THE CELL CYCLE PHENOTYPES OF POLYHOMEOTIC ARE DUE TO LOSS OF DNA DAMAGE CHECKPOINTS AND ARE SUPPRESSED BY THE COHESIN PDS5

by

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Abstract

The Polycomb group (PcG) are well known as epigenetic silencers of many target genes including important developmental regulators such as the Hox genes. PcG mutants also have mitotic phenotypes, though their cell cycle role is not understood. Depletion of the proximal isoform of the PcG protein Polyhomeotic (PhP) results in chromatin bridges during anaphase and telophase in embryos. PhP binds chromatin during S phase but not during mitosis in early embryos. As transcription rates of embryonic genes are low at this time, we suggest that PhP has a direct role in S phase. Time lapse imaging of php mutants reveals an acceleration of S phase timing, indicating that php regulates S phase length. Mutations in php do not affect DNA synthesis rates, but exhibit impaired ability to block cell cycle progression following exposure to x-rays, consistent with a role for PhP in the DNA damage response. PhP likely plays a direct role in the DNA damage checkpoint during S phase. PhP cofractionates and coimmunoprecipitates with the cohesin protein Pds5. Mutations in Pds5 suppress both the chromatin bridge phenotype and the S phase acceleration in php mutants. Other members of the core cohesin complex, with the exception of the cohesion establishment factor eco do not affect the chromatin bridge phenotype of php mutants. Despite this, the suppression of the S phase phenotypes of php is likely indirect, because Pds5 does not suppress the loss of the DNA damage response in php mutants. Instead, php is epistatic to Pds5 in the DNA damage response, which supports a role for php in the DNA damage checkpoint. The function of the complex containing Pds5 and PhP is likely related to silencing, as Pds5 strongly enhances the homeotic phenotypes of php, and Pds5 localizes to the Polycomb Group Response Elements (PREs) and promoter of the PcG target Ubx. This thesis demonstrates that PhP
plays a role in the DNA damage checkpoint during S phase in addition to its role as an epigenetic silencer, and that Pds5 plays a role in silencing of \textit{Hox} genes in addition to its role in the cell cycle.
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CHAPTER 1 Introduction
1.1 Maintenance Proteins

The development of a multicellular organism from a single cell requires a progressive restriction of the genetic information available to a cell lineage. This process of differentiation, whereby the fate of a given cell is gradually restricted, must be coordinated with proliferation. As the DNA sequence in cells does not change, restriction of cell fate, and by implication, gene expression patterns must be epigenetic. The epigenetic restrictions on gene expression patterns must be inherited when a cell undergoes mitosis, just as is the genetic information on which this specialization is based. While we know a great deal about gene regulation during interphase, little is known about the inheritance of gene expression patterns through mitosis.

One family of epigenetic regulators required for the epigenetic inheritance of many gene expression patterns are the Maintenance Proteins (MPs; Beck et al., 2009). MPs fall into three categories: the Polycomb Group (PcG), which maintains target genes in the silent state; the trithorax group (trxG), which maintains target genes in the active state; and the Enhancers of trx and Pc (ETPs) that are required to maintain both silencing and activation. The mechanism by which MPs promote epigenetic inheritance through mitotic cell divisions during development is largely unknown.

Maintenance proteins were discovered due to their role in the regulation of genes critical for anterior/posterior (A/P) axis specification in Drosophila. The Homeotic (Hox) genes are an evolutionarily conserved family of DNA binding transcription factors responsible for assigning segmental fate by combinatorial regulation of a cascade of downstream transcription factors (Degnan et al., 1995; Ferrier and Minguillon, 2003;
Wolpert, 1994). An interesting feature of Hox genes is that their chromosomal position is collinear with their spatial pattern of expression in Drosophila, and with both their spatial and temporal expression patterns in vertebrates (Duboule and Deschamps, 2004).

The spatial pattern of Hox gene expression determines segment fate. A unique code of Hox expression for each segment determines segment identity. In general, few Hox genes are expressed anteriorly, and more Hox genes are expressed posteriorly. Because Hox genes do not determine segment number, only segment identity, misregulation of the spatial pattern of Hox genes results in one or more segments taking on the identity of another, a unique developmental phenotype termed homeosis. Loss of function Hox mutations lead to anterior transformations, because posterior segments acquire the Hox expression pattern, and therefore identity, of more anterior segments. Conversely, gain of function mutations in Hox genes lead to posterior transformations, because anterior segments acquire the Hox expression patterns of more posterior segments.

PcG mutations were first characterized as mutations causing posterior transformations, and therefore it was inferred that PcG genes prevent derepression of Hox genes in anterior parasegments (Lewis, 1978). Similarly, trxG mutations cause anterior transformations in the posterior thorax and abdomen, so it was inferred that these transformations were due to failure to properly activate Hox genes. These inferences were confirmed subsequently (Breen and Harte, 1993; Mazo et al., 1990; McKeon and Brock 1991; Simon et al. 1992; Struhl and Akam, 1985).
This regulation of *Hox* genes by maintenance proteins is epigenetic because the initial boundaries of *Hox* gene expression are determined by the pattern of expression of gap and pair-rule transcription factors, which activate or repress transcription of *Hox* genes. However these gap and pair-rule factors are present only during the early stages of development. Therefore MPs are required for the stabilization of these epigenetic changes through subsequent cell divisions. Consistent with this, PcG and trxG mutations cause changes in the expression patterns of *Hox* genes only during later development (Simon, 1995). Importantly, the loss of silencing of PcG targets occurs within a few cell cycles of their depletion (Breiling et al., 2004) implying that they function directly to direct epigenetic silencing through cell division.

About 20 PcG and 15 trxG genes have been characterized in Drosophila (Beck et al., 2009). Most PcG and trxG proteins have homologs with similar functions in other eukaryotes (Brock and Fisher, 2005). MPs have many target genes, and therefore regulate many developmental processes, including stem cell self-renewal, X-chromosome inactivation, the hedgehog signaling pathway, genomic imprinting, senescence, the cell cycle, and DNA replication (Gil et al., 2005; Hernandez-Munoz et al., 2005; Shindo et al., 2005; Autran et al., 2005; Kohler et al., 2005; Chopra and Mishra, 2005; Voncken et al., 1999; Aggarwal and Calvi, 2004).

Biochemically, Maintenance Proteins are members of multi-protein complexes. In flies, PcG Repressive complex 1 (PRC1) contains the PcG proteins Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), dRing, as well as Sex comb on midleg, the trxG protein Zeste, and other non-PcG subunits in flies (Shao et al., 1999; Levine et al., 2002; Lavigne et al., 2004). The role of PRC1 in regulating transcription during
interphase is likely due to its effects on chromatin structure. PRC1, and Psc by itself, inhibits chromatin remodeling by the SWI/SNF complex *in vitro*, which could prevent access of transcription factors to DNA (Francis et al., 2001). PRC1 also has the non-specific *in vitro* activity of compacting nucleosome arrays (Francis et al., 2004). Ubiquitination of histone H2A lysine 119 is mediated by dRing and its mammalian homologues, and requires Psc or its mammalian homologs (de Napoles et al., 2004; Wang et al., 2004a; Cao et al., 2005; Buchwald et al., 2006), which could function to prevent recruitment of the FACT (Facilitates Chromatin Transcription) elongation complex to promoters (Zhou et al., 2008).

Polycomb Repressive Complex 2 (PRC2) contains Enhancer of zeste (E(z)), Extra Sex Combs (ESC) and Suppressor 12 of Zeste, and the histone binding protein Nurf55/Caf1 (Czermin et al., 2002; Muller et al., 2002). The PRC2 complex is responsible for the classic PcG histone modification, tri methylation of lysine 27 of histone H3 (H3K27me3) found at PcG targets, but the complex is also responsible for mono methylation (H3K27me1) and di methylation (H3K27me2), which are found almost ubiquitously (Ebert et al., 2004). This enzymatic activity is attributed to the SET domain of E(z) and requires *Drosophila* ESC, ESC-Like, or the mammalian ESC homolog EED for binding to the histone H3 substrate (Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Tie et al., 2007). Exactly how PRC2 affects transcription is unknown, but PRC1 binds to H3K27me3 via the chromodomain of Pc (Wang et al., 2004b).

Members of the trxG, responsible for maintenance of the active state, are also part of multi-protein complexes that promote transcription through chromatin remodeling,
histone acetylation or facilitating elongation (Mohrmann et al., 2004; Petruk et al., 2001; Petruk et al., 2004; Nakamura et al., 2002; Smith et al., 2004). The activities demonstrated for PcG proteins thus far support a model in which they antagonize the effects of trxG proteins on transcription, and therefore promote silencing.

