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The Roles of Individual Proteins in De Novo Recruitment of PcG Repressive Complexes to Giant

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THE ROLES OF INDIVIDUAL PROTEINS IN DE NOVO RECRUITMENT OF PcG REPRESSIVE COMPLEXES TO GIANT

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THE ROLES OF INDIVIDUAL PROTEINS IN DE NOVO RECRUITMENT OF PcG REPRESSIVE COMPLEXES TO GIANT

A Thesis Presented to the Graduate Faculty of
Dedman College
Southern Methodist University

in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

in
Molecular and Cellular Biology

by
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May 14, 2022
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Thank you to all of the members of the Jones Lab, I would not be here today if it were not for the help, support, and guidance from you all. I would like to extend a thank you to my lab neighbor, Nick Kurtaneck. Without your comradery, empathy, and encouragement, I would not have been able to finish this program. Thank you also to all of my amazing friends at SMU who I have been able to lean on and who have cheered me on throughout my undergraduate and graduate years.

I would also like to express my deepest gratitude to my parents, C.G. and Vikki, who have always believed in me and supported my dreams. Your support and love have meant the world to me. Thank you also to my siblings, Ryan, Courtney, and Lauren who have given me indispensable advice and support throughout this program.
Epigenetic gene regulation is the process by which external factors regulate the genome. This research studies Polycomb Group (PcG) proteins which function as epigenetic agents that work together in complexes to maintain gene silencing for multiple cellular generations.

*Drosophila melanogaster* PcG proteins can be organized into three canonical complexes: Pho-RC, PRC1, and PRC2. Though there are multiple proposed models for the order of recruitment, it is generally accepted that PhoRC, PRC1, and PRC2 interact with each other to stably recruit to a target gene. Since these proteins are highly conserved, this project studies PcG proteins in the model organism, *Drosophila melanogaster*. In *Drosophila*, these proteins are key regulators of developmental genes, such as giant. In previous work conducted by this lab, it has been shown that a PcG protein Pho, a core component of Pho-RC, binds to giant at two different Polycomb Response Elements (PREs). Through Chromatin Immunoprecipitation experiments, the timing of PcG proteins’ *de novo* recruitment to giant PREs has been established.

To further our understanding of PcG recruitment, my project looks at the roles of individual PcG proteins. Studies have elucidated the activity and function of PcG complexes, but not the behavior and activity of each PcG protein. Through the depletion of one PcG protein at a
time, we were able to independently assay the effect of Pho and Sfmbt on recruitment and assay the effect on other PcG proteins.

This project follows two experimental aims. The first aim is to assay the effect of the depletion of Pho and Sfmbt, respectively, to observe the impact on components of PRC1 and PRC2. Through ChIP analyses at three distinct developmental time points, a pattern emerges when analyzing the presence of PcG proteins at gt PRE1 and PRE2. The data suggest that Sfmbt may play a role in the recruitment of PhoRC and potentially interacts independently of Pho. In addition, the divergence of dRING from Pc suggests its involvement with non-canonical PcG complexes through early developmental time stages. Further research needs to be conducted to see if there is a reciprocal effect of other PcG protein knockdowns on Pho and Sfmbt.

The second aim is to assay the effectiveness of a novel transgene to reduce early embryonic expression of Pcl to determine if this method would allow for effective knockdown of Pcl for future experimentation. Zygotic gene expression occurs post-Midblastula transition (MBT) in embryos, but this vector was designed to induce pre-MBT expression to more effectively deplete zygotically expressed Pcl. Future studies would selectively knockdown Pcl to assay the effect on other PcG recruitment.
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This thesis is dedicated to all the fruit flies that have sacrificed their lives for my research.
1.1 Polycomb Group Proteins

Through highly conserved repressors called Polycomb Group proteins, genetic silencing can be maintained for multiple cellular generations, though they are not the initial repressors (Jones and Gelbart, 1990). PcG proteins work together in complexes to repress transcription of target genes (Di Croce, 2013). They are further organized into three main complexes: Pho-Repressive Complex (Pho-RC), Polycomb Repressive Complex II (PRC2), and Polycomb Repressive Complex I (PRC1) (Figure 1.1). Within the Pho-RC complex are two proteins: Pho and Sfmbt. Pho is the only protein that is able to bind directly to DNA through its DNA binding capabilities (Klymenko 2006). PRC1 is composed of Psc, Ph, dRing, and Pc, proteins and works to ubiquitylate Histone 2A (Francis et al., 2001). PRC1 also works with PRC2, which contains the Polycomb proteins, N55, Su(z)12, E(z), and Esc along with an accessory protein, Pcl. PRC2 functions to methylate Lysine 27 of Histone 3 (Cao et al., 2002).
Figure 1.1 - Canonical PcG Complexes: Pho-RC, PRC2, and PRC1. Blue circles indicate H3K27me3. Yellow stars indicate H2K119K118ub. Histones are pink. Pcl is considered an accessory to PRC2 and not a core component. dRAF and L(3) are non-canonical PRC1 complexes that both contain dRING. dKdm2 is a histone demethylase and therefore removes methyl groups from Histones. dRING and Psc mediate H2K119K118ub as well as dRING and L(3)73Ah.

1.1.1 Pleiohomeotic Repressive Complex, PhoRC

AlHaj Abed et al. (2018) has demonstrated that the PhoRC complex is enriched at giant PRE’s before PRC1 and PRC2. This is evidenced through enrichment of Pho at PRE1 and PRE2 from nc10-12 to nc14b (AlHaj Abed et al., 2018). Despite a nonconsecutive increase in Sfmbt signaling at PRE1 and PRE2 over the same developmental time course, Sfmbt fold-enrichment was well above background throughout these stages (AlHaj Abed et al., 2018). The early presence of Pho and Sfmbt at nc10-12 suggests that Pho-RC is the first PcG complex to stably
bind to PRE1 and PRE2 before other PcG proteins, as stable binding of PRC1 and PRC2 occurs at later developmental stages.

Previous research has shown that Pho directly binds to DNA and recruits complexes containing E(z). This recruitment allows E(z) to methylate histone H3 at lysine 27 (Wang et al., 2004). This lab has also shown that mutations of Pho consensus sites do not completely prevent PRC2 (E(z)) and PRC1 (Pc) from binding at PRE1 (Ghotbi et al., 2020). However, if Pho is depleted, we anticipate weak E(z) enrichment at PRE1 and therefore low methylation signals of histone H3 at lysine 27. Specifically, at PRE2, there is no change in the recruitment of PhoRC, PRC1, and PRC2 if Pho consensus sites are mutated at PRE1 (Ghotbi et al., 2020). Two conclusions could potentially be drawn from the effect of Pho knockdown when looking at PRE2 recruitment. If Pho knockdown leads to decreased binding of E(z) or Pc to PRE2, then this data would suggest that recruitment follows a hierarchical model: the recruitment of Pho allows the subsequent recruitment of PRC1 and PRC2. However, if the depletion of Pho does not strongly inhibit recruitment of E(z) or Pc to PRE2, then PRC1 and/or PRC2 might play a role in the recruitment of Pho. In this scenario, more studies would need to be performed to assess this relationship.

Other studies have depleted Pho by mutating Pho binding sites; however, the individual knockdown of Pho has not been studied. Therefore, the knockdown might indirectly affect stable recruitment of these complexes to PRE1 and PRE2 since the depletion of Pho is directly targeted.

Biochemical analysis has shown that Sfmbt, the non-DNA binding PcG protein of PhoRC, is able to directly bind to Scm (Sex Comb on midleg), a PRC1 accessory protein (Frey et al., 2016). Scm, in turn, binds to Ph, a PRC1 subunit and can also bind directly to PRC2.
Scm has therefore been studied as a link between these two PcG complexes through directly interacting with both (Kang et al., 2015). Through the knockdown of Sfmbt, there are three different scenarios that could occur that would inform our understanding of the individual role it plays. If Sfmbt knockdown results in the loss of PRC1 or PRC2 at PRE’s, but the presence of Pho, then the resultant proposed model would suggest that Pho-RC, through Sfmbt, recruits PRC1 and/or PRC2. However, if PRC1 or PRC2 presence at PRE’s is not lost due to Sfmbt knockdown in the absence of Pho, then PRC1 and/or PRC2 might be responsible for the direct recruitment of Pho-RC to a PRE. Alternatively, if the knockdown of Sfmbt leads to the loss of Pho, PRC1, and PRC2, then we cannot identify the mechanistic relationships of recruitment, except that Pho requires Sfmbt in order to be recruited.

Though previous studies have highlighted the contributions of Pho-RC, our research will demonstrate how Pho and Sfmbt individually perform in order to elucidate each subunit’s role.

1.1.2 Polycomb Repressive Complex 2, PRC 2

E(z) is a core component of PRC2 and is used to identify the presence of PRC2 at a given PRE. It is the only PcG protein to methylate, specifically at histone H3 at Lysine 27 (Wang et al., 2004). Therefore, H3K27me3 signals resemble the fold enrichment of E(z).

