAi efficiently knockdown proteins in Drosophila germline. VALIUM 22 vector expresses better maternally than VALIUM20, however VALIUM20 allows embryo to go through early embryogenesis and in some cases, it's needed for examining PcG protein recruitment in embryos. RSVP Plus is a website made by Harvard medical school. It's available publicly for uploading RNAi validation results.

Pho and Sfmbt are the two proteins of interest in my specific aims. Available RNAi of Pho and Sfmbt from TRiP project has been shown to be effective to knock down proteins in somatic cells using VALIUM 20, however in my case, VALIUM 20 is not expressing well enough maternally to knockdown target proteins in desired developmental stages. By inserting inverted sequences of Pho and Sfmbt RNAi individually into VALIUM 22 vector using cloning protocol provided on TRiP website. Plasmids containing inverted sequences of Pho and Sfmbt RNAi is sequenced to confirm insert is complete and correct. Plasmids are then inserted into SD10 vector, a type of P-ENTR vector, using Gateway cloning technology. Gateway cloning technology is based on site specific recombination properties of bacteriophage lambda. Plasmids are then injected into Drosophila embryos germlines and inserted into attp binding site in Drosophila.

Gal4 driver stock

Gal4 driver stock used in this thesis is a maternal Gal4 driver obtained from Bloomington fly center (BL-7062). I have tested a stronger version of maternal Gal4 driver (BL-7063), but it's not used in data presented. I have also remobilized BL-7062 to construct a stronger maternal Gal4 driver, but it's not needed in data presented in this thesis.

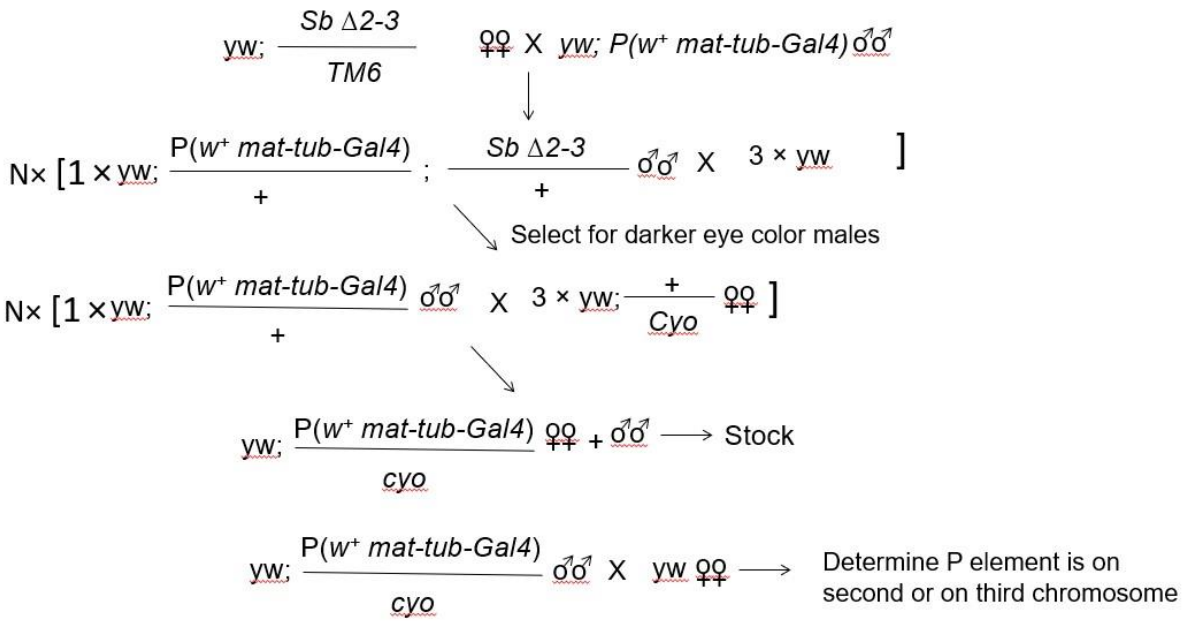

Figure 3 Cross scheme of remobilizing BL-7062 to construct a stronger Gal4 driver

Genetic cross of knocking down target protein using shRNA

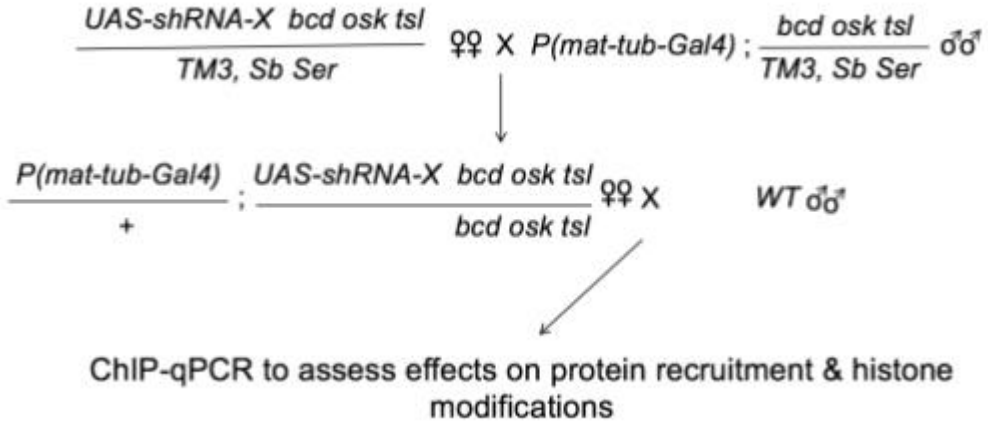

Figure 4 Cross scheme of knocking down target protein using RNAi

Chromatin Immunostaining assay and embryo collections

Embryo collection and embryo developmental stages

6,000-10,000 flies were added to a medium sized cage and fruit juice agar plates covered with yeast paste were placed into the cage for egg lay at 25°C or 28°C. An hour pre-lay is done before any collection for the day.

Embryos in both syncytial and cellular blastoderm stage are collected. Embryo developments are measured in nuclear cycles. Time course embryo collections are based on the timeline in the chart below.

Plates are left in cage for a 30 mins egg lay. Plates were then carefully removed, covered in pair, and left at 25°C or 28°C to age between 80-170 mins depends on developmental stage.

Embryo fixation

Embryos are fixed in 1.75% to 2% formaldehyde for 15 mins based on Shelby Blythe's protocol. Fix solution contains PBS with 0.5% Triton-X to allow formaldehyde permeable better into embryos, heptane allows separation of organic and aqueous phase of solution.

Embryo sorting

After embryo fixation, embryos were stored on ice in PBST containing 1x protease inhibitor cocktail until they are ready to be sorted with 10x brightfield objective of an inverted Primovert microscope. The embryos are placed in cell culture plate lid submerged in ice-cold PBST (0.5% Triton-X). Older embryos were individually removed using forceps. The embryos were surveyed one more time at 20x objective in DIC setting, to ensure all embryos are at the correct stage. Fresh PBST was added occasionally while sorting to ensure the embryos are hydrated. The embryos were sorted while using stages established by Campos-Ortega and Hartenstein, 1985. After sorting, embryos were weighed, flash frozen with liquid nitrogen, and stored at -80°C. Weights of embryos needed for each stage per ChIP is shown below.

Chromatin immunoprecipitation

Embryos are homogenized on ice in RIPA buffer that contains protease inhibitor and DTT. After separating lipid from homogenized embryos, chromatin is sonicated (15 secs on, 45 secs off, total 4mins 30secs). Majority of fragments are between 200bp-1000bp.

Sonicated chromatin is preabsorbed with Salmon sperm DNA beads to lower background. Protein A magnatic beads are blocked with PBST solution containing 3% BSA, followed by incubating with individual antibodies and subsequently incubate with preabsorbed sonicated chromatin.

Chromatin-beads complexes are being washed 5 times in this order below:

Low salt wash buffer (containing 150mM NaCl) x1

High salt wash buffer (containing 500mM NaCl) x1

LiCl buffer x2

TE buffer x1

Chromatin-beads complexes are then eluted with 1%SDS elution buffer in 65°C and vortexing. Eluted chromatin is incubate in 65°C for at least 4hrs to reverse crosslink. DNA fragments in solution is purified with Ampure beads and finally store in PCR water.

Quantitave PCR

Quanta Biosciences SYBR green supermix was used for the reactions. The concentration of primers (upper & lower) per reaction is 0.2 uM. Immunoprecipitated DNA from 100 ug of embryos was used for each PCR reaction. The PCR reactions were performed in triplicates unless otherwise indicated, for each sample. The PCR was carried out in Qiagen Rotor gene.