A major question left unanswered is how Polycomb Group Proteins promote epigenetic inheritance of their target genes through cell divisions. The demonstrated activities of Maintenance proteins are adequate to explain their effects on transcription between cell divisions, but fail to explain how daughter cells are able to inherit the gene expression patterns that dictate their developmental fate.

1.2 The Drosophila Cell Cycle During Development

Proliferation occurs concomitant with the differentiation and determination decisions being made by individual cells during the developmental program. The ultimate choice to divide is dependent on the Cyclin/Cdk system, which responds to both internal and external cues before deciding to progress into the next cell cycle stage (Reviewed by Park and Lee, 2003). The cell cycle is divided into four stages, G1, S, G2, and M. Cyclins and Cyclin dependent kinases (Cdks) drive progression through these stages by phosphorylating of a multitude of target proteins, which in turn enable or are responsible for the mechanism of cell division, such as proteins required for DNA synthesis, chromosome condensation, and the mitotic spindle. Different Cyclin/Cdk complexes govern progression through different cell cycle stages. As the name suggests, the levels of the Cyclin component of the complex oscillate through the cell cycle. Their presence in
complex with their respective Cdk s pushes forward the cycle, provided the inhibitors of cell cycle progression, which include the Cdk Inhibitors (CKIs) and DNA damage checkpoints, do not block their function.

During G1, cells prepare for DNA synthesis and are capable of responding to external growth cues with regards to the choice to proceed or exit the cell cycle. Growth cues are interpreted by D-type cyclins, which promote cell cycle entry from the quiescent G0 stage. D-type Cyclins indirectly activate Cyclin E, which is required for entry into S phase, during which time DNA synthesis occurs. Following DNA replication, a DNA damage checkpoint ensures that proper replication has occurred, and that any damage is repaired before entry into mitosis. Mitotic entry is controlled by Cyclins A and B, which are degraded at the metaphase to anaphase transition to allow for mitotic exit.

During Drosophila development, control of cell division is tuned to the needs of specific tissues (Reviewed in Vidwans and Su, 2001). The first 3 hours of Drosophila embryogenesis are characterized by rapid proliferation, which precedes differentiation and organogenesis. Many of the assays discussed in this thesis involve these unique cycles, which lack Gap phases, and are maternally driven. For 13 cycles, nuclear divisions proceed synchronously and rapidly, with very short or no intervening gap phases. These nuclear cleavage divisions are not accompanied by cytokinesis, and therefore give rise to a syncytial embryo. Cleavage divisions are driven completely by maternally deposited factors, including high levels of maternally deposited Cyclins and constitutively active Cdk s (Edgar et al., 1994). Neither global fluctuations in Cyclin levels, nor inhibitory phosphorylations occur; instead progression to the next cell cycle stage is driven by degradation of cyclins in the region of the nuclei (Su et al., 1998).
Cyclin B regulates mitotic exit, and disappears from spindles as metaphase ends in a wave starting at the poles of the embryo (Huang and Raff, 1999). Cyclin E controls Cdk activity during S phase entry in *Drosophila*, and is constitutively active during syncytial divisions (Knoblich et al., 1994). Unlike in mammalian cell divisions where Cyclin D regulates S phase entry, in *Drosophila* Cyclin D regulates growth but not the G1/S transition (Coqueret, 2002; Datar et al., 2000; Meyer et al., 2002).

*Drosophila* embryos undergo 13 syncytial divisions. The first nine nuclear divisions occur within the interior of the embryo, and are followed by migration of nuclei to the cortex of the embryo, where divisions 10 through 13 occur just under the plasma membrane. These synchronous cortical divisions therefore provide an excellent system for the examination of cell cycle progression. Syncytial cycles slow progressively due to increases in the length of S phase until they arrest at G2 during the mid-blastula transition (MBT) following S phase 13. These delays and the final arrest at the MBT are controlled by the gradual decrease in Cylin B caused by localized degradation, as well as by checkpoint genes, which ensure complete DNA replication (Edgar et al., 1994; Ji et al., 2004). Incomplete DNA replication, due to limiting maternal factors, results in degradation of the Cdk activator, String, which blocks further cell cycle progression until zygotic transcription occurs (Edgar and Datar, 1996).

Following S phase 14, a G2 phase is added, and embryonic divisions 14 through 16 are controlled at the G2/M transition by patterning factors. Larval divisions are coupled to nutrition and growth and are therefore primarily regulated at the G1/S transition. Specialized cycles in larval and some adult tissues serve to create polyploid tissues through nested S phases without accompanying gap phases. Canonical cycles
controlled at both G1/S and G2/M occur in both cells of the nervous system and imaginal disks, which differentiate into adult tissues during pupation.

1.3 The Cell Cycle and Epigenetic Inheritance

Silencing must be established while developmentally programmed cell divisions are still occurring, and both replication fork progression at S phase and chromatin reorganization at mitosis challenge the maintenance of transcriptional states. Consistent with different cell cycle stages affecting the maintenance of gene expression patterns, it has been proposed that cell fate changes involve changes in the cell cycle profile. Experiments in which cells of imaginal disks change fate (“transdetermination”), show that change in gene expression patterns requires a change that occurs in S phase (Sustar and Schubiger, 2005). This link between S phase and change in maintenance of gene expression patterns has been observed in other systems including neuronal cells, vulva precursors in *Caenorhabditis elegans* and the *Drosophila* patterning gene *even-skipped* (McConnel and Kaznowski, 1991; Ambros, 1999; Weigmann and Lehner, 1995). Disruption of the cell cycle upsets Hox regulation in chick somites (Primmet et al., 1989). Intriguingly, the frequency of transdetermination is altered in PcG and trxG mutants (Klebes et al., 2005; Lee et al., 2005). This indicates that there is tight control between entrance into a particular cell cycle stage and maintenance, and that failure to control the timing of cell cycle entry can cause defects in gene expression patterns.

Cell cycle progression, and the plasticity afforded by it, is also important to normal induction of gene expression during development. During *Xenopus* development,
blocking DNA replication prevents expression of the HoxB cluster (Fisher and Mechali, 2003). In the P19 pluripotent embryonal carcinoma cell line, the entire HoxB locus replicates early. Blocking replication of this locus in synchronized P19 cells prevents retinoic acid (RA) induced activation of the locus. Hoxb1 and Hoxb13, which are found at the 3’ and 5’ ends of the locus, respectively, are not sensitive to the replication block, and expression of the locus occurs with temporal colinearity after replication. It is likely that when fate change occurs, nascent DNA allows for the formation of a new chromatin template that is permissive to altered transcriptional activity (Fisher and Mechali, 2003). This implies that something normally prevents nascent DNA from assuming this conformation in differentiated cells. At least part of the answer to this question may lie in preventing differentiated cells from entering the cell cycle.

1.4 PcG Regulation of Genes Involved in Cell Cycle Progression

Given the importance of cell division in reprogramming gene expression, it is not surprising that PcG genes have been implicated in cell cycle regulation. PcG proteins are key regulators of cellular senescence, apoptosis and stem cell self-renewal, and play a key role in hematological malignancies, non-small cell lung cancer, and prostate cancer (Raaphorst et al., 2000; van Kemenade et al., 2001; Varambally et al., 2002; Vonlanthen et al., 2001). Genome-wide ChIP has identified several tumor suppressors as potential targets of PcG mediated silencing (Bracken et al., 2006).

In many cases, stopping proliferation is an important first step to differentiation. If transition of Hox genes from an off to on state requires progression through S-phase
(Fisher and Mechali, 2003; Primmet et al., 1989; Ohsugi et al., 1997), a very simple way for the silent state to be maintained is to prevent progression through this phase of the cell cycle, or to maintain cells in a state of replicative senescence. This predicts that PcG proteins might play a role in maintaining differentiated cells in the senescent state of G0. Some PcG proteins may have this function, however the mammalian Psc homologue B-cell specific Moloney murine leukemia virus integration site 1 (Bmi-1) plays a central role promoting stem cell self-renewal, but plays a very minor role in differentiated daughter cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003).