PRC2 appears to be weakly recruited to PRE1 during nc10-12 and nc13 as demonstrated in E(z) enrichment that is not notably above background levels (AlHaj Abed et al., 2018). However, by nc14a, E(z) enrichment increases at PRE1 and by nc14b is stabilized in this region.

At PRE2, the presence of E(z) does not appear until nc13. By nc14a, it is stably recruited to PRE1 and remains present during nc14b (AlHaj Abed et al., 2018).

Current models propose that despite the presence of an activator, PhoRC is still able to bind to PRE’s, yet PRC2 is prevented from being recruited potentially due to factors like CBP
that acetylate histone H3 at lysine 27 (Ghotbi et al., 2021). The presence of a repressor is sufficient to allow the recruitment of PRC2, even in the presence of an activator (Ghotbi et al., 2021).

1.1.3 Polycomb Repressive Complex 1, PRC 1

Pc is a core PcG protein of PRC1 that is used to detect the presence of PRC1 at various PRE’s. Pc is able to recognize Histone 3 Lysine 27 trimethylation through its chromodomain (Min et al., 2003). Since E(z) deposits trimethylation at Histone 3 Lysine 27, it provides a functional link between PRC2 and PRC1 through the ability of Pc to recognize trimethylation.

dRING, another component of PRC1 has H2A ubiquitin E3 ligase activity, which allows Polycomb-target gene silencing (Wang et al., 2004). dRING, however, has also been shown to interact in a non-canonical PRC1 complex (ncPRC1). This complex, called dRAF, consists of dRING, Psc and the F-box protein, and dKDM2, a demethylase (Lagarou et al., 2008). Lagarou et al. (2018) demonstrate that dRAF contributes to the ubiquitination of Histone 2A at Lysine 118 (H2AK118ub) in Drosophila. Research has also shown that dRING could participate in another ncPRC1 complex with L(3)73Ah, which also functions to distribute H2AK118ub (Lee et al., 2015).

Unlike with PRC2, PRC1 has been shown to not be stably recruited to PRE1 or PRE2 until nc14b (AlHaj Abed et al., 2018). PRC1’s stable association is likely impeded due to the inability of Pho-RC to stably bind to PRE’s in the presence of an activator, such as Caudal (Ghotbi et al., 2021).

1.2 giant gene

Petschek et al. (1987) first identified Giant (gt) as a gap gene through a series of genetic screens. Gap genes are responsible for the segmental pattern of an embryo during development
Giant first was recognized as a genetic target by PcG proteins through the discovery that E(z) suppresses maternal nanos, which leads to giant expression even when hunchback is present (Pelegri and Lehman, 1993).

PcG proteins recognize where to bind to giant through Polycomb response elements (PREs). Two PRE regions have been mapped at giant through observing where Pho binds to DNA (AlHaj Abed et al., 2013). Pho is the only PcG protein with DNA-binding capabilities. Figure 1.2, derived from Ghotbi et al. (2021), describes the positions of both PRE’s as well as the location of giant-specific enhancers. As shown in the Figure, PRE1 corresponds with region four, which is (-0.05 to -1.3kb) upstream from the giant promoter and PRE2 corresponded with region 9, which is (-4.3 to -6.5kb) upstream from the giant promoter (AlHaj Abed et al., 2013). PRE1 contains the giant promoter (AlHaj Abed, 2013). Two other relevant regions include enhancer regions 3 and 6. Region 3 notably encompasses the transcription start site for the giant gene.

Since giant has been studied extensively in our lab and has been characterized for its PRE’s, it was chosen to be studied further for PcG binding. One gap in our current understanding is the relationship of recruitment at PRE1 versus PRE2. PRE2 PcG binding has been shown to occur despite whether Pho is associated with PRE1 or not (Ghotbi et al., 2020). However, we do not know if PRE2 follows a hierarchal model of recruitment or if recruitment of PRE2 impacts recruitment at PRE1.
1.3 Establishment of bcd osk tsl background in Drosophila

The goal of the bicoid oskar torso-like (bcd osk tsl) triple mutant system is to repress giant (gt), a Polycomb group protein target gene universally in the embryo, so that Polycomb group proteins can take over repression (Alhaj Abed et al., 2018). This system has previously been established and utilized in previous research (Alhaj Abed, 2018; Ghotbi et al., 2021). In the typical Drosophila embryo, maternal genes, hunchback (hb) and caudal (cad) are degraded and the zygotic forms of these genes take over giant repression (Niessing et al., 1997). However, in this triple mutant system, zygotic hunchback and caudal do not take over repression, but Polycomb group proteins are able to take over repression (Alhaj Abed et al., 2018). In order to establish this system, flies were generated in a bicoid oskar torso-like (bcd osk tsl) genetic background. All three genes are maternally regulated, meaning that the phenotype of the offspring depends solely on the genotype of the mother.

Bicoid (bcd) is an activator of the giant gene [Eldon and Pirrotta, 1991]. Under this triple mutant system, offspring of these mothers are bcd null. This results in embryos where maternal caudal, another gt activator, is ubiquitously expressed and zygotic hunchback and caudal are unable to be activated (Driever and Nüsslein-Volhard, 1989). However, despite the presence of
maternal *caudal*, *gt* is not activated due to the presence of maternal *hunchback* (Rivera-Pomar et al., 1995).

*Oskar (osk)* is another maternal morphogen whose absence in the triple mutant system offspring results in the inability of *nanos* to localize posteriorly (Ephrussi et al., 1991). Maternal hunchback, as a result, is no longer restricted anteriorly, which leads to ubiquitous repression of *gt*.

*Torsolike (tsl)* is needed for zygotic *hunchback* expression, yet the lack of *tsl* in offspring of *bcd osk tsl* mothers prevents this effect (Deshpande et al., 2004). Another targeted effect that occurs in these embryos is the zygotic *hunchback* and *caudal* cannot be activated.

All three of these morphogens set the stage for the offspring to maintain giant repression through maternal *hunchback* and *caudal* while allowing Polycomb group proteins to take over repression where zygotic forms of *hb* and *cad* normally would in a Wild type background.

### 1.4 Drosophila Melanogaster Embryonic Development

The boundaries of *Drosophila* embryogenesis are characterized by nuclear division cycles (Figure 1.3). After an egg is fertilized, it will undergo 13 nuclear divisions as one cell (Foe and Alberts, 1983). This forms the syncytium, which contains nuclei from 13 mitotic divisions. Prior to this stage, nuclei migrate to the cortex of the embryo (Kotadia et al., 2010). This allows for the simultaneous cellularization of these nuclei at the periphery of the embryo during nuclear cycle 14a, thus generating the cellular blastoderm (Foe and Alberts, 1983).
Early studies of gene expression in *Drosophila* embryos during these stages identifies a transition from maternal expression to zygotic gene expression. Experimental evidence has shown that zygotic gene expression does not occur until nuclear cycle 14 (Merrill et al., 1988). This hand-off from maternal to zygotic gene expression is called the Midblastula Transition (MBT), which occurs simultaneously during the cellularization of nuclear cycle 14 (Farrell and
O’Farrell, 2014). This idea allows us to create a triple mutant system that is maternally regulated and allows for the zygotic transcription and activation of Polycomb group proteins. We also observe the varying presence of PcG proteins throughout these different developmental time points.

1.5 VALIUM Vectors

Through the Harvard TRiP transgene project, several effective vectors have been developed and tested that introduce RNAi knockdown in germline and somatic tissue types. In my project, fly lines derived from VALIUM20, VALIUM22, and VALIUM22z (unpublished vector created in this lab) were used to introduce GAL-4 inducible short hairpin RNA that express a designated transgene.

VALIUM20 is effective for inducing somatic expression, while VALIUM22 is most effective in inducing germline gene expression (Ni et al., 2011). One of the contributors to this difference in expression is through the P-element promoter found in VALIUM22 constructs and the hsp70 promoter found in VALIUM20 constructs (Figure 1.4). For example, P-element promoters are known for driving germline expression; whereas, hsp70 promoters do not work well in the germline.