Normalization is done using delta delta ct method. All ct value normalized against PKA region ct value. 100% DNA input ct value is used to normalized across different PCRs for the same DNA.

Cuticle preparation

Embryos are washed off egg-laying plates, and dechorionated with 50% bleach. To devitellinized, embryos are carried using spatula into 500 ul of methanol in a 1.5mL Eppendorf tube. 500 ul of heptane is added to the tube, then vortex for 30 seconds. Embryos without vitelline membrane will be sink to the bottom due to hydrophilic surface. Transfer embryos at bottom to a microscopic slide, excess liquid is dried up with kimwipes. After drying embryos drop (~80ul) of lactic acid mounting medium is added onto the slide. Cover embryos with a cover slip making sure little to no air bubble between cover slip and slide. The slide is incubated in a 60°C oven overnight. Lactic acid in medium will digest embryos' internal organs and slide will be ready to examine under microscope next day. Cuticle preparation is a crude and fast way to confirm protein knockdowns, as knocking out target proteins often lead to specific cuticle phenotype.

Affinity purification of His6-tagged protein coupled to CNBr beads

Preparing medium

1 g (3.5 ml medium) of sepharose (4B sepharose beads from GE Healthcare) was suspended in 1 mM HCl in a 15 ml tube (up to 5-10 mg of protein/1 ml of medium). After the beads swell, they are washed on sintered glass filter with the vacuum suction with an excess of 1mM HCl, and once with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3).

Coupling Ligand

The beads were added to the ligand solution with an average of 10 mg of protein (5ml coupling: 1g powder), and the mixture was rotated overnight at 4°C or 1 hr at room temperature. The ligand- CNBr coupled beads are spun down at 2200 rpm for 5 min, and then the excess ligand was washed with 5x the medium volume with coupling buffer and spun down. The ligand-CNBr coupled beads are then blocked with blocking solution (0.1M Tris-HCl, pH 8) for 2 hours at room temperature, and then spun down. The medium was washed 3x with the wash buffers, alternating between pH 2 (0.5 M NaCl, 0.1 M NaOAc) and pH 8 (0.5M NaCl, 0.1M Tris-HCl), leaving the ligand-CNBr coupled beads in the pH 8 wash. The medium was then stored at 4°C in Tris-HCl pH 8, 0.5 M NaCl. Aliquots of the supernatant were run on a gel before and after coupling to determine coupling efficiency.

Affinity purification

His-tagged preabsoprtion beads are washed with PBTN (1x PBS, 0.3M NaCl, 0.1% Triton-X-100) and spun down. The anti-serum was diluted 1:1 with PBTN and added to the preabsorption beads and mixed for 3-4 hours at room temperature. The mixture was then poured into a column and the flow through is collected. The previously prepared ligand-CNBr coupled beads is equilibrated with PBTN, mixed and spun down. 1.5- 2 ml of ligand-CNBr coupled beads are used of the 2 mg/ml CNBr-protein. The preabsorbed antiserum is added to ligand-CNBr coupled beads, and incubated overnight at 4°C. Then the antiserum and ligand-CNBr coupled beads are poured into a narrower column. The beads are washed once they are packed with 10x the volume of originally added serum with PBTN. To elute antibody, 1ml of elution

buffer (0.2 M glycine, 0.8 M NaCl pH 2.5-2.75) is added over the beads without disturbing them. After the first ml was discarded, the eluant is collected one ml at a time. The eluant was pooled eventually and pH was equilibrated with 50 ul of 2 M Tris-HCl pH 8 to 1 ml of eluant. The eluant was then dialyzed in dialysis buffer (1x PBS, 0.02% NaN3) at 4°C overnight and was then divided into 100 ul aliquots and flash frozen. The affinity purified antibody is tested with a western blot.

C. Results

Drosophila embryonic develop stages

Drosophila embryos takes 24hrs to hatch into larva. Embryonic development is separated into 16 stages. In my project, I focus on first 3hr and 20mins in development. During that time, the embryo doesn't have individual cells, but it does have a replicating nucleus that is migrating from inside of the embryo to the periphery. Based on the cycles of nucleus replicating, it's divided into 14 nucleus cycles. (Sullivan et al 2000) Nc10-12 is syncytial stage where nucleuses are inside the embryo but rapidly replicating. Nc13-14 is cellular blastoderm where nuclei are migrating from inside the embryo to periphery. Maternally express genes transition to zygotic genes from NC8-9 to NC10-12. Between NC10-12 and NC13 is the transition o syncytial blastoderm to cellular blastoderm.

Bcd osk tsl genetic system

By using *bcd osk tsl* mutant alleles, we constructed Drosophila with *bcd osk tsl* background. This genetic system allows *gt* to be repressed ubiquitously in syncytial embryonic stage while Hb is expressed ubiquitously. Due to the lack of Cad, zygotic Hb will be missing, however *gt* didn't get reactivated, so we assumed Polycomb proteins take over repression in place of Hb. By using Ez mutant embryos and immunostaining, it's been proven that Polycomb proteins indeed are the ones taking over repression. (Abed et al 2018) *bcd osk tsl* genetic system gave us a way to observe what are the individual roles of each Polycomb protein in taking over repression. To examine roles of different protein, I used UAS-Gal4 system and RNAi to knockdown each protein individually in *bcd osk tsl* genetic background.

I previously performed ChIP on embryos with *bcd osk tsl* background in collaboration with previous graduate students to look at where and when Polycomb group proteins bind on *gt* throughout developmental stages of interest. It established a baseline for the changes when individual protein is knockdown (Abed et al 2018). These results (figure 5 and figure 6) show that Hb and Cad are degraded after syncytial blastoderm stage as expected. Pho along with PhoRC weakly associate with *gt* in early stages but didn't increase binding affinity until NC14. PRC2 contains E(z) which mono, di and tri methylate H3K27. As we see in graph below, E(z) also weakly associated with *gt* in early stage but didn't increase binding until NC14a and increase binding even more in NC14b, which is after PhoRC binding to target gene *gt*, and it's consistent with the hierarchical model we had with PhoRC, PRC2 and PRC1 (Wang et al 2004). H3K27me3 shows up alongside PRC2 due to E(z) mono-, di- and tri-methylate H3K27. PRC1 didn't show up until NC14b which is after PRC2 and it's also consistent with our hierarchical model (Wang et al 2004).