*Bmi-1* was first identified as an oncogene involved in the generation of mouse lymphomas and mutations are associated with proliferation defects and premature senescence (van Lohuizen et al., 1991; Haupt et al., 1991; Van der Lugt et al., 1994). In addition to anterior transformations, mice overexpressing *Bmi-1* show a high incidence of lymphomas, and mouse embryonic fibroblasts (MEFs) are immortalized (Alkema et al., 1995; Alkema et al. 1997; Jacobs et al., 1999a). *Bmi-1* is critical for hematopoietic stem cell self-renewal due to its regulation of the *ink4a* locus and possibly other targets (Park et al., 2003; Dimiri et al., 2002). *Bmi-1* promotes proliferation and multipotency primarily through silencing of the *ink4a* locus, which encodes the tumor suppressors p16 and p19arf. Both proteins inhibit CDK activity, and therefore block cell cycle progression (reviewed in Raaphorst, 2003). p16\textsuperscript{ink4a} binds to and inhibits the CDKs that interact with the D type cyclins, and the p19\textsuperscript{ARF} gene product inhibits CDK activity indirectly via MDM2 and p53. This role for Bmi-1 in maintaining plasticity indicates that PcG mediated silencing of cell cycle genes is likely not related to the mechanism by which PcG proteins promotes maintenance of target such as *Hox* genes through cell division.
Other cell cycle regulators targeted by PcG proteins include members of the c-myc/cdc25 pathway in mammalian cells and Cyclin A in Drosophila (Ogawa et al., 2002; Tetsu et al., 1998; Martinez et al., 2006). Different PcG proteins regulate different cell cycle genes, thus as a group they function to both promote and inhibit cell cycle progression. This is unlikely to be an important mechanism for maintaining gene expression states in differentiated cells, given the ability of PcG proteins to drive cell cycle progression. Further, preventing changes in gene expression simply through blocking cell cycle entry does not explain the ways in which maintenance through the cell division cycle does occur.

1.5 The Epigenetic Mark

The mechanism of epigenetic inheritance through cell division has been largely unanswered, though most research has focused on two models, an epigenetic mark, and control of replication timing. The epigenetic mark hypothesis proposes that there is a protein whose binding to chromatin is stable through both DNA replication and mitosis, and that this binding is sufficient to re-initiate silencing or activation of its target genes following these cell cycle stages.

Transcription factors have been proposed to be epigenetic because cytoplasmic inheritance results in the presence of the same factors in the daughter cells as were present in their progenitors (Ptashne, 2007), however, this does not explain stable maintenance of Hox genes. Transcription factors initially establish the expression states of Hox genes. Their effects on transcription are dependent on their own spatial expression
pattern, and therefore cytoplasmic inheritance of these factors through cell division could account for maintenance through a few cell cycles. However, after these transcription factors are gone, *Hox* gene expression is dependent on Maintenance Proteins, which are expressed uniformly and ubiquitously, while the expression patterns of their targets are not (Simon, 1995).

In this way, MPs are different from transcription factors because their ability to repress or activate depends on more than the presence of sequence specific binding activity. This implies the presence of a mark that designates the on/off state, that is stable to both DNA replication and mitosis, and that governs which target genes are repressed or activated within a single cell and its progenitors. Because the depletion of MPs results in mis-expression of their target genes within a few cell cycles, either the presence of this mark or its interpretation must depend on MPs.

The most elegant and well-studied epigenetic mark is DNA methylation, which is usually associated with gene silencing and is critical for development (Klose et al., 2006). Symmetrical methylation of CpGs is transmitted through mitotic cell division because the hemi-methylated state that results from DNA synthesis is the preferential target for the DNA methyltransferase DNMT1 (Bestor, 1992; Pradhan et al., 1999; Vilkaitis et al., 2005). Among other known functions, methylated CpGs affect binding of transcription factors, and proteins containing the methyl CpG binding domain cause transcriptional repression through interactions with histone deacetylases and chromatin remodeling complexes (Tate and Bird, 1993; Zhang et al., 1999; Ng et al., 1999; Ng et al., 2000; Jones et al., 1998; Nan et al., 1998).
DNA methylation could play a role in PcG mediated silencing in mammals. EZH2 interacts with DNA Methyltransferases, and EZH2 knockdown causes loss of DNA methyltransferase binding and loss of DNA methylation at PcG targets (Vire et al., 2006). During in vitro ES cell differentiation, PRC2 targets are more likely then non-PRC2 targets to be de novo methylated (Mohn et al., 2008). A link between PRC2 and establishment of DNA methylation patterns is attractive given that PRC2 members are required during the early stages of maintenance (Simon, 1995). The establishment of both silencing and DNA methylation early by PRC2 could then be maintained during later development by maintenance methyltransferases in cooperation with PRC1.

Disruption of DNMT1 causes improper recruitment of PRC1 members to PcG bodies (Hernandez-Munoz et al., 2005b). A complex containing the maintenance methyltransferase Dnmt1 and PRC1 member Bmi1 and Ring1 has also been observed (Negishi et al., 2007). This interaction is interesting because it also contains the transcriptional repressor Dmap1, which colocalizes with Dnmt1 and HDAC2 at replication foci, and localizes to pericentric heterochromatin during late S phase, presumably to promote DNA methylation as well as histone deacetylation and transcriptional silencing of newly replicated DNA (Rountree et al., 2000). This gives rise to the attractive hypothesis that PRC1 accompanies the maintenance methyltransferase at the replication fork to promote transcriptional silencing of newly synthesized daughter strands.

Despite the attractiveness of DNA methylation as a mark for epigenetic inheritance, it does not consistently explain maintenance in mammals, and is not relevant to epigenetic inheritance in Drosophila. Knockdown experiments in cancer cells have
shown that PcG and DNA methylation dependent silencing mechanisms are largely independent (Kondo et al., 2008). Nuclear transplantation experiments have shown that expression patterns of certain genes can be maintained independent of DNA methylation (Ng and Gurdon, 2008). Importantly, MPs are well conserved between flies and humans, but DNA methylation patterns are not.

_Drosophila_ cytosine methylation is non-symmetrical and no mechanism for inheritance through DNA synthesis is known (Lyko et al., 2000). The vast majority of DNA methylation occurs transiently during embryonic stages and is mediated by the single DNA methyltransferase found in _Drosophila_, DNMT2 (Patel and Gopinathan, 1987; Tweedie et al., 1999; Kunert et al., 2003). RNAi mediated knockdown of DNMT2 in _Drosophila_ embryos results in no major developmental phenotypes (Kunert et al., 2003). Most research in _Drosophila_ therefore focuses on the role of Maintenance Proteins and their accompanying chromatin modifications as potential mediators of epigenetic inheritance.

Many maintenance proteins modify histones, and modified histones in turn are able to recruit maintenance proteins (Grimm et al., 2007; Kuzmichev et al., 2002; Fischle et al., 2003; Hansen et al., 2008). This suggests two models in which either histone modifications, or maintenance proteins themselves, could be the epigenetic mark. If either is stable to DNA replication or mitosis, it could be capable of re-recruiting the other to re-establish silencing in the subsequent cell cycle. I will call the first possibility the “histone modification first” model, in which modified histones are stably inherited through DNA synthesis, and that they in turn recruit Maintenance Proteins. The alternative model is the “Maintenance Proteins first” model, in which Maintenance
proteins are stable to DNA synthesis and they in turn modify newly assembled nucleosomes behind the replication fork.

Histone modifications have been proposed to be epigenetic, and there is no doubt that they are highly stable to multiple rounds of cell division and sometimes to nuclear transplantation and reprogramming (Dean et al., 2001; Santos et al., 2003; Ng and Gurden, 2008). A semi-conservative model of nucleosome inheritance has been proposed as a mechanism for the propagation of modified histones through DNA replication based solely on the ability of histone chaperones to interact with H3-H4 dimers not tetramers (Tagami et al., 2004). If this mechanism were true it would provide an excellent mechanism for inheritance of modified histones through DNA synthesis (“histone modifications first”), however density-labeling experiments have demonstrated that histone inheritance is in fact conservative (Yamasu et al., 1990).