An issue encountered through using these vectors is optimizing knockdown in both soma and germline cells. Through a thought-experiment turned reality, our lab has attempted to create an optimized VAL22 vector, named VAL22z. The goal of creating VAL22z is to induce pre-Midblastula transition (MBT) zygotic expression. The idea is that post-MBT introduces widespread zygotic expression, but if zygotic expression is induced earlier, then there will likely be stronger zygotic expression that will continue from pre- to post-MBT. This is accomplished
through the introduction of 2 TAGteam Zelda binding sites that are added upstream of the promoter. The TATA box and initiator region are also modified to match the pre-MBT promoter more closely (Figure 1.5). TAGteam Zelda binding sites are found to be upstream of genes that are transcribed prior to nc14 in Drosophila embryos (ten Bosch et al., 2006). For this reason, there is evidence that inserting TAGteam Zelda binding sites will induce transcription of a gene pre-MBT, if they are placed upstream of the target gene.
Figure 1.4- VALIUM20 and VALIUM22 expression vectors. Promoters are in shamrock green.
Figure 1.5- VALIUM22z Seq-L primer-Reverse Complement Sequence. This sequence represents the DNA inserted into the conventional VALIUM22 vector. The highlighted regions represent ZELDA binding sites in magenta, the modified TATA box in cyan, and the modified initiator region in yellow.
CHAPTER 2:

METHODS

2.1 Chromatin Immunoprecipitation Experiments

2.1.1 Embryo Collection and Fixation

For each acrylic cage, around 8,000-10,000 females and 8,000-10,000 males were placed inside for large-scale collections. The cage was fully sealed except for an entry point through Nylon tights that allow for access to collection plates. Collection plates contained grape juice infused-agar with a fresh yeast paste spread on the surface. Around 8-10 collection plates were placed inside the cage for egg lay. Before timed collections commenced, a pre-lay of 1-2 hours was conducted to allow for the females to lay and get rid of any older embryos. After the pre-lay, plates were exchanged for fresh agar-yeast plates that were then changed every 30 minutes in a 25°C incubator. Plates were then aged in 25°C outside of the cage depending on the desired developmental stage being studied (Table 2.1).
Table 2.1-Embryo developmental stages and their aging time after egg lay (AEL) based on 30-minute collection intervals.

<table>
<thead>
<tr>
<th>Embryo Developmental Stage</th>
<th>Time AEL (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nc13</td>
<td>110-140</td>
</tr>
<tr>
<td>nc14a</td>
<td>140-170</td>
</tr>
<tr>
<td>nc14b</td>
<td>170-200</td>
</tr>
</tbody>
</table>

Embryos from the plates were washed off and dechorionated using a 50% bleach solution where they were submerged in a mesh basket for 2 minutes. The embryos were then transferred with a spatula to a 15mL conical bottom tube containing a fixation solution of 2mL PBS/0.5% Triton-X-100 (PBST) and 6mL heptane. A timer was set for 15 minutes immediately after 108µL Formaldehyde was added to the fixation tube. The fixation tube with the embryos was vortexed thoroughly and then allowed to rotate at room temperature for 10 minutes. When the 10 minutes was complete, the embryos were centrifuged and all liquid was aspirated out of the tube. Once the 15-minute timer went off, the fixation reaction was quenched using 1mL of PBST/125mM Glycine. The embryos were transferred to fresh tubes and were washed with cold PBST 3 times. 100µL of 100X Sigma Protease Inhibitor were added to the last wash. Using forceps under a Zeiss PrimoVert microscope, the embryos were sorted in order to remove older embryos. Embryos were aliquoted in tubes based on their mass and developmental stage (Table 2.2).
Table 2.2-Mass of embryos per tube with corresponding developmental stages

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Mass per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>nc13</td>
<td>20mg</td>
</tr>
<tr>
<td>nc14a</td>
<td>10mg</td>
</tr>
<tr>
<td>nc14b</td>
<td>10mg</td>
</tr>
</tbody>
</table>

2.1.2 Chromatin Immunoprecipitation Assay

For each pull-down, 50µL of Protein A magnetic beads (Pierce #88846) were added to each Protein LoBind tube. Each tube with the Protein A beads was blocked with PBST-3% BSA for one hour. This method was employed since the antibodies we later use work best in competition and PBST-3% BSA binds nonspecifically to the beads. Once the Protein A beads were blocked for an hour, the blocking solution was taken out and replaced with the antibody dilutions (Table 2.3). The antibody-beads incubated for 3 hours in 4°C on the rotator.
Table 2.3 - Antibody antiserum dilutions in 250µL of PBST-3% BSA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Volume added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pho (Brown et al., 2003)</td>
<td>5</td>
</tr>
<tr>
<td>α-Sfmbt (Alhaj Abed et al., 2018)</td>
<td>5</td>
</tr>
<tr>
<td>α-E(z) (Carrington and Jones, 1996)</td>
<td>10</td>
</tr>
<tr>
<td>α-Pcl (O’Connell et al., 2001)</td>
<td>10</td>
</tr>
<tr>
<td>α-Pc (Wang et al., 2004)</td>
<td>10</td>
</tr>
<tr>
<td>α-dRING</td>
<td>5</td>
</tr>
<tr>
<td>H3 (Abcam #ab1791)</td>
<td>0.5</td>
</tr>
<tr>
<td>H3K27me3 (Millipore #07-449)</td>
<td>0.2</td>
</tr>
<tr>
<td>Mock (Anti-IgG, Cell Signaling #2729)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

While the antibodies incubated with the Protein A beads, previously sorted embryos were prepared for sonication. Embryos were thawed and homogenized using a pestle in 50-100µL of RIPA buffer made of 10mL RIPA, 1:100 Sigma Protease Inhibitor (Sigma-Aldrich #P8340), and 10µL 1M DTT. The volume was brought to 700µL with RIPA buffer and the tubes centrifuged in 4°C at full speed for 5 minutes. Any supernatant was discarded. 100uL of RIPA buffer was added to the embryos so that they could be pounded and homogenized with the same pestle used previously. The volume was brought to a concentration of 10mg per 1mL RIPA buffer for nc14a and nc14b embryos. For nc13, the final volume was brought to 20mg per 1mL RIPA buffer. The embryos were sonicated using a microtip with the settings: 30% power, 15 sec pulses, 45 sec pauses for a total of 4.5 minutes. After every 45 seconds, the machine was paused so that the chromatin could be quickly vortexed and centrifuged to allow for uniform sonication. When the
4.5 minutes of sonication ended, the chromatin was centrifuged in 4°C for 6 minutes. The supernatant (chromatin) was transferred to fresh Protein Lobind tubes and incubated with Salmon sperm DNA/Protein-A beads (Millipore #16-157) for 1 hour at 4°C.

Antibody-bead tubes that had been incubating for 3 hours were placed on magnetic stands in order to pipet out the supernatant liquid. 100µL of sonicated chromatin and 500µL of RIPA buffer was added to each antibody-bead tube. The tubes were allowed to rotate in 4°C overnight. For preparing an input tube, 20µL of chromatin was incubated with 0.5µL of RNase A for 30 minutes in a 37°C heat block. To perform a proteinase K digest and to reverse crosslinks for genomic DNA, the input was treated with 77.5µL elution buffer (50mM Tris-Hcl pH 7.5, 10mM EDTA, 1% SDS, 300mM NaCl) and 2.5µL of Proteinase K (10mg/mL to a final of 0.25 mg/mL). The input was kept in a 65°C water bath overnight.

The next day the beads underwent several washes (each lasting a maximum of two minutes) in order to prepare the beads to separate from the antibody. This process functions to separate the DNA from the protein and antibody. The beads were washed in the following order: Low salt wash buffer x3 (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), High salt buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 500mM NaCl), LiCl buffer x2 (0.25M LiCl, 1% NP-40, 1% SDC, 1mM EDTA, 10mM Tris-HCl pH 8.0), and TE buffer (20mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). After washing with TE buffer, 100uL of Elution buffer (50mM Tris-HCl pH 7.5, 10mM EDTA, 1% SDS, 300mM NaCl) was added to each antibody-bead tube. The tubes were vigorously vortexed and incubated at 65°C in the hot water bath for 15 minutes. Every 5 minutes within that interval, the tubes were vortexed and centrifuged. After incubation, the tubes were placed on a magnet and the DNA supernatant to fresh protein lobind tubes. In each tube, 95µL TE buffer and 5µL Proteinase K
(10mg/mL) were added. The input tube was taken out of the 65°C water bath and prepared with 100µL TE.

10µL of 3M Sodium Acetate was added to all IP tubes and the input. Next, for the phenol chloroform extraction, 200µL of phenol chloroform isoamyl alcohol was added to each tube. The tubes were vigorously shaken. All tubes were centrifuged at room temperature for 3 minutes on top speed. The top layer of each tube was transferred to new corresponding Protein Lobind tubes. An equal amount of chloroform was added, then all the tubes were vigorously shaken. Tubes were then centrifuged for 30 seconds at top speed. The top aqueous layer was extracted slowly and placed into new corresponding Protein Lobind tubes. Then for the ethanol precipitation step, each tube was prepared with 2µL of Glycogen (20µg/µL) and 2x volume of 100% ethanol. The reaction was allowed to run at least two hours. After letting the tubes defrost at room temperature, they were centrifuged at top speed for 15 minutes to pellet the DNA. After the spin, the supernatant was removed with a pipette. Next, a wash of 70% ethanol was done, after which the tubes were inverted several times. The tubes were centrifuged again for 30 seconds at top speed and the supernatant removed. They were then placed in the SpeedVac for at least 15 minutes and resuspended in 40µL of ChIP/PCR water.