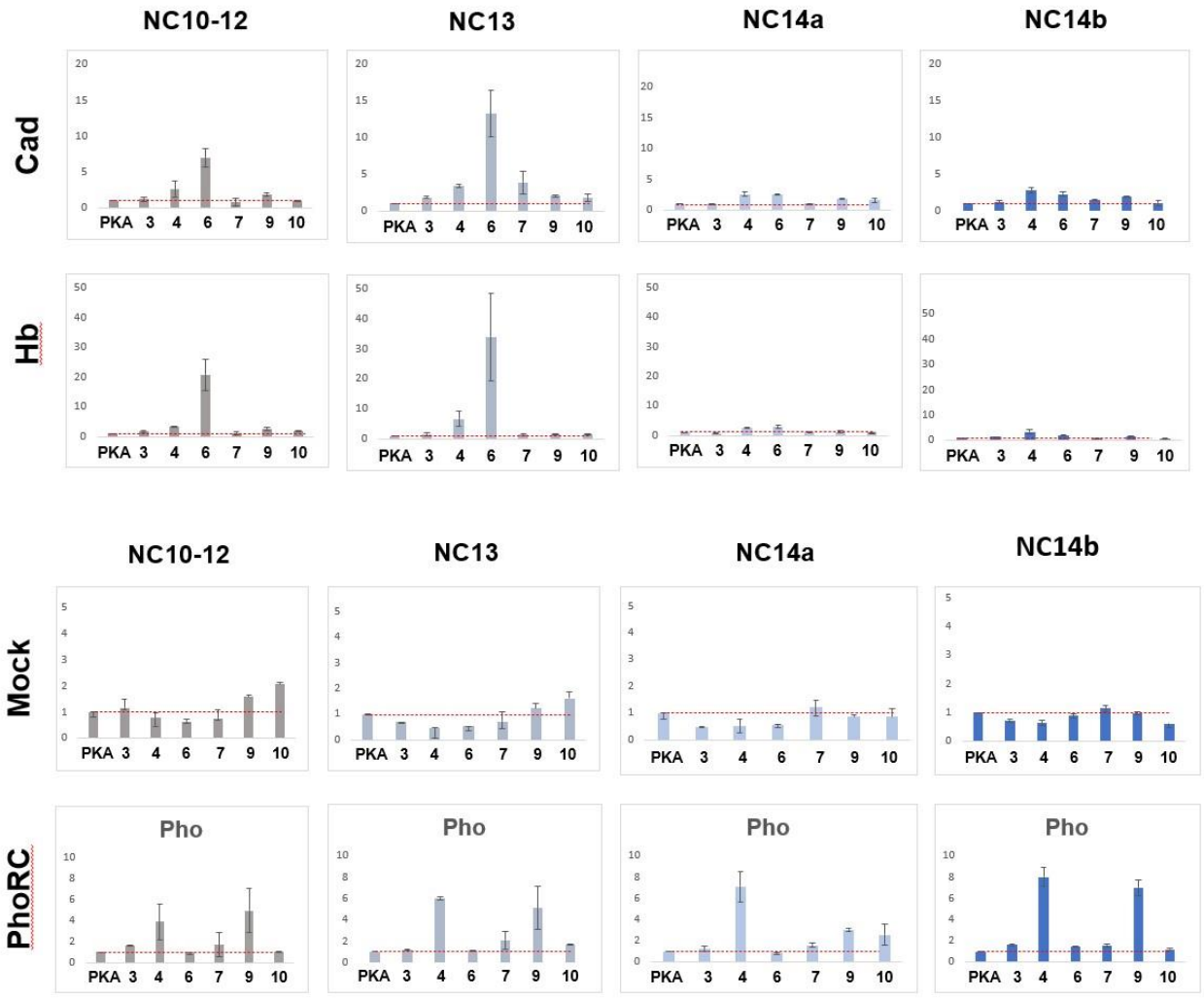

Figure 5 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC10-12, NC13, NC14a and NC14b. It shows binding of PhoRC, Mock, Hb and Cad. (Abed et al 2018)

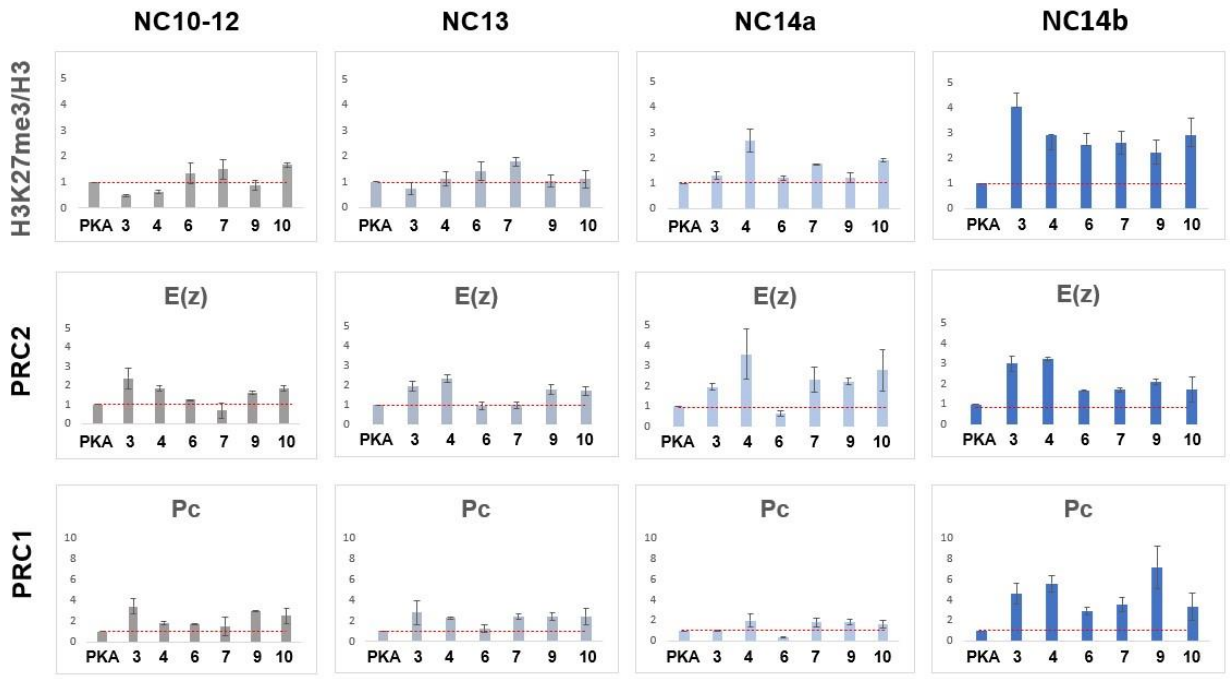

Figure 6 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC10-12, NC13, NC14a and NC14b. It shows binding of PRC1, PRC2 and H3K27me3. (Abed et al 2018)

Results on Individual Protein Knockdown

Pho

Pho transgenic RNAi line is made in *bcd osk tsl* background. Pho is knockdown efficient using BL-7062 maternal Gal-4 driver in *bcd osk tsl* background (Figure 5) shRNA-Pho is crossed with BL-7062 maternal Gal-4 driver. Females that are heterozygous of shRNA and Gal-4 driver are back crossed to males that carry shRNA. The said embryos are collected in different stages, i.e., nc13, nc14a, nc14b.

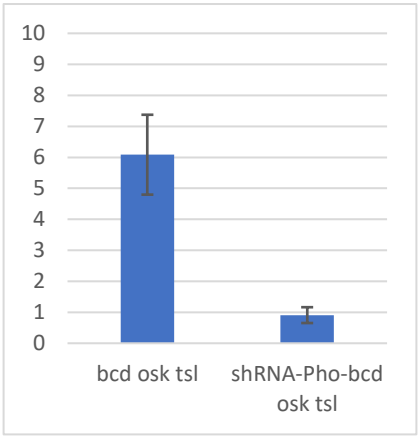

Figure 7 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC14b showing Pho has been sufficiently knocked down.

sfmbt

sfmbt transgenic RNAi line is made in *bcd osk tsl* background. sfmbt is knockdown efficient using BL-7062 maternal Gal-4 driver in *bcd osk tsl* background.

shRNA-sfmbt is crossed with BL-7062 maternal Gal-4 driver. Females that are heterozygous of shRNA and Gal-4 driver are back crossed to males that carry shRNA. The said embryos are collected in different stages, i.e., nc10-12, nc13, nc14a, nc14b.

Figure 7 shows the result of knocking down Sfmbt in *bcd osk tsl* background in NC14b. BOT WT is wild type embryos in *bcd osk tsl* background, and results are comparable to experiment previously done in 2018 in Dr. Jones lab. Sfmbt KD in orange shows what happens when we knock down sfmbt in *bcd osk tsl* background. Sfmbt is greatly reduced compared to WT embryos. Along with sfmbt, Pho is also missing. Unsurprisingly Pcl, which is an accessory protein of PRC2, and E(z) are also missing, so are Pc and Ring in PRC1. This indicating PcG proteins failed establishing recruitment in the absent of Sfmbt. However, H3K27me3 still show up strongly and it's comparable to its level in wild type embryos. PRC2 is the only methyltransferase in this case, so it's possible PRC2 is recruited to gt in earlier stage through other recruiting mechanism, but it's removed by NC14b.

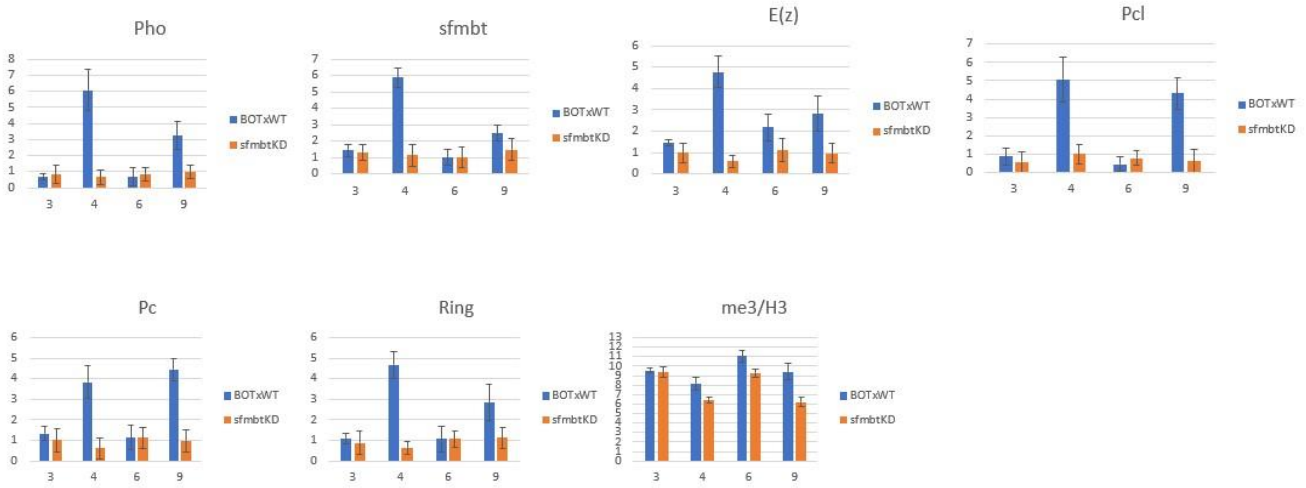

Figure 8 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC14b. It shows binding of Sfmbt is sufficiently knocked down as well as binding levels of other Polycomb group proteins and histone modification