Conservative histone inheritance suggests that, as nucleosomes are shuffled between daughter strands immediately following DNA synthesis, any marks they carry will be diluted due to de novo nucleosome assembly. It is possible that conservative histone inheritance could allow for propagation of histone modifications if histone modifying enzymes are preferentially recruited to chromosomal regions containing the mark they are responsible for in order to modify neighboring newly synthesized nucleosomes. This is supported by the binding of PcG members to PcG mediated modifications (Grimm et al., 2007; Kuzmichev et al., 2002; Fischle et al., 2003; Hansen et al., 2008).
H3K27me3 and PRC2, the complex that mediates this modification, are stable to mitosis, though PRC1 binding is not (Aoto et al., 2008; Buchenau et al., 1998). PRC1 binds to H3K27me3 via the chromodomain of Pc, and therefore the methylation mark has been proposed to mediate re-recruitment of PRC1. This is an attractive model, however in vitro binding activities do not agree with in vivo binding patterns, indicating that factors other than the methylation mark play a role in localization of PcG proteins to their targets (Papp and Muller, 2006; Ringrose et al., 2004; Bernstein et al., 2006; Wang et al., 2004b). Therefore, the “histone modifications first” model can not entirely explain re-recruitment of PcG proteins following cell division, because binding of PcG proteins does not solely depend on histone modifications.

At best, evidence supports a role for PRC2 mediated histone modifications as an epigenetic mark stable to mitosis, but there must be an epigenetic mark that is also stable to replication fork progression during S phase. Immunofluorescence experiments in a human cell line have shown that H3K27me3 decreases as cells progress through S phase, and ChIP experiments in yeast have shown that H3K9me at some genomic regions is not propagated through DNA synthesis (Hernandez-Munoz, 2005b; Gullerova and Proudfoot 2008). Histone modifications therefore do not fulfill the requirements for an S-phase specific epigenetic mark because they are not stable to DNA synthesis.

It is possible that MPs themselves could be the epigenetic mark that is stable to DNA synthesis, and their presence following replication allows for modification of histones. Groundbreaking work by Mazo et al (unpublished) has shown that MPs are present directly behind the replication fork, as are unmodified histones, but not modified histones. That MP binding, but not histone modifications, is stable to replication strongly
suggests that MPs are the epigenetic mark, and that new histones deposited during DNA replication are modified anew each cell cycle (Maintenance Protein first model). Because binding of some PRC1 members is not stable to mitosis (Buchenau et al., 1998; Voncken et al., 1999), it has long been thought they are not the epigenetic mark. However, there may be separate epigenetic marks for mitosis and DNA synthesis. Histone modifications might play the role of the mitotic epigenetic mark, or the small level of Maintenance Protein that remain bound to chromatin through mitosis could be a sufficient mark (Buchenau et al., 1998; Voncken et al., 1999).

If Maintenance Protein binding, together with their corresponding histone modifications, function together as epigenetic marks, this implies that simple PcG binding together with silencing modification vs. trxG binding, together with activating modifications, ultimately should determine the silent vs. active state of a particular target gene. In fact, genome wide ChIP studies have recently called into question the simple model that PcG binding vs. trxG binding determines transcriptional silencing vs. activation, respectively. 10-20% of PcG target genes are actively transcribed in human and mouse ES cells, Drosophila cell lines, and human embryonic fibroblasts, (Schwartz et al., 2006; Tolhuis et al., 2006; Bracken et al., 2006 Boyer et al., 2006; Lee et al., 2006).

PRC1, PRC2 as well as Trx binding has been shown in both wing discs, where Ubx is on, and leg/haltere discs where Ubx is off (Papp and Muller, 2006). In Drosophila S2 cells, Ringrose et al (2004) observed no correlation with either Pc binding, H3K27me3, or H3k9me3 and transcription as assayed by RT-PCR.

Discrepancies in the correlations betweentrxG binding and transcriptional activation, and PcG binding and transcriptional repression, could be explained by the fact
that these studies were done in a population of cells in which the transcriptional states of the target genes were not uniform. At the single cell level, the simple model does in fact appear to be true. In a single cell of the salivary gland, PcG and trxG binding to a Ubx transgene is mutually exclusive, and correlates with the transcriptional status of the gene (Petruk et al., 2008). However polytene chromosomes are a unique tissue. When possible, single cell studies in different tissues and developmental stages will be very informative.

It remains to be determined whether Maintenance Proteins and their histone modifications satisfy the model for epigenetic marks, because both their ability to re-recruit one-another in vivo and the relationship between their binding and the transcriptional states of their targets are more complex than simple models predict.

### 1.6 Replication Timing

A simple model to account for maintenance of transcriptional states through DNA synthesis has evolved from observations that there are replication timing differences between transcriptionally active and inactive regions of the genome (for review see Gilbert, 2002). It is hypothesized that early replication occurs while factors are available to facilitate the establishment of a chromatin state that allows for active transcription to occur. These factors are no longer available during late S-phase, but factors that assemble late replicating regions into a repressive chromatin structure are.

Correlations between replication timing and transcriptional status were first observed in the 1960s (Braun et al., 1965), and have since been confirmed in mammals, *Drosophila*, and *Xenopus* (Taljanidisz et al., 1989; Hatton et al., 1988; Schubeler et al.,
The best example of a developmentally regulated region whose transcriptional activity and replication timing are correlated is the beta-globin locus, whose replication timing appears to be determined by a genetically defined origin (For review see Aladjem, 2004).

Replication timing could be controlled through restriction of origin firing or by fork elongation though passively replicated regions. The timing of origin firing is more easily and often studied and will be discussed later. Passive replication through regions between origins also has the potential to delay the replication timing of a chromosomal region (Ermakova et al., 1999). Passive replication has been shown to be epigenetically important for control of the Schizosaccharomyces pombe mat locus. Lagging strand synthesis through the region originating from a centromere proximal replication origin results in a double strand break, due to failure to remove an RNA primer. This double strand break acts as an imprint to induce mating type switching in one daughter cell (Dalgaard and Klar, 1999).

The most commonly studied control over early vs. late replication is through choice of origin location and control of the timing of origin firing. DNA replication begins from many origins (Huberman and Riggs, 1968), some firing early during S phase, and others firing late (Ferguson et al., 1981). During early Drosophila syncytial cycles, a large number of origins allow for rapid duplication of the genome (Shinomiya and Ina, 1991). As development progresses, however, origin use becomes restricted (Sasaki et al., 1999), concomitant with differentiation decisions.
Origin preference and/or origin firing are dependent on both chromatin states and transcription. In many systems, histone acetylation has been linked to replication timing and origin firing. The human beta-globin contains a replication origin that fires early in erythroid cells but late in nonerythroid cells. Early and late firing are correlated with histone acetylation and deacetylation, respectively, and tethering of a histone deacetylase (HDAC) delays replication (Goren et al., 2008). Injection of a transcription inducible template into Xenopus eggs causes origin specification upon TBP and GAL4-VP16 binding, concomitant with histone H3 acetylation (Danis et al., 2004). Treatment of HeLa cells with the HDAC inhibitor TSA causes an S phase acceleration, due either to early firing of late origins or increased firing of latent origins as both were observed (Kemp et al., 2005).

In Drosophila, the gene amplification that occurs in follicle cell nuclei has been studied extensively as a model for replication origin specification and initiation, and has been used to implicate a PcG protein in origin specification. Tethering of Pc to the amplified origin inhibited origin activity (Aggerwal and Calvi., 2004). This could be due to affects on chromatin structure, as PcG proteins interact with Histone Deacetylases. Recruitment of a Histone Deacetylase to a replication origin could disrupt replication directly, because replication requires histone acetylation directed by the Origin Recognition Complex (Iizuka et al., 2006). However it should be noted that rae28, the mammalian homolog of PH, Co-IPs with the licensing factor geminin, which inhibits origin firing through dup/cdt1 (Luo et al., 2004), allowing for the possibility of a direct role for PcG proteins in replication origin licensing.
In *Drosophila*, the SuUR (*Suppressor of Under Replication*) gene controls late replication. The SuUR protein localizes to PcG targets on polytene chromosomes, and a 300kb region of underreplication (and hence late replication) is found in the Bithorax Complex from the Ubx to Abd-B loci. Mutation of the SuUR gene suppresses late replication of this region (Moshkin et al., 2001). SuUR binds to this region, as well as many sites on polytene chromosomes that overlap with binding sites for PcG proteins (Makunin, 2002). However, Mutations in Pc or E(z) do not cause early replication of this or other genomic regions (Zhimulev et al., 2003), suggesting that they do not function to regulate replication timing of their target genes as a mechanism of epigenetic inheritance.

At a fine scale, correlations between transcription and replication are not strong (MacAlpine et al., 2004), suggesting that the long-range chromosomal structure is more relevant to replication timing than is transcription itself (Donaldson, 2005). Importantly, some loci do not change replication timing as a result of a differentiation-induced change in transcriptional status (Azuara et al., 2003; Hiratani et al., 2008). However, the major challenge to the model of replication timing as a mechanism for epigenetic inheritance comes not from lack of evidence for correlation between transcriptional state and replication timing, but from lack of evidence that the timing of replication actually directs silencing or activation.