Each tube then received 50µL of Agencourt magnetic beads for DNA purification ((Beckman Coulter #A63880)). The tubes were incubated for 30 minutes on a rotator at room temperature. After this incubation, they were placed on the magnetic stand for 15 minutes and the supernatant discarded as the DNA was still attached to the beads. A quick wash with 80% Ethanol was administered twice and the beads were allowed to dry on the magnetic stand for 5 minutes. Post-dry-time, 113µL of ChIP/PCR purified water was added to each tube and allowed to incubate off the magnet for 2 minutes. Subsequently, the tubes were placed on the magnetic
stand for 5 minutes. 112uL of the supernatant were each transferred to new tubes, respectively. The Input tube received an additional 112uL of purified ChIP PCR water.

2.1.3 Polymerase Chain Reaction

For PCR analysis, each set of tubes was analyzed using 5 different primer mixes (Table 2.4). PKA, gt4, and gt17 all share an annealing temperature of 55°C. The other primers—gt3 and gt8—had an annealing temperature of 58°C.

Table 2.4—Primers alongside their sequence read from 5’ to 3’

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA-C1</td>
<td>Forward: CAATCAGCAGATTCTCCGGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCCGCACTCGCGCTTC</td>
</tr>
<tr>
<td>gt4</td>
<td>Forward: AACGCAAACGGATTTTCCTCTCG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAGAAGCAAAGCCAGAATCC</td>
</tr>
<tr>
<td>gt3 (3-endogenous)</td>
<td>Upper: GGAGTCTTTCTGGGTGTCTCTACGC</td>
</tr>
<tr>
<td></td>
<td>Lower: CCACTTGCGCGACAGCCAAT</td>
</tr>
<tr>
<td>gt8</td>
<td>Forward: CGTATAGCCCAAGCCAATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCATTATGGCGAAGGAACA</td>
</tr>
<tr>
<td>gt17</td>
<td>Forward: CCGGGCCATGCAATAAGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGCTTCTCCAACCTCCATATTC</td>
</tr>
</tbody>
</table>

For each PCR reaction, a Master Mix was prepared and 20µL MM was distributed into each PCR reaction tube (Table 2.5). Subsequently each tube received 5µL of DNA. Each PCR test was conducted in three biological replicates, which each had three internal technical replicates.
Table 2.5- **Master Mix Recipe**

<table>
<thead>
<tr>
<th></th>
<th>1 tube (x1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR ChIP Certified Water</td>
<td>7uL</td>
</tr>
<tr>
<td>Primer Mix (20uM of F/R primers)</td>
<td>0.5uL</td>
</tr>
<tr>
<td>Super Mix</td>
<td>12.5uL</td>
</tr>
</tbody>
</table>

Data was normalized to PKA, a negative control where no Polycomb recruitment occurs. The DNA Inputs--10%, 1%, and 0.2%--where averaged and adjusted to find the average of concentration of PKA. Each technical replicate for each antibody was averaged in order to determine the signal across the data (**Table 2.6**). Fold Enrichment was tabulated against control experiments in order to determine if there was statistically significant knockdown of the target protein and what effects that had on the recruitment of the rest of the proteins. This was analyzed for each region that the antibodies were being tested in (PKA, gt4, gt3, gt8, and gt17).

**Table 2.6- Data analysis formulas for PCR:**

\[
\Delta \Delta CT^{**} = \left[ \text{INPUT of (+) region} - \text{INPUT of PKA} \right] - \left[ \text{signal of (+) region} - \text{signal of PKA} \right]
\]

\[2\Delta \Delta CT = \text{Fold Enrichment}\]

**CT is defined as the cycle time**

(Rao et al., 2013)
Statistical significance was computed using the GraphPad unpaired student $t$ test calculator.

### 2.2 Cuticle Preparation

Flies were bred (female: male; 2:1 ratio) in small perforated beakers attached to agar-yeast petri dishes (Figure 2.1). Embryos were collected from these agar-yeast petri dishes for 24 hours in a 25°C incubator. These embryos were aged off the beaker for an additional 24 hours at 25°C. Embryos were washed off agar petri dishes and collected through a sieve. The embryos within the sieve were exposed to a 50% bleach solution in order to remove their chorion membranes. After this dechorionation step, embryos were washed 3 times in 0.1% Triton DD H.O in 1.5mL tubes. The embryos were transferred to microscope slides and any liquid was dried with Kim wipes carefully around them. Once the slide was completely dry, CMCP-10 high viscosity mountant (Polysciences #16300) was applied directly on the embryos and a cover slip was applied. The embryos were placed in a 70°C oven with weights for 24 hours in order to crack the cuticle and allow the internal access of the mountant to digest internal organs. This allowed for greater visibility of the cuticle under the microscope.
2.3 Cloning

2.3.1 Preparing the Vector-Digesting VALIUM22 with NheI and EcoRI

13uL of ChIP/PCR water, 2uL EcoRI buffer, 4uL VAL22 DNA, and 2uL EcoRI enzyme for a total of 20uL were combined in a sterile tube, flicked and centrifuged lightly. The EcoRI digestion commenced at 37°C for 2 hours. The EcoRI enzyme was heat-inactivated in a 65°C water bath for 20 minutes. Then, 42µL of ChIP/PCR water, 7µL CutSmart buffer, 1µL NheI-HF
enzyme for a total of 70µL were added to the tube. The NheI-HF digestion ran for 2 hours in
37°C. The enzyme was then heat-inactivated in a 65°C water bath for 20 minutes. In order to
precipitate the DNA, 70µL of ammonium acetate, 2µL glycogen and 2x (the volume of DNA)
100% ethanol, were incubated with the tube in room temperature overnight.

2.3.2 Ethanol Precipitation

The next day, centrifuge the tube of DNA for 15 minutes at room temperature at
maximum RPM (15,000). Supernatant was discarded and the white pellet was undisturbed.

450µL of 80% ethanol was added. The tube was then centrifugated at maximum speed for 20
seconds. The ethanol supernatant was discarded and the tube was dried in a SpeedVac at room
temperature until the tube was completely dry. The DNA was then resuspended in 10µL
ChIP/PCR water.

2.3.3 DNA extraction using a 1.5% agarose gel

In order to prep the VALIUM22 DNA for the agarose gel, 1µL of DNA loading buffer
was mixed into the DNA tube. Meanwhile, a 1.5% agarose gel was prepared and the
electrophoresis chamber assembled. The gel ran with the sample at 0.56V for one hour. After the
gel ran for a sufficient amount of time, it was briefly destained in 1X TAE (tris-acetate-EDTA)
buffer. A razor was used to cut out the DNA band with minimal gel. This was accomplished
using a UV light wand in a dark room. The DNA band was transferred to a 0.6mL tube and the
band was melted in the tube at 65°C. ChIP/PCR water was added to the tube in order to get a
final volume of 50µL after the gel was sufficiently melted in the tube. 1µL of gelase buffer was
mixed into the solution and then incubated in 65°C. The tube was placed in 42°C for at least 30
seconds and 1µL of gelase was added to the sample and immediately was incubated for an hour
in 42°C in order to activate the enzyme. After centrifugation and testing to confirm the gelase
digested the gel, an equal volume of 5M ammonium acetate was added. After thorough mixing, the tube was centrifuged for 5 minutes. Then an ethanol precipitation was performed and incubated overnight at room temperature.

2.3.4 Annealing DNA

To determine the amount of DNA present, a 0.8% gel was run the next day. The sample was run in a 0.5µL and 0.05µL dilution compared to a 24kb DNA ladder. After the amount of DNA was confirmed, the DNA was annealed. 1µL of the top oligo, 1µL of the bottom oligo, 1µL of PNK buffer, and 0.5µL T4 PNK enzyme (tube contained 10µL altogether). The tube was then placed in the thermocycler with a setting of 37°C for 30 minutes, 95°C for 5 minutes for one cycle.

2.3.5 Ligating DNA

After annealing, the reaction was put on ice to defrost and then concentrated to make a 1µM solution. On ice, 6µL of the annealing products was combined with 2µL 10X ligation buffer and 1µL DNA ligase. The reaction tube was thoroughly mixed and centrifuged. It was then incubated for 1 hour at 16°C.

2.3.6 Transformation

5µL of Stellar competent cells, was dispensed into three separate vector tubes (VAL20, VAL22, and VAL22z). 100µL was quickly added to each tube so as to not heat up the temperature-sensitive bacteria. Then 0.5µL of vector DNA was added to each tube correspondingly. All tubes received 2-2.5µL of ligated DNA (previously described above). The tubes were kept on ice for 30 minutes. After the 30-minute incubation, the bacteria underwent heat shock in a 42°C water bath for exactly 45 seconds. The tubes were then placed on ice for 1-
2 minutes to end the heat shock. Heated LB media was added to each tube in order to bring their individual volumes to 500µL. The tubes were then ready to be incubated in a 37°C shaker at 220 RPM for one hour. After one hour on the shaker, the bacteria was plated on Ampicillin LB-agar at two different concentrations. For the first concentrations, 5µL was taken from each tube and put into 100µL of fresh LB and directly plated on an LB agar plate. For the second concentration group, the bacteria was pelleted using a centrifuge for 2-3 seconds on, followed by 2-3 seconds off for a total of 10 times. The supernatant was discarded and the bacteria was resuspended in the remaining 100µL LB media. This more concentrated stock was then plated. All sets of plates were then incubated in 37°C overnight upside-down to prevent condensation from dropping on the bacteria.