Hunchback (Hb)

hb transgenic RNAi line is made in *bcd osk tsl* background. Hb is knockdown efficient using BL-7062 maternal Gal-4 driver in *bcd osk tsl* background in NC13. Shown as graph below

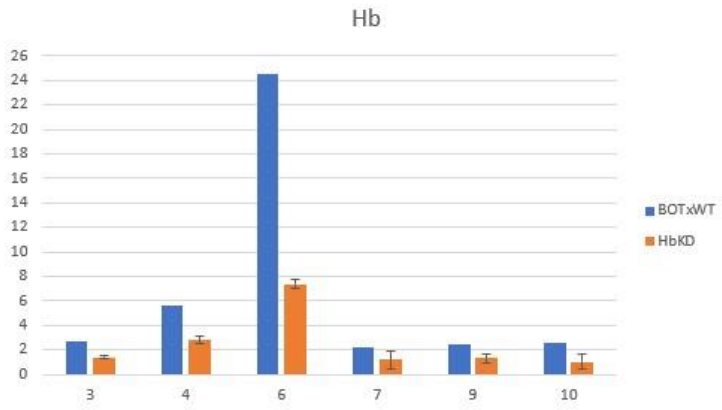

Figure 9 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC13 showing Hb has been sufficiently knocked down.

shRNA-hb is crossed with BL-7062 maternal Gal-4 driver. Females that are heterozygous of shRNA and Gal-4 driver are back crossed to males that carry shRNA. The said embryos are collected in different stages, i.e., nc13, nc14a, nc14b.

gt is transcriptionally active ubiquitously in syncytial blastoderm when Hb is knocked down. Figure 9 shows what happens to polycomb group proteins in NC 14a and NC14b when Hb is knocked down. Pho represents PhoRC, it shows a similar trend as wild type embryos, showing that PhoRC remain on *gt* whether it's repressed or activated. E(z) and Pcl represents PRC2, and it shows E(z) and Pcl are positive on *gt* but level of binding didn't increase in NC14b as it did in

wild type. E(z) and Pcl might not be able to establish stable binding due to various reasons, one of them can be H3K27ac. H3K27ac as an active histone mark can be inhibiting PRC2 binding. Pc represents PRC1, which also lack of an increased level of binding in NC14b. PRC2 signal didn't increase in NC14b even though it's present on *gt* in NC14a, and PRC1 signal remain weak in NC14b is consistent with PcG protein take over but not initiate repression.

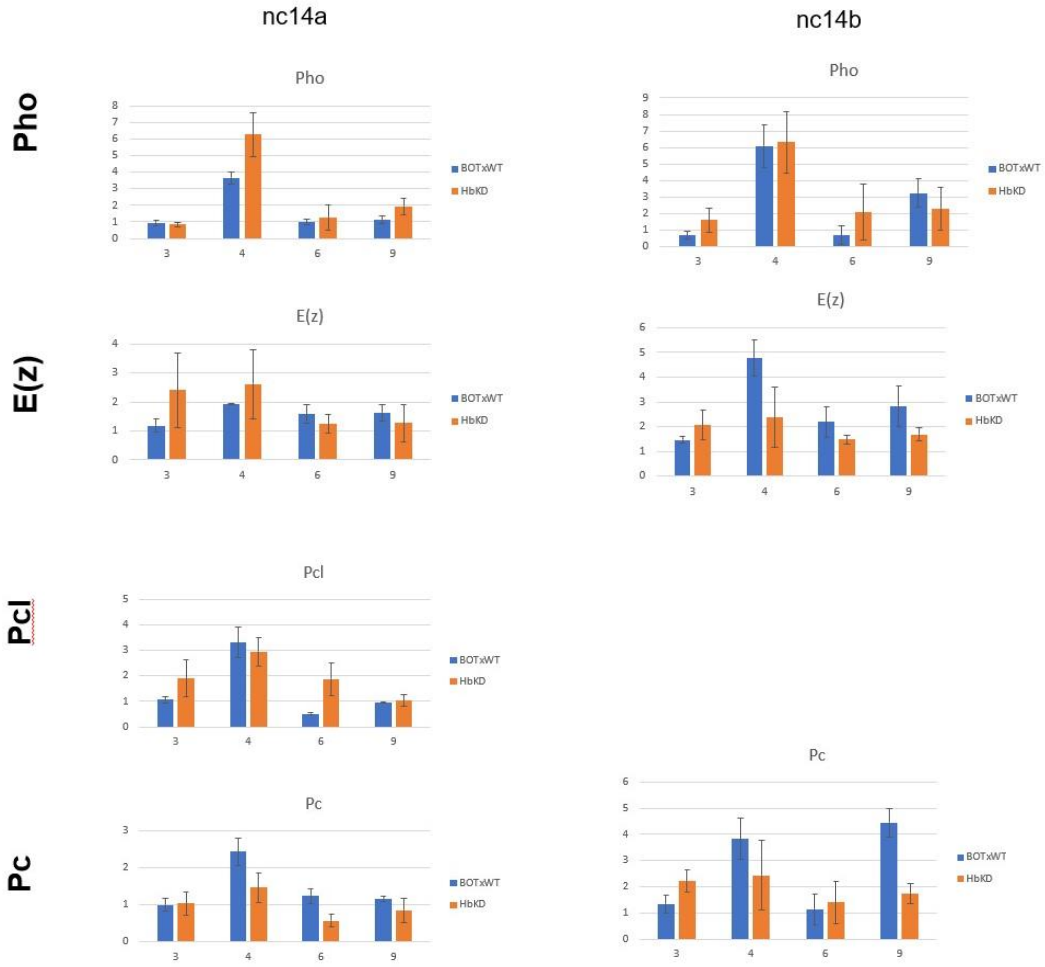

Figure 10 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC14a and NC14b. It shows the binding levels of Polycomb group proteins.

Caudal (Cad)

Maternal Cad is ubiquitously present in *bcd osk tsl* embryos as an activator of *gt*, without maternal Cad, *gt* will be repressed from the beginning. Cad as an activator might be inhibiting Polycomb Group proteins binding, removing Cad might allow PhoRC and subsequently other Polycomb group proteins to bind to target gene earlier.

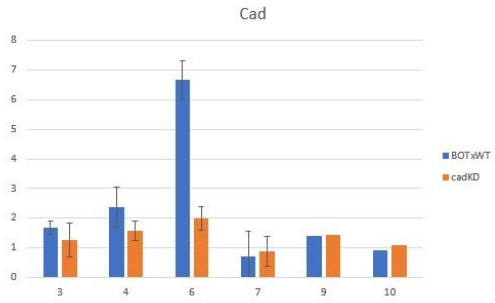

Figure 11 ChIP has been done on Drosophila embryos with *bcd osk tsl* background on developmental stage NC13 showing Cad has been sufficiently knocked down.

D. Discussion

Polycomb group proteins are conserved epigenetic regulators that do not initiate but rather take over repression. *bcd osl tsl* genetic system is constructed so *gt* is repressed ubiquitously in syncytial blastoderm by Hb and repression is taken over by PcG proteins in cellular blastoderm (Abed et al 2018).

Chromatin immunoprecipitation shows that PhoRC binds to *gt* as early as NC10-12, but it doesn't increase binding until NC14a. PRC2 signal is weak across all regions in NC10-13 and doesn't increase until NC14a. PRC1 is negative until NC14b. Overall PcG proteins signals start increasing in NC14a. NC14a is the first nucleus cycle after Hb degrades and it's when Polycomb group proteins taking over repression, so NC14b maybe the beginning of PcG proteins recruitment. It's not clear why PhoRC is present on gt as early as NC10-12, but signal is not increased until NC14a and b. One possibility is nucleus cycles before NC14a are short and rapid cycles. Rapid replicating nucleus maybe preventing more PhoRC binding on gt as well as recruiting the rest of PcG proteins (Abed et al 2018). It's not clear what are the individual roles of each PcG protein complexes. It's also not clear how active and repress state of target gene will affect *de novo* PcG proteins recruitment.