Replication timing as an epigenetic mechanism is not universal, and may occur as a consequence of transcriptional state, but not play a role in epigenetic inheritance. The location of origins and timing of origin firing is regulated by many processes, and the result is that the chromatin state, transcriptional status, and DNA sequence are differentially important at different origins. Further, there is little evidence that the
advancement of replication timing is sufficient to cause transcriptional activation, and, as discussed, the correlations have broken down under scrutiny. If two nuclear environments existing during S phase promote assembly of active or repressive chromatin domains depending on replication timing, replication of active and silent regions at the same time would never be observed. Replication timing is therefore an inadequate hypothesis for the mechanism for the epigenetic inheritance of the silent state of PcG targets.

1.7 Coordination of Maintenance with Cell Cycle Progression

Proposed models for maintenance do not sufficiently explain how genetic and epigenetic inheritance are coordinated, and how Maintenance Proteins stabilize gene expression states through DNA synthesis and mitosis. One overlooked hypothesis is that PcG proteins are directly involved with the stabilization of the silent state of their targets during the different cell cycle stages, and that they therefore function differently within each cell cycle stage to meet the silencing challenges of that specific phase.

This coordination would require that PcG proteins interact with cell cycle regulators in order to regulate cell cycle phase specific challenges to maintenance, which has been observed in flies and mammals. PCNA, the DNA replication processivity factor that participates in the inheritance of chromatin structure in yeast, co-localizes with the PcG protein Cramped during S-phase (Zhang et al, 2000; Yamamoto et al., 1997). Rae28, a mammalian ph homologue, interacts with the replication licensing factor Geminin (Luo et al., 2004). This interaction could be significant to Hox gene silencing because over expression of geminin inhibits Hoxb9 transcription in chick embryos. The interaction
between Rae28 and geminin has been proposed to cause poly-ubiquitination and degradation of geminin, and loss of Rae-28 affects the proliferative capacity of stem cells (Ohtsubo et al., 2008). However, geminin was named for its duality of function. During neurogenesis, geminin promotes maintenance of the undifferentiated state by antagonizing the SWI/SNF chromatin remodeling protein Brg1 (Seo et al., 2006). The interaction between the two could affect transcription directly, rather than affecting inheritance of the transcriptional state through cell division.

Precision coupling of genetic and epigenetic inheritance through direct interactions between regulators of the two implies that disruption of one process would result in disruption of the other. As such, many cell cycle regulators have epigenetic defects, and vice versa (Zhang et al., 2000; Shareef et al., 2003; Hallson et al., 2008; Inoue et al., 2008). Deficiencies in maternal deposits of a wide variety of PcG, TrxG, and ETPs cause mitotic defects in early syncytial embryos, such as mitotic bridges and nuclear fallout (Yamamoto et al., 1997; Lupo et al., 2001, O’Dor et al., 2006).

Normal cell cycle progression in syncytial embryos is shown in Figure 1-1A (Left Panel). Embryos from mothers mutant for the PcG genes indicated have bridges between separating nuclei (Fig. 1-1A). These bridges are the result of a failure by sister chromatids to properly segregate. All other cell cycle stages in these embryos appear identical to wild type, with the notable exception that embryos derived from mutant mothers have higher than normal nuclear fallout, a negligible amount of which is due to the presence of the balancer chromosomes (Fig 1-1B). Fallout results from cell cycle delays in individual nuclei, which are removed form the cortex, preventing them from becoming somatic nuclei (Sullivan et al., 1993a). This work was done as part of the
graduate research of E. O’Dor. My contribution to the figure is the right panel of Fig. 1-1A, as well as the balancer chromosome data in Fig. 1-1B.

Mitotic defects are also observed in larval brain squashes from *php* mutants, indicating the PcG role in the cell cycle is not unique to S-M cycling cells (O’Dor and Brock, unpublished data). These cell cycle phenotypes indicate that PcG proteins play a role in cell cycle progression. Epigenetic maintenance requires close coordination with cell cycle progression. Therefore, closer examination of the cell cycle phenotypes observed in PcG is an important step in the elucidation of the mechanism of maintenance through cell division.
**Figure 1-1.** PcG proteins play a role in cell cycle progression. (A) Wild type (OR) embryos at telophase with properly segregated sister chromatids (arrows) are shown in the left panel. PcG mutants have chromatin bridges at anaphase and telophase (Arrows), including embryos mutant for *php* (right panel). (B) PcG mutants also have a high level of nuclear fallout. Low severity nuclear fallout occurs to a small degree in all embryos (grey bars). High severity fallout, in which more than 5 nuclei have internalized into the embryo (black bars) is more prevalent in PcG mutants of the indicated genotypes.
1.8 Thesis Aims

These mitotic phenotypes are evidence that PcG proteins play a role in cell cycle progression, which is very important to the elucidation of their role in epigenetic inheritance. Direct interactions with cell cycle regulators would be expected if these phenotypes are the result of PcG proteins playing a direct role in the cell cycle. Although cell cycle phenotypes have been observed in a wide variety of PcG mutants, the PcG gene polyhomeotic (ph) was chosen as the focus of this study. This was due to its well-defined role within PRC1, as well as reports of its physical association with both S phase and mitotic proteins (Luo et al., 2004; Lupo et al., 2001). These direct physical associations made ph a strong candidate for having a direct role in a cell cycle process, and though it is well characterized in its requirement for silencing and as a subunit of silencing complexes, its role within these complexes is largely unknown.

The ph locus is duplicated and contains two functional units, polyhomeotic proximal (php), and polyhomeotic distal (phd). Most phenotypic description in this thesis deals with a homozygous viable mutant, ph410. Unlike the ph504 mutants used in the initial characterization of the chromatin bridge phenotype, which carry disruptions of both proximal and distal transcripts, the ph410 mutants contain an inversion disrupting only the proximal repeat. The Proximal (PhP) and Distal (PhD) isoforms are 85% identical. However PhP but not PhD is in a complex that contains Pc, but not Posterior Sex Combs or dRing (Wang and Brock 2003). Consistent with differing roles for PhP and PhD, mutations in php but not phd cause mitotic defects in embryos (O’Dor et al., 2006).
As such, this thesis will focus on the further dissection of the cell cycle role of polyhomeotic proximal. Mitotic bridging at anaphase and telophase is a common defect that can arise from defects in multiple cell cycle processes (Su et al., 1999; Ji et al., 2004; Xu and Du, 2003; Sibon et al., 1999; Frenz and Glover, 1996; Sibon et al., 2000; Rogers et al., 2004; Donaldson et al., 2001; Giet and Glover, 2001; Stratmann and Lehner, 1996; Philp et al., 1994; Stumpff et al., 2004). Defects occurring during DNA synthesis can carry over to mitosis producing the chromosome bridge phenotype, especially in early embryos whose cell cycles have fewer checkpoints than canonical cell cycles (Su et al., 1999; Ji et al., 2004; Xu and Du, 2003; Sibon et al., 1999; Frenz and Glover, 1996; Sibon et al., 2000). Therefore, Chapter 2 will address what cell cycle stage, and what cell cycle process, is disrupted in PcG mutants. Chapter 3 will address whether the cell cycle phenotypes are the result of a novel PcG complex containing the cell cycle protein Pds5, and whether Pds5 contributes to PhP dependent silencing or the cell cycle role of PhP.
CHAPTER 2 Polyhomeotic functions in the DNA damage checkpoint during S phase
2.1 Introduction

The presence of mitotic defects in PcG mutants, and in particular in php mutants, provides a key link between PcG mediated silencing and cell cycle progression. The major question addressed in this chapter is where during the cell cycle PcG proteins, and PhP in particular act. Understanding the stage at which PcG proteins act could provide a clue to the stage at which their presence is required for maintenance. Different PcG proteins could promote maintenance of epigenetic memory during DNA synthesis or mitosis, as the challenges to memory at these two stages are very different.

During S phase, plasticity of PcG target genes is most obvious, probably due to changes in chromatin structure induced by replication fork progression (Fisher and Mechali, 2003; Sustar and Schubiger, 2005). The stability of PcG dependent silencing through S phase requires that a mechanism exists to promote epigenetic silencing during replication fork progression in both DNA replication and repair. A MP that plays a role in S phase progression is a likely candidate for this role.