Use a sterile toothpick to select a single colony from the overnight plates in order to keep a backup stock in 4°C. The toothpicks were then distributed into round-bottom test tubes each containing 5mL of 1:1,000 AMP/LB. The test tubes containing the toothpicks were placed in a 37°C shaker at 180 RPM overnight in order to grow the bacteria.

2.3.6 Minipreps

The following protocol was derived from Qiagen.

Between 1-5mL of the bacteria cultures was centrifuged at greater than 8,000RPM for 3 minutes at room temperature. The pellet should be loosened through vortexing if necessary in order for the buffers to be able to penetrate the pellet. The supernatant was discarded and the bacteria was resuspended in 250µL of Buffer P1 and placed in sterile centrifuge tubes. 250µL of Buffer P2 was added to each tube. The tubes were inverted until the reaction solution was no longer opaque. 350µL of Buffer N3 was added and each tube was inverted to mix the reagents. The tubes were centrifuged at 13,000 RPM for 10 minutes at room temperature. 800µL of the
supernatant from the tubes was filtered through a QIAprep 2.0 spin column. The spin columns were centrifuged for about a minute and the flow-through was removed. After the flow-through was discarded, the spin columns were washed with 0.5mL of Buffer PB each. The spin columns were then centrifuged for about a minute at room temperature and the flow through subsequently discarded. Another wash was conducted with 0.75mL of Buffer PE to each spin column. The spin columns were centrifuged for about a minute and the flow-through subsequently discarded. Any remaining buffer was removed after additional centrifugation for one minute at room temperature. The spin columns were placed in 1.5mL microcentrifuge tubes prior to DNA elution. DNA was eluted using 50µL of ChIP/PCR water. The spin columns were allowed to stand for one minute and then centrifuged for another minute.

The DNA samples were then analyzed for DNA sequencing to confirm successful transformation and DNA isolation. Depending on the tube with the highest concentration of DNA, that original colony was selected and grown in a fresh culture. To ensure the DNA vector was correct, a DNA digest using Pst1 was performed. Figure 2.7 shows the Pst1 digest performed and the corresponding fragment lengths.

The DNA samples were then sent for injection into Drosophila embryos. Once these flies were received, they were allowed to lay down embryos, which were dechorionated before further use.

2.4 Embryo Viability Assay
Flies were crossed in a beaker containing an agar yeast petri dish as described above in Cuticle Preparation (Figure 2.1). Following an overnight egg lay, the agar-yeast petri dish now containing embryos were aged for 5 hours off the beaker in 29°C. Embryos were washed with DI water and transferred to a fresh agar petri dish. Exactly 100 embryos were transferred to each
fresh agar dish using a needle. The embryos were aged at least 24 hours in 29°C. The following
day, the embryos were counted for the percentage that survived as larvae compared to the
percentage that died as embryos.

2.5 Generation of Fly Lines

2.5.1 Control cage- *bcd osk tsl* stock generation for crosses

Our control utilizes the *bicoid oskar torso-like (bcd osk tsl)* triple mutant system
previously developed in this lab. The goal is to repress *giant (gt)*, a Polycomb group protein
target gene universally in the embryo, so that Polycomb group proteins can take over repression.
This serves as a functional control because PcG protein knockdown does not occur. Flies with
*bcd^7 osk^6 tsl^4* over a TM3 balancer were readily available in our fly lab. These flies were
originally created by crossing flies with *tsl^4* (Bloomington Drosophila Stock Center #3289) with
*bcd^7 osk^6* (Bloomington Drosophila Stock Center #3252). They were then balanced over a third
chromosome balancer, *TM3*. Flies were gifted from Dr. Leslie Stevens *bcd^6 osk^6 tsl^{PZRev32}* and
crossed to container the *TM3* balancer. Before experimental crosses, preliminary studies
confirmed and verified the presence of the triple mutant through cuticle preparation analysis.

Females were generated in the F1 generation to be homozygous for the triple mutant
(Figure 2.2). However, different *bcd osk tsl* alleles were used due to unidentified recessive lethal
mutations. Since this triple mutant system utilizes maternal expression, the progeny of the
females would have the triple mutant null phenotype and be analyzed through ChIP and PCR for
our control.
Figure 2.2 - Genetic cross scheme for the experimental control. Flies with \( bcd^7\ osk^6\ tsl^4/\text{TM3} \) and \( bcd^6\ osk^6\ tsl^{PZRev32}/\text{TM3} \) were previously tested and available in our fly lab. All females used in these crosses were virgins.
2.5.2 Genetic cross to deplete embryos of Sfmbt in a bcd osk tsl genetic background

The generation of fly lines with Sfmbt short-hairpin RNAi (shRNAi) in a bcd osk tsl background were confirmed and tested by Piao Ye. The GAL4 driver used in this cross contains the *mat-tub-GAL4* on the second chromosome (Bloomington Drosophila Stock Center #7062).

*TM3* is a balancer that is phenotypically characterized by stubble on the backs of the fruit flies. Therefore, in order to ensure that the *UAS-shRNA sfmbt bcd osk tsl* and maternally expressed driver are present in the next generation, *TM3* cannot be there, evidence by stubble+ flies (Figure 2.3). These females of the F1 generation will determine the phenotype of their progeny due to maternal gene expression. The males used in the F1 generation introduce additional zygotic knockdown of *Sfmbt*.

Figure 2.3- Genetic cross scheme to deplete embryos of Sfmbt in a bcd osk tsl genetic background in preparation for ChIP experimentation. All females used in crosses were virgins.
2.5.3 Genetic cross to deplete embryos of Pho in a bcd osk tsl genetic background

The generation of fly lines with Pho short-hairpin RNAi (shRNAi) in a bcd osk tsl background were confirmed and tested by Piao Ye. The GAL4 driver used in this cross contains the mat-tub-GAL4 on the second chromosome (Bloomington Drosophila Stock Center #7062).

The crossed utilized for Pho depletion is similar in concept to that of Sfmbt (Figure 2.4). The difference, however, is the shRNA for Pho, not Sfmbt. The females of the F1 generation will determine the phenotype of their progeny due to maternal gene expression. The males used in the F1 generation introduce additional zygotic knockdown of Pho.

Figure 2.4- Genetic cross scheme to deplete embryos of Pho in a bcd osk tsl genetic background in preparation for ChIP experimentation. All females used in crosses were virgins.
CHAPTER 3:

RESULTS

3.1 ChIP Results for sfmbt and pho Knockdown

Through Chromatin Immunoprecipitation Experiments (ChIP), three different crosses using *Drosophila* (described in METHODS) were conducted in cages to create a control (*bcd osk tsl*), to selectively knockdown Sfmbt, and to selectively knockdown Pho. Flies used in the control cage were crossed and their embryos collected by me and Piao Ye. ChIP experiments and data analysis for *bcd osk tsl nc13* were completed by me. ChIP experiments and data analysis for *bcd osk tsl nc14a* and *nc14b* were completed by Piao Ye.

Flies bred and crossed for the *Sfmbt-KD-bcd osk tsl* cage were crossed and their embryos collected by Piao Ye. ChIP experiments for *Sfmbt-KD-bcd osk tsl nc14b* were completed by Piao Ye. ChIP experiments for *Sfmbt-KD-bcd osk tsl nc14a* and *nc13* were completed by me. All data analysis for *Sfmbt-KD-bcd osk tsl* ChIP experiments was completed by me.
Flies bred and crossed for the Pho-KD-bcd osk tsl cage were crossed and their embryos collected by Piao Ye. All Pho-KD-bcd osk tsl ChIP was completed by me (nc13, nc14a, and nc14b) along with all of the data analysis for these time stages.

All sorting of the Drosophila embryos for all three cages was completed by Piao Ye.

The following ChIP/PCR data describes the enrichment of specified Polycomb group proteins in the control, Sfmbt-KD-bcd osk tsl, and Pho-KD-bcd osk tsl embryos at four different regions of giant at the developmental time stages indicated.

3.1.1 nc13 Analysis

The earliest embryonic developmental time stage investigated in this experiment is nc13. Previous work in this lab has shown that specifically at region 4, Pho and Sfmbt are stably bound initially following the stable binding of these proteins at PRE2 (region 9) during this developmental stage (AlHaj Abed et al., 2018). It is not until nc14b that Pho and Sfmbt appear to be stably bound to (PRE2) region 9, following their reduced presence at PRE2 during nc14a (AlHaj Abed et al., 2018).

Compared to the control where no knockdown occurs (bcd osk tsl), we observe successful knockdown of Pho through decreased enrichment in both instances where either Sfmbt or Pho are depleted (Figure 3.1). This sets the stage for being able to analyze the effect of knockdown on other PcG proteins one by one by specifically looking at PRE1 and PRE2 binding.