To further examine the roles of individual protein in *de novo* recruitment of Polycomb group proteins, I use UAS-Gal4 system to knock down individual protein in *bcd osk tsl* background. I successfully constructed Drosophila stocks to knock down Pho, Sfmbt and Cad. Inverted sequences that transcribe into RNAi are designed by TRiP project from Harvard medical school. VALIUM22 and VALIUM20 are two vectors express maternally and are also designed by TRiP

project. VALIUM 22, which contains a p element promoter express better maternally and can result in a more robust knock down in embryo. Cad is knocked down using VALIUM20, but Hb, Pho and Sfmbt are knocked down using VALIUM22 vector. All target proteins are knocked down maternally and have been confirmed they are knocked down efficiently. Hb and Cad are knocked down efficiently using BL-7062 maternal Gal4 driver in 25c°. Embryos survive past NC14b, so PcG recruitment can be observed when Hb or Cad is missing. There are Pho and Sfmbt RNAi that have been shown to be effective, however these RNAi transgenic stocks are only available in VALIUM20 vector. These RNAi transgenic stocks have been tested with different maternal Gal4 drivers as wells as different temperatures. It's confirmed that they can't knock down Pho and Sfmbt effectively. VALIUM 22 is a vector that express better maternally, so I have inserted effective inverted sequence for Pho and Sfmbt RNAi into VALIUM22 vector and construct Drosophila stocks in *bcd osk tsl* background. Pho, Sfmbt are embryonic lethal if either of them was completely depleted maternally, so I used BL-7062, which is a weaker version of the two available maternal Gal4 driver to knock down Pho and Sfmbt individually. Pho is successfully knocked down at 25c°, and Sfmbt is successfully knocked down at 28c°.

ChIP has been performed on embryos in *bcd osk tsl* background that are absent of Sfmbt in NC14b. It shows Pho is missing in the absence of Sfmbt, so are PRC2 and PRC1, however H3K27me3 is positive and signal level is comparable to WT embryos in *bcd osk tsl* background. It's possible PRC2 is recruited through mechanism independent of PhoRC in earlier nucleus cycle and H3K27me3 is then established. However, PRC2 is not able to stay binding to *gt* due to the lack of PhoRC, so PRC2 signal is low in NC14b.

ChIP has been performed on embryos in *bcd osk tsl* background that are absent of Hb in NC14a and b. In NC14a, signals of Pho, E(z), Pcl and Pc in Hb knock down are comparable to the signals in WT embryos. PhoRC is recruited to *gt*, PRC2 signals are low but present on *gt*, and PRC1 is missing. In NC14b Pho signals increase as it does in WT background, but PRC2 signals remain low and so do PRC1 signals. This indicates PRC2 failed to establish stable binding on *gt* and PRC1 is not recruited to *gt*. Knocking down maternal Hb results in *gt* ubiquitously active in embryo. PcG proteins take over but not initiate repression is consistent with ChIP results in Hb knock down.

Conclusion

Knocking down individual proteins in *bcd osk tsl* background gives us a better understanding of *de novo* PcG proteins recruitment. Knocking down Hb results in ubiquitously active *gt* expression in embryos. ChIP results of Hb knock down is consistent with PcG proteins taking over but not initiating repression. Knocking down Sfmbt suggests PRC2 have been recruited to *gt* by mechanism independent of PhoRC, however PRC2 is removed before NC14b. It's not clear why H3K27me3 is present in NC14b when PRC2 is not present on *gt*. Performing ChIP on Sfmbt knocked down embryos in earlier nucleus cycles in *bcd osk tsl* background will give a better insight into PRC2 recruitment when Sfmbt is missing. Other interesting targets for knock down experiments are E(z), Pcl and Pc. Depleting E(z) maternally is embryonic lethal, while depleting maternal Pcl and Pc have no obvious effect on embryo development. Different strengths of maternal Gal4 drivers may be needed to sufficiently knock down individual proteins. The remobilized maternal Gal4 driver may be useful in future knock down experiments.

E. References:

Abed,J.,E Ghotbi, P Ye., Frolov, A., Benes, J., Jones, R. 2012 *De novo* Recruitment of Polycomb Proteins in Drosophila Embryos, Development volume 145 issue 23

Americo, J., Whiteley, M., Brown, J. L., Fujioka, M., Jaynes, J. B., Kassis J. 2002. A Complex Array of DNA-Binding Proteins Required for Pairing-Sensitive Silencing by a Polycomb Group Response Element From the Drosophila engrailed Gene, Genetics 160: 1561-1571

Almeida, M., Pintacuda, G., Masui, O., Koseki, Y., Gdula, M., Cerase, A., Brown, D., Mould, A., Innocent, C., Nakayama, M., Schermelleh, L., Nesterova, TB., Koseki, H., Brockdorff, N. 2017. PCGF3/5-PRC1 initiates Polycomb recruitment in X chromosome inactivation. Science 356: 1081-1084

Aranda, S., Mas, G., Di Croce L. 2015. Regulation of gene transcription by polycomb proteins. Science

Alfieri, C., Gambetta, MC., Matos, R., Glastt, S., Sehr, P., Fraterman, S., Wilm, M., Muller, J., Muller, CW., 2013. Structural basis for targeting the chromatin repressor Sfmbt to Polycomb reponse elements. Genes&Development 27: 2367-2379

Beuchle, D., Struhl, G., Muller, J. 2001. Polycomb group proteins and heritable silencing of Drosophila Hox genes. Development 128: 993-1004

Bornemann, D., Miller, E., Simon, J. 1996. The Drosophila Polycomb group gene Sex comb on midleg (Scm) encodes a zinc finger protein with similarity to polyhomeotic protein. Development 122: 1621-1630

Bornemann, D., Miller, E., Simon, J. 1998. Expression and Properties of Wild-Type and Mutant Forms of the Drosphila. Genetics 150: 675-686.

Boyer, LA., Kathrin, P., Zeitlinger, J., Brambrink, T., Medeitos, LA., Lee, TI., Levine, SS., Wernig, M., Tajonar, A., Ray, MK., Bell, GW., Otte, AP., Vidal, M., Gifford, DK., Yound, RA., Jaenisch, R. 2006. Nature 441:349

Bracken, AP., and Helin, K. 2009. Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nature review 9: 773

Blackledge, NP., Rose, NR., Klose, RJ. 2015. Targeting polycomb systems to regulate gene expression: modifications to a complex story. Nature review 16: 643-649

Bernstein, E., Duncan, EM., Masui, O., Gil, J., Heard, E., Allis, D. 2006. Mouse Polycomb Proteins Bind Differentially to Methylated Histone H3 and RNA and Are Enriched in Facultative Heterochromatin. Molecular and Celluar Biology p.2560-2569

Brown, JL., Mucci, D., Whiteley, M., Dirksen, ML., Kassis, J. 1998. The Drosophila Polycomb Group Gene pleiohomeotic Encodes a DNA Binding Protein with Homology to the Transcription Factor YY1. Molecular Cell vol1: 1057-1064

Brown, JL., Fritsch, C., Muller, J., Kassis, J. 2003. The Drosophila pho-like gene encodes a YY1-related DNA binding protein that is redundant with pleiohomeotic in homeotic gene silencing. Development 130:285-294

Brown, JL and Kassis, JA. 2010. Spps, a Drosophila Sp1/KLF family member, binds to PREs and is required for PRE activity late in development. Development. 137, 2597-2602

Blackledge, NP., Farcas, AM., Kondo, T., King, HW., McGouran, JF., Hanssen, LLP., Ito, S., Cooper, S., Kondo, K., Koseki, Y., Ishikura, T., Long, HK., Sheahan, TW., Brockdorff, N., Kessler, BM., Koseki, H., Klose, RJ. 2014. Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2 recruitment and polycomb domain formation. Cell 157: 1445-1459

Bauer, M., Trupke, J., Ringrose, L. 2015. The quest for mammalian Polycomb response elements: are we there yet?. Springer Chromosoma

Breiling, A., Bonte, E., Ferrari, S., Becker, PB., Paro, R. 1999. The Drosphila polycomb proteins interacts with nucleosomal core particles in vitro via its repression domain. Molecular and cellular biology. p. 8451-8460

Breen, TR and Duncan, IM 1986. Maternal expression of genes that regulate the bithorax complex of Drosphila melanogaster. Developmental biology. 118

Birve, A., Sengupta, A., Beuchle, D., Larsson, J., Kennison, JAM., Lestander, AR., Muller, J. 2001. Su(z)12, a novel Drosophila polycomb group gene that is conserved in vertebrates and plants. Development 128. 3371-3379

Cheutin, T., and Cavalli, G. 2014. Polycomb silencing: from linear chromatin domains to 3D chromosome folding. Current opinion in Genetics & Development 25: 30-37

Casanova, M., Preissner, T., Cerase, A., Poot, R., Yamada, D., Li, Xiangzhi., Appanah, R., Bezstarosti, Demmers, J., Koseki, H., Brockdorff, N. 2011. Polycomblike2 facilitates the recruitment of PRC2 polycomb group complexes to the inactive X chromosome and to target loci in embryonic stem cells. Development 138: 1471-1482

Cunningham, MD., Brown, JL., Kassis JA. 2010. Characterization of the Polycomb group response elements of the Drosophila melanogaster invected locus. Molecular and Cellular Biology. p. 820-828

Calo, E and Wysocka, J. 2014. Modification of Enhaner Chromatin: what, how and why?. Molecular cell 49

Ciabrelli, F., Comoglio, F., Fellous, S., Bonev, B., Ninova, M., Szabo, Q., Xuereb, A., Klopp, C., Aravin, A., Paro, Renato., Bantignies, F., Cavalli, G. Stable 2016 polycomb-

dependent transgenerational inheritance of chromatin states in Drosphila. Nature.