A mechanism also must exist for the inheritance of epigenetic states and the higher order chromatin structure of silenced regions through the massive chromatin changes that occur during mitosis (Michelotti et al., 1997; Martinez-Balbas et al., 1995; Kruhlak et al., 2001). The presence of mitotic phenotypes arising in mitosis could identify which PcG proteins act at this stage to promote maintenance.

A previous study showed chromosome segregation defects in ph mutants. Based on observations that Ph associated with condensins in vivo, the authors concluded that Ph
was required during prophase (Lupo et al., 2001). Unpublished results from our laboratory (O’Dor) were unable to replicate the observation that Ph associates with the condensin encoded by Barren. Further, demonstration of an interaction between the two proteins is not equivalent to demonstration that the interaction is required for proper cell cycle progression.

PCG proteins mediate condensation of a nucleosomal template in vitro (Francis et al., 2004). However, this activity appears to be non-specific, as no Polycomb Group Response Elements were present on the template. Non-specific assembly of structured chromatin following S phase could be mediated by PCG proteins, and mutants could have defects in chromosome segregation as a direct result of this, or as a result of a failure to integrate this chromatin structure with the mitotic chromosome condensation process.

Polycomb group proteins play a direct role in the DNA damage response in mammals and possibly in *Drosophila*, and disruption of DNA damage checkpoints leads to similar mitotic phenotypes to those observed in *php* mutants (Stumpff et al., 2004; Su et al., 1999). The mammalian PCG protein RYBP stabilized p53 via MDM2 following DNA damage (Chen et al., 2009). A Polycomb-like (Pcl) mammalian homologue localizes to double strand breaks, and may promote homologous end joining over homologous recombination as a repair mechanism (Hong et al., 2008). A similar role for extra sex combs (esc) and Enhancer of Polycomb (E(Pc)) in choice of repair process has been observed in *Drosophila*, and is likely dependent on histone acetylation by Rpd3 (Holmes et al., 2006).
Ring1B, a member of the mammalian PRC1 complex that also contains Ph and Pc, mediates ubiquitination of H2A in response to DNA damage in a manner dependent on ATR but not ATM (Bergink et al., 2006). The role of histone ubiquitination is unknown but may mediate chromatin relaxation due to the large size of the modification, which could facilitate access of repair enzymes to damaged DNA (Guerrera-Santoro et al., 2008). Other enzymes also ubiquitinate H2A in response to DNA damage. Ubiquitination of the H2A variant H2AX by UBC13 releases H2AX from chromatin (Ikura et al., 2007; Guerrera-Santoro et al., 2008). Bergink et al (2006) estimate that H2Aub could extend over a 10-30kb region spanning a lesion, which could function to pause transcription in the region while damage is repaired.

The first goal of this chapter is to identify if S or M phase is disrupted in PcG mutants. The second goal of this chapter is to identify the specific cell cycle process within S or M phase for which PcG proteins are required. In S phase, PcG proteins could be required during DNA synthesis and/or DNA repair. Maintenance of the transcriptional state of PcG targets must be stable not only during replication fork progression, but also during fork progression and chromatin changes occurring in the repair of DNA damage accrued spontaneously and during synthesis. In addition, PcG proteins could be required for normal function of DNA damage checkpoints, or in repair of DNA damage. Defects in either synthesis, or in the DNA damage checkpoints and DNA repair can lead to anaphase bridges. In mitosis, epigenetic inheritance through mitosis could affect chromosome resolution, chromatin condensation, and sister chromatid cohesion. All of these processes are interdependent and defects in any one of them can result in chromatin bridging similar to that observed in PcG mutants when disrupted.
2.2 Results

2.2.1 Bridged nuclei from in php mutants resolve, fail to divide, or result in fallout

In order to further examine the cell cycle role of php, the cell cycle phenotypes were examined through live imaging. One prediction made based on images of fixed nuclei from php mutants was that chromatin bridges resolved (O’Dor et al., 2006). This prediction was based on the observation that the number of bridged nuclei observed in the mutants is far greater than the number of fallout nuclei, or the number of fragmented nuclei that should be observed if chromatin bridges are not resolved (O’Dor et al., 2006). Defects in DNA synthesis and damage checkpoints result in bridges that do not resolve (Frenz et al., 1996; Su et al., 1999; Stumpff et al., 2004). A failure to resolve could therefore indicate the bridges are the result of defects in these processes.

In order to visualize the outcome of chromatin bridging, I used time-lapse imaging to directly observe chromatin dynamics during progression through syncytial cycles in embryos from heterozygous ph410 in a white background. Chromosomes were visualized through the use of a transgene on chromosome 3 expressing H2A.z fused to GFP, a kind gift from Dr. Shelagh Cambell (University of Alberta). Image stacks were collected every 60s in embryos progressing through mitosis 10 through to mitosis 13.

Through time lapse imaging of mitotic progression in ph410 mutants, I was able to confirm that some chromosome bridges do resolve. Fig. 2-1A shows an example of a resolving telophase bridge. The chromatin between the two daughter nuclei separates as the nuclei re-circularize, and subsequent divisions occur normally (Fig 2-1A; data not shown).
Figure 2-1. Chromatin bridges resolve or result in nuclear fallout. (A) Chromatin bridges occur at anaphase (panel three, arrowhead), and the previously bridged nuclei continue into subsequent stages normally. (B) Chromatin bridges (panel three, arrowhead) cause nuclei to snap back and fuse. (C) Chromatin bridges (first and second panel, arrowhead) result in chromosome breakage, which later results in internalization of defective nuclei into the embryo (last panel).
Some nuclei with bridges that resolved were observed to fail at subsequent mitotic divisions, indicating that chromosome breakage may have occurred in one cell cycle, but the fallout is delayed until a later cell cycle (data not shown). Due to the number of images and amount of nuclear movement this data is difficult to present two dimensionally.

Occasionally, telophase bridging immediately results in fragmented chromosomes and nuclear fallout, or nuclei which fail to divide altogether, and snap back to form polyploid nuclei (Fig. 2-1B,C). Nuclei that failed to divide were most frequently observed during mitosis 12 and 13 (Fig. 2-1B,C). Nuclear fallout also occurred during the interphase immediately following chromosome bridging, likely as a result of chromosome breakage. Fig. 2-1C shows the formation of a chromatin bridge that appears to resolve; however one of the daughter nuclei subsequently over-condenses and is internalized into the embryo. Embryos were not observed beyond cellularization, so it is unknown whether they failed or were delayed in subsequent divisions.

The inability of some bridges to resolve is consistent with either an S phase or a mitotic role for *php*. Occasionally, embryos were observed in which massive bridging and death occurred, similar to that observed in DNA damage checkpoint mutants (Stumpff et al., 2004). However, this phenotype was also occasionally observed in wild type embryos, therefore embryos that did not survive through 13 nuclear divisions were not included in the data set.
2.2.2 PhP binds chromatin during S phase in the absence of transcription

If PhP acts in S phase, it must be chromatin associated at this stage. 0-2.5 hr embryos are controlled by maternally deposited protein and RNA, and transcription of the embryonic genome does not occur until the completion of S phase 13 (Edgar et al., 1996). Therefore, binding of PhP to chromatin in S phase would presumably be for reasons other than for the regulation of transcription. It has been described previously that Polycomb Group proteins leave chromatin during mitosis in older embryos (Buchenau et al., 1998), which would suggest that they do not have a direct effect on mitotic progression. In order to address whether PhP is present in syncytial embryos on chromatin during S phase or mitosis and therefore limit its time of action, 0-2.5 hr embryos were immunolabeled with an antibody against PhP.

Fig. 2-2 shows that PhP is present on chromatin during S phase, prior to the onset of transcription following S phase 13, but is absent during mitosis in these cycles, consistent with earlier reports on PcG behavior during cell division (Bucheneau et al., 1998; Dietzel et al., 1999). Binding is punctate during S and prophase (Fig. 2-2A,B), but PhP leaves chromatin before chromosomes align at metaphase (Fig 2-2C), and does not return until chromosomes decondense at telophase (Fig. 2-2D). PhP is not present during anaphase when the chromatin bridge phenotype occurs, but is instead present during S phase. Therefore, if PhP does play a direct role in cell cycle progression, this role must be during S phase.
Figure 2-2. PhP is not localized to chromatin during mitosis. Projections of confocal images through cortical nuclei of embryos stained with anti-PhP during syncytial cycles. (A) During S phase, PhP is localized to the region of the nucleus, as determined by DAPI staining, and has a punctate pattern. (B) During prophase, the punctate pattern is lost and PhP begins to leave the nucleus. (C,D) During metaphase and anaphase PhP is outside of the nuclear periphery. (E) PhP returns to chromatin during telophase. Bar = 10um
2.2.3 Loss of PhP binding at mitosis is not due to phosphorylation of serine 28 of histone H3

Similar to PhP, heterochromatin protein 1 (HP1) chromatin binding is cell cycle regulated (Fischle et al., 2005). HP1 binding to chromatin is in part due to interaction of the HP1 chromodomain with trimethylated H3K9 (Stewart et al., 2005; Peters et al., 2002; Thiru et al., 2004; Nielsen et al., 2002; Jacobs et al., 2002; Lachner et al., 2001). During mitosis, phosphorylation of Serine 10 of histone H3 by Aurora B kinase disrupts binding of HP1 to H3K9me3 causing the mitosis specific dissociation of HP1 from chromatin (Fischle et al., 2005; Hirota et al., 2005; Terada et al., 2006).