What we observe at PRE1 is similar in both knockdowns, except for with E(z). The levels of Pcl and Pc remain unchanged compared to the control, which aligns with the observation that it is too early for stable binding of these complexes, though their signals are above background (AlHaj Abed et al., 2018). In sfmbt-KD bcd osk tsl embryos, we see a very significant decrease in the signal of E(z) when compared to the control along with a decrease in dRING levels (Figure
E(z) and dRING are both components of PRC2 and PRC1 respectively. In other words, the depletion of Sfmbt affects PRC2 and PRC1 recruitment, but not Pho depletion. This could indicate the potential divergent activities of Pho and Sfmbt though they are in the same complex, PhoRC. It has been shown that Sfmbt is required for the recruitment of Pho to PREs, which would suggest that they do not operate uniformly (Kahn et al., 2016). Interestingly, the signal for Pho decreases to near background one fold more than Sfmbt when Sfmbt is knocked down (Figure 3.1). This could provide evidence that Sfmbt recruitment is necessary for the recruitment of Pho. Other research in this lab has shown that mutant PREs affect both Pho and Sfmbt binding, but at different levels in each (Ghotbi et al., 2020). For example, in Pelican-gt-mut transgene signals, the fold enrichment of Pho is decreased to background and although Sfmbt enrichment is decreased, it is still well above background (Ghotbi et al., 2020).

Another observation is that through statistical analysis, we observed a difference between the control (bcd osk tsl) at region 3 for Pho and Sfmbt when comparing their depletions (Figure 3.1). Region 3 is associated with the Transcription Start Site (TSS) for the giant gene, in which case we anticipate no difference in signal when either protein is knocked down. We are unable to definitively explain this change in signal at region 3.

It is important to compare the binding differences of PRE1 and PRE2. However, looking closer at PRE2, we don’t see strong enrichment of any PcG protein during nc13. Previous research in this lab by Jumana et al. (2018) has shown weakly positive signals of Pho, E(z), and Pc which are reflected in their enrichment at PRE2 (Figure 3.1).

The enrichment for dRING is interesting in this region. We find that dRING signals are significantly decreased compared to bcd osk tsl when Sfmbt is depleted at regions 4, 6, and 9 (Figure 3.1). When we explore the differences in dRING signals between Sfmbt knockdown and
Pho knockdown, we see that the enrichment of dRING is lower in Sfmbt knockdown than in Pho knockdown. Although through statistical analysis, the levels of dRING in Sfmbt knockdown compared to Pho knockdown are not quite statistically significant, we assume this is due to the lack of biological replicates in the control. Unfortunately, due to the strenuous task and time constriction of repeating the experiment, it was not possible to acquire more biological replicates. However, in substituting the average of the two biological replicates for a hypothetical third biological replicate, we see the results are statistically significant. This makes it possible to speculate that under different circumstances, these results could be statistically significant.

Since PRC1 and PRC2 have not been shown to stably recruit to PRE1 or PRE2 during nc13, it is important to look at later time stages in order to get a fuller understanding of recruitment.
Figure 3.1 - Effect of Pho and Sfmbt Knockdown on individual PcG proteins during nc13 developmental stage in Drosophila embryos. Row 1: Pho-RC, Row 2: PRC2, Row 3: PRC1. Background levels standardized to PKA are indicated with a dotted line at fold enrichment 1. Fold enrichment for each IP is shown for three different genotypes: bcd osk tsl, Sfmbt-KD-bcd osk tsl, and Pho-KD-bcd osk tsl. ChIP signals from bcd osk tsl represent two biological replicates. ChIP signals from sfmbt-KD-bcd osk tsl and pho-KD-bcd osk tsl represent three biological replicates. Error bars indicate standard deviation. Statistical significance was calculated using GraphPad unpaired two-tailed Student’s t-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****P ≤ 0.0001. N.q.s. denotes not quite significant.
3.1.2 nc14a Analysis

By nc14a, PRC2, through the detection of E(z), has been shown to stably bind to both PRE1 and PRE2 (AlHaj Abed et al., 2018). Pc, or PRC1, is different in that its signal remains low during nc14a (AlHaj Abed et al., 2018). Pho-RC on the other contrary is stable at PRE1 and PRE2 by nc14a (AlHaj Abed et al., 2018).

When zeroing in on Pho-RC levels, we observe that Pho is very significantly reduced to background levels in both cases where Sfmbt and Pho are depleted at region 4 (Figure 3.2). Pho appears at background levels at every other region, which follows previous reports that Pho levels are lower at nc14a, compared to at region 4 (AlHaj Abed et al., 2018). The graphical data for Sfmbt signals, however, is different from Pho. At region 4, Sfmbt is significantly knocked down; whereas, its level during Pho knockdown is not (Figure 3.2). In other words, Pho is reduced to background without the presence of Sfmbt, yet when Pho is knocked down, Sfmbt enrichment remains above background (Figure 3.2). When comparing Sfmbt-KD-bcd osk tsl, at regions 3, 6, and 9, we find that Sfmbt levels in pho-KD-bcd osk tsl are higher than Sfmbt levels during its depletion. If Sfmbt is acting independently of Pho, then it makes sense that we see Pho depletion not reduce Sfmbt signals to background level.

It is important to note certain experimental limitations that affect analysis. In region 9 looking at Sfmbt, though statistical analysis indicates the enrichment between Sfmbt-KD bcd osk tsl and Pho-KD-bcd osk tsl Sfmbt is not statistically different, this is likely due to n=2 for Sfmbt-KD-bcd osk tsl. A greater number of biological replicates increases the odds of statistical significance, which was unfortunately prevented due to limited embryos for further testing. However, despite this lack of replicates, we can continue to look at how depletion affects other PcG protein recruitment.
What trends do we see regarding the presence of PRC1 and PRC2 at the PRE’s when Sfmbt or Pho is depleted? When Sfmbt is depleted, we see that E(z) is reduced to background, specifically at region 4. Unfortunately, due to error bars and not enough biological replicates, we cannot claim that the knockdown of Sfmbt significantly depleted Pcl, Pc, H3K27me3 or dRING, levels at region 4. However, if this were not the case, there is a possibility that Sfmbt depletion would lead to the loss of signal of these proteins in this region. This is probable because we notice differences in the levels of certain proteins when comparing Sfmbt and Pho knockdown experiments. For example, there is a very significant difference between the signal of E(z) at region 4 when comparing Sfmbt to Pho knockdown (Figure 3.2). E(z) levels decrease when Sfmbt is knocked down, but remain the same when comparing the control to Pho knockdown. This begs the question: how would PhoRC be able to recruit PRC2?

Interestingly, other labs have shown that Sfmbt might link PRC1 and PRC2 through an accessory protein, Scm (Kang et al., 2015). In an experiment conducted by Kang et al. (2015), the lab discovered that Scm was enriched during Pc (PRC1) and E(z) (PRC2) pull-down assays. Through ChIP analysis, they found Pho binding was unaffected by Scm knockdown which recapitulates the idea that Pho and Sfmbt have different roles in PhoRC. Similarly, when they conducted a pull-down assay of Scm, they found Sfmbt was highly enriched. Frey et al. (2016) further demonstrated Sfmbt and Scm binding through their shared SAM domains by identifying the crystallized structure of these two proteins in complex. Synthesizing this data, we find that Sfmbt binds to Scm through their shared SAM domains, which leads to the activity of Scm interacting with PRC1 and PRC2.

When observing enrichment at PRE2, we see that in both knockdowns (Pho and Sfmbt), all PcG signals are at background or near background levels (Figure 3.2). These PcG signals
reflect a change not statistically different from the control. In nc13 we also find that levels at PRE2 are also generally not different from the control (Figure 3.1). The results from nc14a are not definitive on PRE2, which requires further analysis of nc14b.

Since we find that Pho-RC, PRC1, and PRC2 are not all stably recruited by nc14a, it is important to look 30-minutes later in development during nc14b to assess the impacts of Sfmnb and Pho knockdown.
Figure 3.2- Effect of Pho and Sfmbt Knockdown on individual PcG proteins during nc14a developmental stage in Drosophila embryos. Row 1: Pho-RC, Row 2: PRC2, Row 3: PRC1. Background levels standardized to PKA are indicated with a dotted line at fold enrichment 1. Fold enrichment for each IP is shown for three different genotypes: bcd osk tsl, Sfmbt-KD-bcd osk tsl, and Pho-KD-bcd osk tsl. ChIP signals from sfmbt-KD-bcd osk tsl represent two biological replicates. ChIP signals from bcd osk tsl and pho-KD-bcd osk tsl represent three biological replicates. Error bars indicate standard deviation. Statistical significance was calculated using GraphPad unpaired two-tailed Student’s t-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****P ≤ 0.0001. N.q.s. denotes not quite significant.
3.1.3 nc14b Analysis

This lab has previously shown that by nc14b, PhoRC, PRC1, and PRC2 are stably bound to both PRE1 and PRE2 (AlHaj abed et al., 2018). Therefore, we anticipate the greatest disruption of binding during our knockdown experiments.