Carrington, EA and Jones, RS. 1996. The Drosophila Enhancer of zeste gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. Development 122: 4073-4083

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., Pirrotta, V. 2002. Drosohphila enhancer o zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal polycomb sites. Cell. Vol 111, 185-196

de Napoles, M., Mercoud, JE., Wakao, R., Tang, YA., Endoh, M., Appanah, R., Nesterova, TB., Silva, J., Otte, AP., Vidal, M., Koseki, H, Brockdorff N. 2004. Polycomb Group Proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing. Developmental cell vol 7: 663-676

Decoville, M., Giacomello, E., Leng, M., Locker, D. Dsp1,2001. an HMG-like protein, is involved in the regulation of homeotic genes. Genetics 157: 237-244

Dejardin, J., Rappailles, A., Cuvier, O., Grimaud, C., Decoville, M., Locker, D., Cavalli, G. 2005. Recruitment of Drosophila polycomb group proteins to chromatin by Dsp1. Nature vol 434

DeVido, SK., Kwon, D., Brown, JL., Kassis, JA. 2008. The role of polycomb-group response elements in regulation of engrailed transcription in Drosophila. Development 135: 669-676

Dorsett, D and Kassis, JA. 2014. Checks and Balances between cohesion and polycomb in gene silencing and transcription. Current biology 24

Di Croce, L and Helin, K. Transcriptional regulation by Polycomb group proteins. Nature vol 20

DeCamillis, M., Cheng,N., Pierre, D., Brock, HW. 1992. The polyhomeotic gene of Drosphila encodes chromatin protein that shares polytene chromosome-binding sites with polycomb. Gene and development 6

Egan, B., Yuan, CC., Craske, ML., Labhart, P., Guler, GD., Arnott, D., Maile, TM., Busby, J., Henry, C., Kelly, TK., Tindell, CA., Jhunjhunwala, S., Zhao, F., Hatton, C., Bryant, BM., Classon, M., Trojer, P. 2016. An alternative approach to ChIP-Seq normalization enables detection of genome wide changes in histone H3 lysine 27 trimethylation upon Ezh2 inhibition. PLOS one

Erokhin, M., Elizar'ev, P., Parshikov, A., Schedl, P., Georgiev, P., Chetverina, D. Transcriptional read- through is not sufficient to induce an epigenetic switch in the silencing activity of Polycomb response elements. PNAS. Vol112 no. 48

Eldon, E and Pirrotta, V. 1991. Interactions of the Drosophila gap gene giant with maternal and zygotic pattern-forming genes. Development 111: 367-378

Entravan, M., Schuettengruber, B and Cavalli, G. 2016 Regulation of genome architecture and function by polycomb protein. Trends in Cell biology. vol 26.

Eskeland, R., Leeb, M., Grimes, GR., Kress, C., Boyle, S., Sproul, D., Gilbert, N., Fan, Y., Skoultchi, AI., Wutz, A., Bickmore, WA. 2010. Ring 1B compacts chromatin structure and represses gene expression independent of histone ubiquitination. Mol.cell 38(3): 452-464

Farcas, AM., Blackledge, NP., Sudbery, I., Long, HK., McGouran, JF., Rose, NR., Lee, S., Sims, D., Cerase, A., Sheahan, TW., Koseki, Haruhiko., Brockdorff, N., Ponting, CP., Kessler, BM., Klose, RJ. 2012. Kdm2b linksthe polycomb repressive complex1 to recognition of cpg islands. Elife

Francis, NJ., Saurin, AJ., Shao, Z., Kingston RE. 2001. Reconstitution of a functional core

polycomb repressive complex. Molecular cell vol 8: 545-556

Francis, N., Kingston, RE., Woodcock, CL. 2004, Chromatin compaction by a polycomb group protein complex. Science. Vol 306

Frey, F., Sheahan, T., Finkl, K., Stoehr, G., Mann, M., Benda,C., Muller, J. 2016. Molecular basis of PRC1 targeting to polycomb response elements by PhoRC. Genes&development 30

Fritsch, C., Beuchle, D., Muller, J. 2003. Molecular and genetic analysis of the polycomb group gene sex combs extra/ Ring in Drosophila

Fritsch, C., Brown, JL., Kassis, JA., Muller, J. 1999. The DNA- binding polycomb group protein pleiohmeotic mediates silencing of a Drosophila homeotic gene, development 126

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., Reinberg, D. 2012. PCGF homologs, CBX proteins and RYBP define functionally distinct PRC1 family complexes. Molecular cell 45

Grimm, C., Matos, R., Ly-Hartig, N., Steuerwald, U., Lindner, D., Rybin V, Muller J, Muller CW. 2009. Molecular recognition of histone lysine methylation by the polycomb group repressor Sfmbt. The EMBO journal 28

Goldberg, ML., Colvin, RA., Mellin, AF., 1989. The Drosophila zeste locus is nonessential. Genetics.123:145-155

Herzog, VA., Lempradl, A., Trupke, J., Okulski, H., Altmutter, C., Ruge, F., Boidol, B., Kubicek, S., Schmauss, G., Aumayr, K., Ruf, M., Pospisilik, A., Dimond, A., Senergin, HB., Vargas, ML., Simon, JA., Ringrose, L., 2014. A strand-specific switch in noncoding transcription switches the function of a Polycomb/Trithorax response element. Nature. Vol46

Huang, DH, Chang, YL, Yang, CC, Pan, IC, King, B. 2002. Pipsqueak encodes a factor essential for sequence-specific targeting of a polycomb group protein complex. Molecular and celluar biology.

Jiao, L and Liu, X. 2015. Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex2. Vol350. issue 6258

Jones, RS and Gelbart, WM. 1990. Genetic analysis of the enhancer of zeste locus and its role in gene regulation in Drosophila melanogaster. Genetic 126

Jurgens, G, 1985 A group of genes controlling the spatial expression of the bithorax complex in Drosophila. Nature vol 316

Jones, RS and Gelbart, WM. 1993. The Drosohpila polycomb-group gene enhancer of zeste contains a region with sequence similarity to trithorax. Molecular and cellular biology. p. 6357-6366

Juven-Gershon, T., Hsu, JY., Kadonaga JT. 2008. Caudal, a key developmental regulator, is a DPE- specific transcriptional factor, genes & development 22:2823-2830

Kaneko, S., Son, J., Bonasio, R., Shen, SS., Reinberg, D. 2014. Nascent RNA interaction keeps PRC2 activity poised and in check. Genes & development 28

Kanhere, A., Viiri, K., Araujo, CC., Rasaiyaah, J., Bouwman, RD., Whyte, WA., Pereira, CF., Brookes, E., Walker, K., Bell, GW., Pombo, A., Fisher, AG., Young, RA., Jenner, RG. 2010. Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. Mol cell 38(5)

Kassis, JA and Kennison, JA. 2010. Recruitment of polycomb complexes: a role for SCM. Molecular and cellular biology.

Kasis, JA and Brown, JL. 2014. Polycomb group response elements in Drosophila and

Vertebrates. Adv Genet. 81: 83-118

King, IFG., Francis, NJ., Kingston, RE. 2002. Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Molecular and cellular biology

King, IFG., Emmons, RB., Francis, NJ., Wild, B., Muller, J., Kingston, RE., Wu, CT. 2005. Analysis of a polycomb group protein defines regions that link repressive activity on nucleosomal templates to in vivo function. Molecular and cellular biology.