The trimethylated lysine binding site on histone H3 bound by PRC1 (H3K27me3) via the chromodomain of Pc is also adjacent to a highly conserved serine residue that is phosphorylated in a mitosis specific manner by the Aurora B kinase (Goto et al., 2002). In order to determine whether the mitotic dissociation of PhP correlates with the phosphorylation of H3ser28, 2.5hr Oregon R embryos were collected, fixed and stained with anti H3ser28P and anti PhP. Mitosis in syncytial embryos occurs in waves beginning at the poles, allowing visualization of multiple nuclei at slightly different mitotic stages. Fig. 2-3A shows an embryo in which nuclei on the left side are in early prophase, while nuclei on the right are in late prophase or metaphase. Phosphorylation of serine 28 of histone H3 occurs during late prophase (Fig. 2-3A), the same approximate stage that dissociation of PhP occurs (Fig. 2-2B,C).

Double immunolabeling with antibodies against H3ser28P and PhP was used to determine whether colocalization of the two occurs. If phosphorylation of H3ser28
Figure 2-3. Phosphorylation of Serine 28 of histone H3 does not preclude binding of PhP. (A) Immunostaining of embryos demonstrates that phosphorylation of serine 28 of histone H3 occurs between prophase (P) and metaphase (M). (B) A single slice (<0.1 u) through nuclei at prometaphase shows overlap between PhP staining and that of the phosphorylated histone. Bar = 10 um
destabilizes binding of PRC1 to H3K27me3, colocalization of phosphorylated H3ser28 and PhP should not occur. Fig. 2-3B shows that nuclei with strong H3ser28 phosphorylation signals also have strong nuclear localization of PhP. At the resolution of immunohistochemistry, it does not appear that phosphorylation of H3ser28 immediately destabilizes PRC1 during mitosis.

### 2.2.4 Embryos from php mutant mothers have accelerated S phases

The function, if any, of PhP in cell cycle progression is limited to S phase when it is present on chromatin (Fig. 2-2). Visible defects during S phase were not observed in $p h^{410}$ mutants; however at the level of fixed nuclei this is not surprising. To determine whether embryos derived from php mutant mothers progressed differently through S phase I used time-lapse imaging to observe directly progression through S phase and mitosis in embryos derived from homozygous $p h^{410}$ mutant mothers as compared to control embryos.

A representative set of images from mothers with the genotypes $w/w; H2A.z$-$GFP$ and $p h^{410}, w/w; H2A.z$-$GFP$ progressing through S Phase and Mitosis 12 are shown in Fig. 2-4A. Entrance into mitosis or completion of S phase was determined by the onset of chromosome condensation, and entrance into S phase or completion of mitosis was determined by completion of chromosome decondensation. Similar images were collected from multiple (n= 4-6 per stage) embryos. Embryos that did not survive to the end of mitosis 13 were not included in the data set to reduce artifacts produced by the imaging process. The average timing of each cell cycle stage from Mitosis 10 to mitosis
Figure 2-4. See legend on following page.
Figure 2-4. S phase is accelerated in the syncytial divisions of php mutant embryos.

Time lapse confocal images of syncytial cell divisions in *Drosophila* embryos were taken every 60 seconds using a fly strain expressing histone H2A.Z fused to GFP and cell cycle staging was determined by chromatin condensation. (A) Representative images of progression through S phase in a control (Con; w/w) and *php* (*ph*410) mutant embryo. The length of S phase is reduced in *php* mutants. (B) Representative images of progression through mitosis in a control (Con; w/w) and *php* (*ph*410) mutant embryo. The duration of mitosis does not differ between the two. (C) Cell cycle timing was determined from 5 nuclei per embryo, and 5 embryos per genotype were used to determine the mean (black and red columns). Error bars represent standard error of the mean. For simplification, genotypes do not include the H2A.z-GFP transgene. Mitosis has a similar duration between control embryos (black bars) and *ph410* mutants (red bars) at all stages. S phases at during cycles 11 and 12 were significantly accelerated in *ph410* mutants compared with control embryos (P <0.05, determined using unpaired Student’s t test).
13 were determined for each embryo by averaging the times from 5 individual nuclei. The average length of each stage was then determined for each genotype (Fig. 2-4B).

Embryos from \( ph^{410},w/w; H2A.z\)-GFP mothers completed S phase faster than the control embryos. They completed S phase between 28 - 42% faster than embryos from \( w/w; H2Az\)-GFP mothers. \( ph^{410} \) mutants therefore showed the greatest acceleration during S phases 11 and 12 (Fig. 2-4B). The timing difference in \( php \) mutants occurs only during S phase. There was no significant difference between \( ph^{410} \) mutants and control embryos during any mitotic stages (Fig. 2-4). This indicates that \( php \) plays a role in controlling S phase length, and loss of \( php \) results in aberrant S phases. There is no change in the timing of the stage during which visible defects occur.

2.2.5 Mutations in known S phase regulators do not interact genetically with mutations in \( php \)

If the cell cycle role of PhP occurs through direct interaction with or regulation of a known S phase regulator, mutation in the gene encoding that regulator could lead to synergistic enhancement of the chromatin bridge phenotype. Syncytial cell cycles 10 through 13 progressively lengthen in duration, leading to an extended delay in G2 of cycle 14, the mid-blastula transition (MBT), after which time transcription of embryonic genes is required for further divisions and development (Edgar et al., 1986). These delays in cell cycles 10-14 are the result of the activation of a checkpoint pathway involving the grapes/checkpoint-1 (grp/chk1) kinase, and mei-41/ATM, which are also required for the MBT (Sibon et al., 1997; Sibon et al., 1999). Both mei-41 and grp mutants have accelerated S phases during syncytial divisions, and are characterized by chromatid
bridges during anaphase 12 that do not resolve (Sibon et al., 1999; Fogarty et al., 1997). The inability of sister chromatids to resolve in grp mutants leads to mitotic catastrophe, a phenotype we occasionally observe in ph mutants (Sullivan et al., 1993b; O’Dor et al., 2006).

The similarity between the mutant phenotypes of these checkpoint genes and the phenotypes observed in ph410 mutants suggested that mutations in these checkpoint genes would enhance the mitotic bridge phenotype of ph410 mutants. Double heterozygotes of grp or atm and ph410 did not show a synergistic enhancement of the chromatin bridge phenotype. Instead we observe an additive effect as chromatin bridges are also observed in single mutants of these checkpoint genes (Fig. 2-5; Sibon et al., 1999; Fogarty et al., 1997).

Experiments in vitro have shown that the processivity factor PCNA can accelerate rates of DNA synthesis (Moldovan et al., 2007), and mutations in the gene mus209, which encodes PCNA cause defects in syncytial division cycles (Henderson et al., 2000). PCNA colocalizes with the PcG protein cramped during S phase (Yamamoto et al., 1997). During S phase while PhP is localized to chromatin, it too colocalizes with PCNA (O’Dor and Brock, unpublished). In order to determine whether the chromatin bridge defect observed in ph410 mutants are the result of an interaction between PhP and PCNA, the frequency of severe chromatin bridging was quantitated in double heterozygotes for ph410 and mus209902. The frequency of severe mitotic bridging did not differ in double mutants from the frequency observed in single mutants for ph410 (Fig. 2-5).
Figure 2-5. Genes with known roles in checkpoints and DNA synthesis do not interact genetically with ph410. 0 - 2.5 hr embryos from mothers of the specified genotype were collected and stained with DAPI to visualize DNA. Embryos at anaphase or telophase were scored with regard to bridges between sister chromatids. The number of embryos in which greater than 25% of nuclei were bridged out of the total population of embryos (n=10-12) was then determined for each genotype. See Materials and Methods for crossing schemes.
The origin licensing factor geminin co-immunoprecipitates with murine rae-28 (Luo et al., 2004). An involvement of PhP in the inhibition of replication licensing via geminin in Drosophila could explain the accelerated S phase phenotype, and over-replicated DNA could carry over as bridges or chromosome breakage during mitosis, as has been observed in geminin depleted human cells (Melixetian et al., 2004). 0-2.5 hr embryos from double heterozygous mothers with mutations in both php and geminin were stained with DAPI and examined, and the frequency of severe chromatin bridging (>25% of nuclei bridged) was determined. The frequency of severe chromatin bridges is roughly double that of embryos from mothers with a single mutation in php (Fig. 2-5). This indicates that there is no synergistic interaction between the two, and that this phenotype is unlikely to be related to a protein complex containing geminin and PhP.