Looking at Pho enrichment, we find a very significant decrease in the level of Pho at all regions, especially in Pho-KD-bcd osk tsl embryos (Figure 3.3). This logically tracks as Pho should be thoroughly depleted in its own depletion assay. In region 6, there is a significant increase of Pho signal compared to the control, however, this is irrelevant as both signals are at or below background. Unlike in nc14a, we see a decrease in Pho enrichment at both PRE’s (Figure 3.2 and Figure 3.3.). When looking at Pho’s binding partner, Sfmbt, we find that the knockdown of Sfmbt produces less significant reduction of Sfmbt than of its signal during Pho knockdown. However, this is likely due to the high error bar for the Sfmbt signal when Sfmbt is knocked down. The same pattern likely occurs at region 9 when assessing Sfmbt signal due to high Sfmbt knockdown error bars as well (Figure 3.3). What we find when looking at PRE1, is that both Pho and Sfmbt are strongly depleted. At PRE2, we also find a depletion of both Pho and Sfmbt, except for Sfmbt (likely due to error bars). How then does that affect the signals for other PcG proteins?

At PRE1, E(z) is very significantly depleted and decreased at regions 3, 6, and 9 (Figure 3.3). In the Pho knockdown genotype, E(z) is also very significantly depleted. When examining the H3K27me3 data, we would anticipate that along with E(z) depletion, we would see correspondingly low levels of HeK27me3 since E(z) is the only PcG protein to methylate Histone 3 at Lysine 27 (Wang et al., 2004). However, we do see that with Pho depletion, E(z) levels are weaker and H3K27me3 levels are at background levels. Pcl also is at or near
background levels in all four regions compared to the control. However, when Pho is knocked down, there is not a clear, consistent pattern in how the levels of Pcl are behaving.

PRC1 signals are extremely weak at both PRE’s (Figure 3.3). dRING levels at PRE1 are extremely reduced for the Sfmbt knockdown and very reduced for Pho knockdown. The impact of Pho and Sfmbt knockdown appear to be greater on PRC1 levels than on PRC2 levels, generally. When comparing Sfmbt and Pho knockdown for Pc levels, we find a significant difference between the two genotypes. For example, Pc is reduced to background when Sfmbt is knocked down, but when Pho is knocked down Pc remains above background. This might indicate that Sfmbt may have a greater role in PRC1 stability than Pho.

dRING appears to be affected similarly to Pc when either Sfmbt or Pho is knocked down. Earlier in nc13 and nc14a it appeared that dRING was affected differently than Pc. A possible explanation for dRING acting independently is that dRING is involved in a noncanonical PRC1 complex early on. However, by nc14b, it appears that dRING is involved in canonical PRC1 since it does not act independently of Pc.
Figure 3.3- **Effect of Pho and Sfmbt Knockdown on individual PcG proteins during nc14b development stage in Drosophila embryos.** Row 1: Pho-RC, Row 2: PRC2, Row 3: PRC1. Background levels standardized to PKA are indicated with a dotted line at fold enrichment 1. Fold enrichment for each IP is shown for three different genotypes: *bcd osk tsl*, *Sfmbt-KD-bcd osk tsl*, and *Pho-KD-bcd osk tsl*. ChIP signals from *bcd osk tsl* and *pho-KD-bcd osk tsl* represent three biological replicates. ChIP signals from *sfmbt-KD-bcd osk tsl* represent three biological replicates, except for Pho, Pc, H3K27me3, and Mock which represent four biological replicates. Error bars indicate standard deviation. Statistical significance was calculated using GraphPad unpaired two-tailed Student’s t-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. N.q.s. denotes not quite significant.
3.2 VAL22z Pcl cloning

Embryos were injected with a Pcl RNAi transgene within a VAL22z vector through GenetiVision. From GenetiVision, we received three different fly lines: \textit{m2m1 x v1}, \textit{m15m1 x v1}, and \textit{m9m1 x v1}. All three lines should be identical, but in case the injection was unsuccessful in one line, there are two others to use instead. For the sake of simplifying the nomenclature, \textit{m2m1 x v1} will be called the “red” line, \textit{m15m1 x v1} will be called the “pink” line, and \textit{m9m1 x v1} will be called the “yellow” line.

The presence of Pcl was confirmed in the VALIUM22z vector through sequencing analysis (Figure 3.4). In order to test whether these flies would successfully be able to induce pre-MBT zygotic expression of Pcl RNAi, genetic crosses were constructed in order to observe knockdown through an embryo viability assay and cuticle preparation. First, upon receiving flies from injection of our VALIUM22z containing the Pcl RNAi transgene, a stock homozygous for the transgene needed to be constructed (Figure 3.5). Then, using this homozygous stock, the homozygous stock could be used in a series of crosses to test the knockdown efficiency of Pcl (Figure 3.6).
Figure 3.4 - **Sequence analysis confirming the insertion of Pcl RNAi into our VALIUM22z vector.** DNA was sequenced by Lone Star Labs. Data above reflects the reverse sequence of the Pcl-VAL22z vector and the yellow-highlighted region reflects the Pcl RNAi antisense oligo. ([https://www.flyrnai.org/cgibin/DRSC_gene_lookup.pl?gname=HMS00897](https://www.flyrnai.org/cgibin/DRSC_gene_lookup.pl?gname=HMS00897)).

Figure 3.5 - **Genetic cross scheme for constructing a stock homozygous for UAS-shRNA-Pcl.** Males in the parental generation contain the VALIUM22z-Pcl transgene inserted in their genome. Females from the parental generation were tested and readily available for use in our lab. All females used were virgins to ensure the correct phenotype in the next generation.
Figure 3.6- **Genetic cross scheme for analyzing the knockdown efficiency of Pcl.** Females in the parental generation are the homozygous stock produced in Figure 3.5. All females used in these crosses are virgins. The Embryo Viability Assay is described in *Methods*.

If the knockdown was successful, we would observe lethality in the embryos as a consequence of knocking down Pcl (Breen and Duncan, 1986). In order to test this, we conducted an Embryo Viability Assay (*Methods 2.4* and Figure 3.6). By counting the embryos that survived (hatched), those that died (unhatched), and those unfertilized, we found relatively similar percentages in all three lines (Figure 3.7). In *m9m1 x v1* (yellow) flies, 38% hatched, 55% were unhatched, and 7% were unfertilized. In *m2m1 x v1* (red) flies, 38% hatched, 61% were unhatched, and 1% were unfertilized. In *m15m1 x v1* (pink) flies, 37% hatched, 63% were unhatched, and 0% were unfertilized. Among the three different fly lines, we see that of the fertilized embryos, between 59% and 63% of embryos die. From these observations, we then wanted to understand if these embryos were dying due to the knockdown of Pcl or other causes.
In order to determine the cause of death, we performed Cuticle preparations on the progeny of the F1 generation from Figure 3.6, the same progeny used for the Embryo Viability Assay for \textit{UAS-shRNA-Pcl} (Figure 3.7). In Figure 3.6, the F2 generation is either homozygous for \textit{UAS-shRNA-Pcl} or heterozygous for the driver. Since the driver is maternally expressed, it should not matter if there is a copy of the driver in the genotype of the next generation. However, these genotypes could account for the almost 50% survival rate of embryos in the three lines. In other words, the two paternal copies of the \textit{UAS-shRNA-Pcl} could account for the lethality of half the embryos and the ones that survived could be the ones heterozygous \textit{UAS-shRNA-Pcl}.
Therefore, it is important to continue experimentation to understand how the genotypes are affecting lethality in the embryos.

The knockdown of *Pcl* has previously been studied by Breen and Duncan (1986) through the analysis of cuticle preparations. We anticipated that our *UAS-shRNA-Pcl* knockdown embryos, if knockdown was successful, would appear similar to those previously described in Figure 3.8B. In Figure 3.8B, we see the phenotype of an embryo that is heterozygous for *Pcl*. The morphology of this embryo indicates that, unlike in wild type embryos, the sixth (A6) and seventh (A7) abdominal segments are homeotically transformed, appearing like the eighth abdominal segment (A8). A8 is characteristically rectangular and thicker than the other abdominal segments, which is apparent also in A6 and A7 (Breen and Duncan, 1986).