Klose, RJ., Cooper, S., Farcas, AM., Blackledge, NP., Brockdorf, N. 2013. Chromatin sampling- an emerging perspective on targeting polycomb repressor proteins. PLOS genetics. Vol 9. Issue 8

Kraut, R and Levine, M. 1991. Spatial regulation of the gap gene giant during Drosophila development. Development 601-609

Kahn, TG., Stenberg, P., Pirrotta, V., Schwartz, YB. 2014. Combinatorial interactions are required for the efficient recruitment of Pho repressive complex (PhoRC) to polycomb response elements. PLOS genetics

Kahn, TG., Dorafshan, E., Schultheis, D., Zare, A., Stenberg, P., Reim, I., Pirrotta, V., Schwartz, YB. 2016. Interdependence of PRC1 and PRC2 afor recruitment to polycomb response elements. Nuleic acids research vol 44. No 21

Kehle, J., Beuchle, D., Treuhei, S., Christen, B., Kennison, JA., Bienz, M., Muller, J. 1998. dMi-2, a Hunchback- interacting protein that fuctions in polycomb repression. Science. Vol 282

Lewis, EB. 1978. A gene complex controlling segmentation in Drosophila. Vol 276

Lagarou, A., Mohd-Sarip, A., Moshikin, YM., Chalkley, GE., Bezstarosti, K., Demmers,

JAA., Verrijzer, CP. 2008. dKdm2 couples histone H2A ubiquitylation to histone H3 demethylation during polycomb group silencing. Gene&development 22

Lau, MS., Schwartz, MG., Kundu, S., Savol, AJ., Wang, PI., Marr, SK., Grau, DJ., Schorderet, P., Sadreyev, RI., Tabin, CJ., Kingston, RE. 2017. Mutation of a nucleosome compaction region disrupts polycomb mediated axial patterning. Sicence 355

Lavigne, M., Francis, NJ., King, IFG., Kingston, RE. 2004. Propagation of silencing: recruitment and repression of naïve chromatin in trans by polycomb repressed chromatin. Molecular cell vol 13

Lee, TI., Jenner, RG., Boyer, LA., Guenther, MG., Levine, SS., Kumar, RM., Chevalier, B., Johnstone, SE., Cole, MF., Isono, K., Koseki, Haruhiko., Fuchikami, T., Abe, K., Murray, HL., Zucker, JP., Yuan, BB., Bell, GW., Herbolsheimer, E., Hannett, NM., Sun, K., Odom, DT., Otte, AP., Volkert, TL., Bartel, DP., Melton, DA., Gifford, DK., Jaenisch, R., Young, RA. 2006. Control of developmental regulators by polycomb in human embryonic stem cell. Cell 125:301-313

Li, XY., Harrison, MM., Kaplan, T., Eisen, MB.,2014. Establishment of regions of genomic activity during the Drosophila maternal to zygotic transition. bioRxiv

Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, CL., Dynlacht, BD., Reinberg, D. 2008. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Molecular cell 32: 503-518

Margueron, R., Justin, N., Ohno, K., Sharpe, ML., Son, J., Drury, WJ., Voigt, P., Martin, SR., Taylor, WR., De Marco, V., Pirrotta, V., Reinberg, D., Gamblin, SJ. 2009. Role of the polycomb protein EED in the propagation of repressive histone marks. Nature. Vol 46

Margueron, R and Reinberg, D. 2011. The polycomb complex PRC2 and its marks in life.

Nature 469

Min, J., Zhang, Y., Xu, RM. 2003. Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes&Dev.

Minoux, M., Holwerda, S., Vitobello, A., Kitazawa, T., Kohler, H., Stadler, MB., Rijli, FM. 2017. Gene bivalency at polycomb domains regulates cranial neural crest positional identity. Science. 355

Muller, J and Kassis, JA. 2006. Polycomb response elements and targeting of polycomb group proteins in Drosophila. Current opinion in genetics and development 16:476-484

Muller, J, 1995. Transcriptional silencing by the polycomb proteins in Drosophila embryos. The EMBO journal vol 14 no. 6

Muller, J., Hart, CM., Francis, NJ., Vargas, ML., Sengupta, A., Wild, B., Miller, EL., O' Connor, MB., Kingston, RE., Simon, JA. 2002. Histone methyltransferase activity of a drosphila polycomb group repressor complex. Cell vol. 111. 197-208

Nekrasov, M., Wild, B., Muller, J. 2005. Nucleosome binding and histone methyltransferase activity of Drosophila PRC2. The EMBO reports vol6. No.4

Nekrasov, M., Klymenko, T., Fraterman, S., Papp, B., Oktaba, Katazyna,, Kocher. T., Cohen, A., Stunnenber, HG., Wilm, M., Muller, J. 2007. Pcl-PRC2 is needed to generate high levels of H3K27 trimethylation at polycomb target genes. The EMBO journal. 26.

Ni, JQ., Zhou, R., Czech, B., Liu, LP., Holderbaum, L., Zhou, DY., Shim, HS., Tao, R., Handler, D., Karpowicz, P., Binari, R., Booker, M., Brennecke, J., Perkins, LA., Hannon, GJ., Perrimon, N. 2011. A genome scale shRNA resource for transgenic RNAi in Drosophila. Nature vol8

Nowak, AJ., Alfieri, C., Stirnimann, CU., Rybin, V., Baudin, Ly-Hartig, N., Lindner, D.,

Muller, CW.. 2011. Chromatin modifying complex component nurf55/p55 associates with histones h3 and h4 and polycomb repressive complex 2 subunit su(z)12 through partially overlapping binding sites. Journal of biological chemistry. Vol 286

Negre, N., Henetin, J., Sun, LV., Lavrov, Bellis., White, K., Cavalli, G. 2006. Chromosomal distribution of PcG proteins during Drosophila Development. PLOS biology. vol 4 Issue 6

O'Meara, MM and Simon, JA. 2012. Inner workings and regulatory inputs that control polycomb repressive complex 2. Chromosoma 121

Ohno, K., McCabe, D., Czermin, B., Imhof, A., Pirrotta, V. 2008. Esc, Escl and their roles in polycomb group mechanisms. Mechanisms of development 125

Pelegri, F and Lehmann, R. 1994. A role of polycomb group genes in the regulation of gap gene expression in Drosophila. Genetics. 136

Papp, B and Muller, J. 2006. Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins.

Petruk, S., Sedkov, Y., Riley, KM., Hodgson, J., Schweisguth, F., Hirose, S., Jaynes, JB., Brock, HW., Mazo, A. 2006. Transcription of bxd noncoding RNAs promoted by trithorax represses ubx in cis by transcriptional interference. Cell. 127.

Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canaani, E., Croce, CM., Mazo, A. 2001. Science. Vol294

Peterson, AJ., Mallin, DR., Francis, NJ., Ketel, CS., Stamm, J., Voeller, RK., Kingston, RE., Simon, JA. 2004. Requirement for Sex Comb on Midleg protein interactions in Drosophila Polycomb Group repression. Genetics. 167:1225-1239.

Poux, S., McCabe, D., Pirrotta, V., 2001. Recruitment of components of Polycomb Group chromatin complexes in Drosphila. Development. 128:75-85

Riising, EM., Comet, I., Leblanc, B., Wu, X., Johansen, JV., Helin, K. 2014. Gene silencing triggers polycomb repressive complex 2 recruitment to CpG islands genome wide. Molecular cell. 55

Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H., Jackle, H. 1995. Activation of posterior gap gene expression in the Drosophila blastoderm. Nature vol 376

Rastelli, L., Chang, CS., Pirrotta, V. 1993. Related chromosome binding sites for zeste, suppressors of zeste and polycomb group proteins in Drosophila and their dependence on enhancer of zeste function. The EMBO journal. Vol12 no. 4

Simon, J., Peifer, M., Bender, W, O'connor, M. 1990. Regulatory elements of the bithorax complex that control expression along the anterior- posterior axis. The EMBO journal vol. 9, no. 12

Simon, J., Chiang, A., Bender, W. 1992. Ten different polycomb group genes are required for spatial control of the abdA and AbdB homeotic products. Development. 114: 493-505

Simon, J., Bornemann, D., Lunde, K., Schwartz, C., 1995, The extra sex combs product contains WD40 repeats and its time of action implies a role distinct from Polycomb group products. Mechanisms of development. 53: 197-208

Simon, JA and Kingston, RE. 2013. Occupying chromatin: polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. Molecular cell. 49

Schulz, C and Tautz, D. 1995. Zygotic caudal regulation by hunchback and its role in abdominal segment formation of the Drosophila embryo. Development. 121, 1023-1028

Struhl, G and Akam, M. 1985. Altered distributions of Ultrabithorax transcripts in extra sex combs mutant embryos of Drosophila. The EMBO journal vol 4 no 12

Staller, MV., Yan, D., Randklev, S., Bragdon, MD., Wunderlich, ZB., Tao, R., Perkinds, LA., DePace, AH., Perrimon, N. 2013. Depleting gene activities in early Drosophila embryos with the “maternal gal4 shRNA” system. Genetics vol 193

Schaaf, CA., Misulovin, Z., Gause, M., Koenig, A., Gohara, DW., Watson, A., Dorsett, D. 2013. Cohesin and polycomb protiens functionally interact to control transcription at silenced and active genes. PLOS genetics vol 9 issue6.