Another possible phenotype discovered by Duncan and Breen (1986) was the partial deletion of segments in embryos (Figure 3.8B). In the image on the right of Figure 3.7B, we see the partial deletion of segment A6, indicated by the arrow. Normally, A6 is continuous from its end to end (Figure 3.8A). Due to the observation by Duncan and Breen (1986) that *Pcl* mutations caused deletions in even segments, their experimental data provided evidence that *Pcl* might be necessary for the proper regulation of pair-rule segmentation genes.
(see Figure Description next page)
Figure 3.8- The phenotypic effects of Pcl knockdown on Drosophila segmentation. (A) Representative posterior, wild type image displayed from m9m1 x v1, “yellow” flies. Segments A6-A8 are denoted with arrows and have a wild type morphology. (B) Images are adapted from Breen and Duncan (1986). Original arrows and A8 labeling were kept. The image on the left characterizes the homeotic transformation of A6 and A7 (labeled with arrows) into A8 (posterior-most segment) of embryos with partial maternal knockdown of Pcl and complete zygotic knockdown of Pcl. The image on the right shows another defect (partial deletion of segments) of embryos with partial maternal knockdown of Pcl and complete zygotic knockdown of Pcl. (C) The image on the left demonstrates the homeotic transformation and segment deletion observed in UAS-shRNA-Pcl knockdown flies. A7 and A6 have the phenotype of A8 observed through their compressed, stockier appearance. The image on the right shows an example of a partial deletion found in UAS-shRNA-Pcl knockdown flies.

Interestingly, when we observed the embryos under the microscope, we noticed heterogeneous phenotypes in the three lines. Among the three lines, we found four different categories of phenotypes: wild-type, homeotic transformation, deletions of segments, and both homeotic transformation coupled with deletion of segments. We would anticipate that all three lines would behave similarly, but found varying percentages of the four categories of phenotypes observed. For example, in the yellow line of flies, all had a wild-type appearance, as in Figure 3.7A. This could potentially suggest that the transgene was not inserted at the attP2 docking site where it was supposed to. In the red line of flies, 40% were wild-type. 20% had weak homeotic transformation, and 40% had deletions of segments. There were not any flies from the red line that had homeotic transformation coupled with deletions of segments. Lastly, in the pink line of flies, 33% were wild-type, 11% had both homeotic transformation coupled with deletions of segments, and 56% had only deletions of segments. However, there were not any flies from the pink line that only had a homeotic phenotype. Surprisingly, the deletions found were particularly in segments A4 and A5 (Figure 3.7C). Partial deletions as well appeared in segments A5 as well, which departs from what Duncan and Breen (1986) have previously seen.
Table 3.1- Percentages of different phenotypes among the three UAS-shRNA-Pcl lines.

<table>
<thead>
<tr>
<th></th>
<th>m9ml x vl (yellow)</th>
<th>m2ml x vl (red)</th>
<th>m15ml x vl (pink)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type</td>
<td>100%</td>
<td>40%</td>
<td>33%</td>
</tr>
<tr>
<td>Homeotic Transformation</td>
<td>-</td>
<td>20%*</td>
<td>-</td>
</tr>
<tr>
<td>Deletions of Segments</td>
<td>-</td>
<td>40%</td>
<td>56%</td>
</tr>
<tr>
<td>HT and DS</td>
<td>-</td>
<td>-</td>
<td>11%</td>
</tr>
</tbody>
</table>

The observed phenotypes and percentage of these phenotypes in the three different lines do not quite align with other studies conducted by Duncan and Breen (1986). Though the embryos in this experiment have deletions of segments and some homeotic transformation, it appears that the causes for these phenotypes might be different. Duncan and Breen describe partial deletions in segments during Pcl depletion in even-numbered segments. This data suggests that Pcl depletion leads to pair-rule gene segmentation defects (Duncan and Breen, 1986). Alternatively, in our data, we see the deletion of adjacent A4 and A5 segments, which suggests that Pcl deletion creates a gap-gene mutant phenotype. Specifically, our results could demonstrate the potential role of Pcl in some Krüppel misexpression. Krüppel is a gap gene whose misregulation alters the expression of adjacent segments in the embryo, particularly in the central domain of the embryo and anterior segments (Jacob et al., 1991; Nüsslein-Volhard and Wieschaus, 1980). Further experimentation through molecular analysis would be able to conclusively describe the effect of Pcl on gap gene expression in order to further understand our findings.
CHAPTER 4:

DISCUSSION

4.1 Conclusions

In observing the results, the selective knockdown of Sfmbt and Pho, two trends appeared from the ChIP data. Both of these observations introduce potential non-canonical involvement of proteins during nc13 and nc14a that later return to association with their respective canonical complexes by nc14b (Figure 3.8).

(See Figure Description next page)
Figure 3.9- Proposed model of Recruitment to giant PRE1 and PRE2. ncPRC1 is representative of the potential non-canonical PRC1 complex that dRING could be acting with in the early stages, though it is unconfirmed if dRAF or L(3)73Ah-RING1 is responsible. Weak signaling at PRE2 during nc13 and nc14a is demonstrated with a dashed arrow. PRC2 recruitment in the early stages appears to be affected by Sfmbt depletion, though it is unclear if canonical or non-canonical Sfmbt is causing this. By nc14b, signals appear to follow previously published data in this lab (AlHaj Abed et al., 2018).

The first interesting observation is that Sfmbt appears to be present separately from Pho-RC early in nc13 and nc14a. We find that the knockdown of Pho in these time periods does not significantly reduce the presence of Sfmbt, even though the reverse occurs. This agrees with a finding in work conducted in this lab by a former graduate student where Sfmbt signals are reduced when Pho-binding sites are mutated, but do not get to background like Pho levels (Ghotbi 2020). However, by nc14b, Sfmbt appears to act in Pho-RC due to its similar signals to Pho in both cases of depletion.

The second interesting observation is that of dRING. dRING appears to act in a non-canonical PRC1 complex early in nc13 and nc14a because it was positive when Pc was not. We also find that the knockdown of Sfmbt specifically (opposed to Pho) has some kind of effect on dRING presence, though we cannot conclusively determine through what mechanism.

In the second study of this research project, the effectiveness of a novel vector, VALIUM22z with a Pcl RNAi transgene was analyzed. Through testing lethality and observing the agent, we could observe the presence of unique mutations in the embryos of three Drosophila lines. This data suggests that Pcl may affect gap gene expression, though previous studies have only identified the effect of Pcl on pair rule gene expression (Duncan and Breen 1986). In order to understand our results better and to have a clearer picture on the role of Pcl, molecular analyses will be crucial to further elucidate this gap in understanding.
4.2 Future Directions

Further experimentation is necessary in order to comprehensively understand the individual contributions of each PcG protein in order to assay the impact of its depletion on other PcG proteins and complexes. Three other essential proteins need to be studied in order to understand the roles of proteins within PRC1 and PRC2.

4.2.1 Enhancer of Zeste, E(z)

In order to assay the effect of E(z) depletion, experimental conditions and fly stocks will need to be fully tested and confirmed. First, flies will need to be tested for the ability of the driver to knockdown the RNAi induced transgene. A second test will be utilized in order to confirm that bcd osk tsl are present in the genetic background of this stock.

Originally one of my research plans was to knockdown the PcG protein, E(z) using flies constructed in the triple mutant background that had confirmed evidence of E(z) knockdown and the presence of the triple mutant. However, upon preliminary work with these flies, there was evidence of contamination. After decontamination of the stock, unusual phenotypes continued to appear, which led us to determine that the stock was not reliable and would not be used for a knockdown assay.

4.2.2 Polycomb, Pc

Flies containing the Pc RNAi transgene for use in a knockdown cage were constructed in a genetic background that was not suitable for further testing. Smaller scale experiments showed that a novel vector would be unable to produce sufficient knockdown resulting in a mutant phenotype or lethality. In order to demonstrate sufficient knockdown of Pc in the embryos, there should be the appearance of a homeotic phenotype (Lewis, 1978).
Originally my research plans included working with the \textit{Pc} PcG protein and to assay the effect through ChIP and PCR experimentation. I inherited these flies from another graduate student who constructed this stock. The \textit{Pc} triple mutant stock was constructed in a VALIUM22z background, an unpublished vector created by Dr. Richard Jones. Before a large cage could be done to analyze genetic knockdown of \textit{Pc}, some experimental conditions needed to be tested and confirmed. Embryos lacking a maternal and paternal copy of \textit{Pc} will not survive (Lewis, 1978). Therefore, an embryo viability assay was conducted using three different male fly lines to assess which conditions led to the best knockdown of \textit{Pc}, as evidenced through lethality. Figure 4.1 compares the presence of unhatched (dead) to hatched (alive). Out of 100 embryos, 20% died and 80% lived when BL:33964 VAL20 males were used. When BL: 33622 VAL20 males were crossed lethality was 29\% compared to 71\% that survived. Most surprising was a 56\% lethality to 44\% that survived when BL: 36070 VAL22 males were crossed. This result was unexpected because VAL22 typically works well maternally, but not well zygotically (Harvard TRiP website). However, since none of the tests produced at least 80\% lethality, the \textit{Pc} triple mutant flies were not usable for a knockdown assay.
4.2.3 *Polycomb-like, Pcl*

Though the inception of *Pcl* testing was initiated in this project, experimental conditions need to be further explored in order to identify the correct genotypes of flies needed for the effective knockdown of *Pcl*. As mentioned in previous sections, molecular analysis through ChIP experimentation is necessary to determine if our VALIUM22z vector and Pcl RNAi transgene could be effective for use in a knockdown experiment.
BIBLIOGRAPHY