Schmitages, FW., Prusty, AB., Faty, M., Stutzer, A., Lingaraju, GM., Aiwazian, J., Sack, R., Hess, D., Li, L., Zhou, S., Bunker, RD., Wirth U, Bouwmeester T, Bauer A, Ly-Hartig N, Zhao, K., Chan, H., Gu, J., Gut, H., Fischle, W., Muller, J., Thoma, NH. 2011. Histone Methylation by PRC2 is inhibited by active chromatin marks. Cell. 42, 330-341

Schoeftner, S., Sengupta, AK., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., Wutz, A. 2006. Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. The EMBO Journal. 25, 3110-3122

Schroeder, MD., Pearce, M., Fak, J., Fan, HQ., Unnerstall, U., Emberly, E., Rajewsky, N., Siggia, ED., Gaul, U. 2004. Transcriptional control in the segmentation gene network of Drosphila. PLOS. Vol2. Issue9

Schuettengruber, B., El-kayam, NO., Sexton, T., Entrevan, Stern, S., Thomas, A., Yaffe, E., Parrinello, H., Tanay, A., Cavalli, G. 2014. Cooperativity, specificity, and evolutionary stability of Polycomb targeting in Drosophila. Cell. 9, 219-233

Schuettengruber, B and Cavalli, G. 2009. Recruitment of Polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development. 136, 3531-3542

Schwartz, YB., and Pirrotta, V. 2013. A new world of polycombs: unexpected partnerships and emerging functions. Nature. Vol14

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, JR., Wu, CT., Bender, W., Kingston, R. 1999. Stabilization of Chromatin Structure by PRC1, a Polycomb Complex. Cell. Vol98: 37-46

Shen, X., Liu, Y., Hsu, YJ., Fujiwara, Y., Kim, J., Mao, X., Yuan, GC., Orkin, SH. 2008. Ezh1 mediates methylation on histone H3 lysine 27 and complements Ezh2 in maintaining stem cell identity and executing plurpotency. Molecular cell, 32, 491-502

Shir-Shapira, H., Sharabany, J., Filderman, M., Ideses, D., Ovadia-Shochat, A., Mannervik, M., Juven-Gershon, T. 2015. Structure-Funcion analysis of the Drosophila melanogaster Caudal transcription factor provides insights into core promoter-preferential activation. The Journal of Biological Chemistry. Vol290

Soto, MC., Chou, TB., Bender, W. 1995. Comparison of germline mosaics of genes in the polycomb group of Drosophila melanogaster. Genetics 140: 231-243

Stanojevic, D., Small, S., Levine, M. 1991. Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. Science. 254:5036

Struhl, G. 1981. A gene product required for corrct initiation of segmental determination in Drosophila. Nature. Vol 293

Struhl, G. 1983. Role of the esc+ gene product in ensuring the selective expression of segment-specific homeotic genes in Drosophila. J. Embryol. Exp. Morph. 76: 297-331

Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E., Harte, PJ., The Drosophila Polycomb Group proteins ESC and E(z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. Development. 128: 275-286

Tie, F., Prasad-Sinha, J., Birve, A., Rasmuson-Lestander, A., Harte, PJ. 2003. A 1-Megadalton Esc/E(z) complex from Drosophila that contains polycomblike and RPD3.

Molecular and cellular biology

Tie, F., Stratton, CA., Kurzhals, RL., Harte, PJ. 2007. The N terminus of Drosophila ESC binds directly to histone H3 and is required for E(Z)-dependent trimethylation of H3 lysine 27

Tie, F., Banerjee, R., Stratton, CA., Prasad-Sinha, J., Stepanik, V., Zlobin, A., O. Diaz, M., Scacheri, PC., Harte, PJ. 2009. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila polycomb silencing. Development. 136:3131-3141

Tolhuis, B., Muijrers, I., de Wit, E., Teunissen, H., Talhout, W., Steensel, Bv., Lohuizen, Mv., Genome-wide profiling of PRC1 and PRC2 polycomb chromatin binding in Drosophila melanogaster. Nature.

Tracey, WD,Jr., Ning, X., Klingler, M., Kramer, SG., Gergen, JP., 2000. Quantitative analysis of gene function in the Drosophila embryo. Genetics 154: 273-284

Tyler, JK., Bulger, M., Kamakaka, RT., Kobayashi, R., Kadonaga, JT., 1996. The p55 Subunit of Drosophila chromatin assembly factor 1 is homologous to a histone deacetylase-associated protein. Molecular and cellular biology. p. 6149-6159

Urmi, S., Benes, J., Zhang, J., Jones, RS. 2008. Recruitment of Drosophila Polycomb-group proteins by polycomblike, a component of a novel protein complex in lavae. Development 135, 813-817

Usui, H., Ichikawa, T., Kobayashi, K., Kumanishi, T., 2000. Cloning of a novel murine gene Sfmbt, Scm-related gene containing four mbt domains, structurally belonging to the polycomb group of genes. Gene. 248:127-135

Walker, E., Chang, WY., Hunkapiller, Julie., Cagney, G., Garcha, K., Torchia, J., Krogan, NJ., Reiter, JF., Stanford, WL., Polycomb-like 2 associates with PRC2 and regulates

transcriptional networks during mouse embryonic stem cell self-renewal and differentiation.

Wang, C., Dickinson, LK., Lehmann, R., 1994. Genetics of nanos localization in Drosophila. Developmental dynamics. 199:103-115

Wang, LJ., Brown, JL., Cao, Ru., Zhang, Y., Kassis, JA., Jones, RS., 2004. Hierarchical Recruitment of Polycomb Group silencing complexes. Molecular cell, vol14

Wang, LJ., Jahren, N., Vargas, ML., Andersen, EF., Benes, J., Zhang, JY., Miller, EL., Jones, RS., Simon, JA., 2006. Alternative ESC and ESC-like subunits of a polycomb group histone methyltransferase complex are differentially deployed during Drosophila development. Molecular and cellular biology. p.2637-2647

Wang, LJ., Jahren, N., Miller, EL., Ketel, CS., Mallin, DR., Simon, JA., 2010. Comparative analysis of chromatin binding by sex comb on midleg and other polycomb group repressors at a Drosophila Hox gene. Molecular and cellular biology. p. 2584-2593

Wilkinson, F.H., Park, K, Atchison, M.L, Polycomb recruitment to DNA in vivo by the YY1 REPO domain. Proc. Natl. Acad. Sci. 103(2006),PP. 19296-19301

Wu, SC., Zhang, Y. 2011. Cyclin-dependent Kinase1(CDK1)-mediated phosphorylation of enhancer of zeste2(ezh2) regulates its stability. The journal of biological chemistry. Vol 286

Xu, C., Bian, C., Yang, W., Galka, M., Ouyang, H., Chen, C., Qiu, W., Liu, H., Jone, AE., MacKenzie, F., Pan, P., Li, SSC., Wang, H., Min, J., 2010. Binding of different histone marks differentially regulates the activity and specificity of polycomb repressive complex 2 (PRC2). PNAS. Vol107, no.45

Yan, D., Neumulller, RA., Buckner, M., Ayers, K., Li, H., Hu, Y., Yang-Zhou, D., Pan, L.,

Wang, X., Kelly, C., Vinayagam, A., Binari, R., Randklev, Perkins, LA., Xie, T., Cooley, L., Perrimon, N. 2014. A regulatory network of Drosophila germline stem cell self-renewal. Dev Cell 28(4)

Yuan, W., Wu, T., Fu, H., Dai, C., Wu, H., Liu, N., Li, X., Xu, M., Zhang, Z., Niu, T., Han, Z., Chai, J., Zhou, XJ., Gao, S., Zhu, B., 2012. Dense chromatin activates polycomb repressive complex 2 to regulate H3 lysine 27 methylation. Science. Vol 337.

Zeng, X., Han, L., Lin, X., Hou, SX.2015. Genome wide RNAi screen identifies networks involved in testinal stem cell regulation in Drosophila. Cell

Zhao, J., Sun, BK., Erwin, JA., Song, JJ., Lee, JT., 2008. Polycomb proteins targeted by a short repeat RNA to the mouse x chromosome. Science. Vol 322.

Zhang, Y., Cao, R., Wang, L., Jones, RS., 2004. Mechanism of polycomb group gene silencing. Cold spring harbor symposia on quantitative biology. vol LXIX