2.2.6 php mutants do not have accelerated rates of DNA synthesis

A. Catching performed the experiments described in this section.

The faster progression through S phase observed in php mutants could arise because DNA replication is faster, perhaps because the altered chromatin structure in mutants allows use of cryptic origins of replication or because altered chromatin structure allows greater processivity of the DNA polymerase. To test this hypothesis DNA synthesis rates were measured in imaginal disks of wild type and php mutants. Wing disks were cultured in tissue culture medium containing tritiated thymidine for 40 min. Half the sample was used to spectrophotometrically quantitate the amount of DNA, and the remainder was used to determine thymidine incorporation. As shown in Fig. 2-6A, there is no significant difference in DNA replication rates in wild type and php mutants.
**Figure 2-6.** *php* mutants are unable to arrest cell division in response to DNA damage and do not have accelerated rates of DNA synthesis. (A) The amount of thymidine incorporated in 40 minutes does not differ between imaginal discs from Oregon R (OR) and *php410* mutants. Error bars represent Standard Deviation. (B) The number of mitotic nuclei was determined by staining with an antibody specific to mitotic maker phosphorylated serine 28 of Histone H3 (AbCam) after irradiation by a 60Co source. Following irradiation, discs from *php410* mutants have more cells which failed to arrest before entry into mitosis than do discs from OR. Error bars represent Standard Deviation.
2.2.6 *php* mutants lack DNA damage checkpoints

Acceleration of S phase accompanied by chromatin bridging at mitosis are phenotypes shared by mutants in DNA damage checkpoint genes (Su et al., 1999; Ji et al., 2004; Xu and Du, 2003; Sibon et al., 1999; Stumpff et al., 2004; Sibon et al., 1997; Sibon et al., 1999). No genetic interaction between *php* and the checkpoint genes *grp* or *atm* were observed, however PhP might be required for a different stage of DNA damage repair, or have a role in regulation of other genes required for DNA damage checkpoints. Therefore DNA damage response in wild-type and *php* mutants was determined using an assay that determines the ability of imaginal disc cells to arrest before entrance into mitosis following exposure to x-rays from a $^{60}$Co source (Laurencon et al., 2003). Third instar larvae were collected and exposed to 5 Gy of radiation. About 2 hours later, imaginal discs where dissected and fixed, and the number of cells in mitosis was determined by staining with the mitotic marker anti phospho serine 28 of Histone H3. As shown in Fig. 2-6B, in wild-type disks the number of cells per disc able to enter mitosis is less than 40. However in *ph*<sup>d10</sup> mutant larvae, the ability to arrest the cell cycle before entrance into mitosis is impaired relative to wild-type, and over 150 mitotic cells per disc are observed (Fig. 2-6B). This decrease in the ability to arrest the cell cycle in response to DNA damage supports a role for *php* in the DNA damage checkpoint.
2.2.7 PhP is not recruited to sites of DNA damage repair

The inability to arrest the cell cycle in response to DNA damage suggests that \textit{php} plays a role in the DNA damage checkpoint (Fig. 2-6B). However it does not address whether this effect is direct, or an indirect result of misregulation of PcG target genes. If the effect is direct, then PhP might localize to regions of DNA damage. In order to address this, early embryos were permeabilized and treated with the DNA damaging agent bleomycin to induce double strand breaks. This was followed by double immunolabeling with PhP and an antibody specific to the phosphorylated form of histone H2Ax. Early in the double strand break response, histone H2Ax is phosphorylated in the region of the break (Takada et al., 2007). Overlap of PhP with phospho H2Ax would therefore indicate recruitment of PhP to the break site.

Fig. 2-7 shows a single z-slice (~0.1 μm) through embryonic nuclei after exposure to the DNA damaging agent. Embryos were permeabilized, followed by exposure to bleomycin, resulting in multiple damage foci per nucleus (Fig. 2-7). Multiple PhP staining regions are also visible (Fig. 2-7). A merge is shown in the right panel, with regions of H2Az ser139P and PhP overlap shown in yellow. Some regions of DNA damage show strong overlap with PhP signal (Fig. 2-7 arrowhead), however some DSB regions do not overlap with PhP (Fig. 2-7 arrow), showing that PhP is occasionally present at regions of double strand breaks in early embryos, but is not actively recruited to them.
Figure 2-7. PhP is not recruited to sites of double strand breaks. A single slice (<0.1μm) through nuclei stained with anti PhP and the double strand break marker phosphorylated serine 139 of histone H2A.X is shown. PhP is found in the location of some double strand breaks (arrowhead), but not at others (arrow). Bar = 10μm
2.3 Discussion

2.3.1 *php* mutations affect S phase progression, not mitosis

The fact that the timing of S phase is altered in *ph*<sup>410</sup> mutants, but the timing of mitosis is not (Fig. 2-4), strongly suggests that *php* has a role in S but not M phase. Therefore, I suggest that the chromosome bridging which occurs at anaphase is a carry over from a defect occurring during S phase in *php* mutants. There is precedence for visible defects in mitosis arising from mutations for factors involved in S phase. Human *Orc6* mutants have segregation defects as well as replication defects, though unlike in *php* mutants, bridges do not resolve and segregation fails completely (Prasanth et al., 2002). Chromatin bridges are most commonly observed in *Drosophila* syncytial embryos with replication checkpoint defects (Su et al., 1999; Ji et al., 2004; Xu and Du, 2003; Sibon et al., 1999; Stumpff et al., 2004).

The lack of chromatin association of PhP during syncytial mitoses indicates that PhP does not play a direct role in mitotic processes (Fig. 2-2). This mitotic dissociation may be a common characteristic of many PcG proteins in many species, though its function is unknown (Miyagishima et al., 2003; Akasaka et al., 2002). If PhP is required directly for cell cycle arrest in response to DNA damage (Fig. 2-6B), perhaps its presence on chromatin is refractory to mitotic progression.

The mechanism of PRC1 dissociation from mitotic chromosomes is unknown. However direct phosphorylation by MAPKAP kinase 3 (3pK) controls chromatin association of PRC1 member Bmi-1, and possibly mammalian Ph (Voncken et al., 2005), and the phosphorylation status of Bmi-1 correlates with its mitotic loss from
chromosomes (Voncken et al., 1999). Mitosis specific phosphorylation, perhaps even by mitotic Cdks, of Ph and/or other PRC1 members could cause chromatin dissociation. Phosphorylation of serine residues adjacent to dimethylated lysines allows for formation of a hydrogen bond between adjacent modified residues, which could directly promote chromatin condensation, or promote the binding of condensins (Eberlin et al., 2008). That phosphorylation of H3ser28 does not preclude PhP binding suggests that chromatin structure is not the sole determinant of PRC1 binding (Fig. 2-3B).

It has been suggested recently that PRC2 together with H3K27me3 are stable to mitosis, and promote recruitment of PRC1 members in G1 (Aoto et al., 2008). However the methylation mark is unlikely to be the sole determinant of PRC1 binding as H3K27me is spread through larger domains than PRC1 binding (Papp and Muller, 2006; Kahn et al., 2006). The chromatin structure of Drosophila syncytial embryos is largely uncharacterized, and it is unknown whether methylation of H3K27 occurs.

A mitotic role for PhP has been suggested previously by the association of PhP with Topoisomerase II (TopoII) and the condensin barren (barr), which also localize to PREs (Lupo et al., 2001). However this interaction is likely not the basis for the chromosome bridge phenotype observed in php mutant embryos because double mutants for both php and barr are indistinguishable from single mutants of php, indicating that php is epistatic to barr (O’Dor and Brock, unpublished). This is consistent with a role for PhP in S phase, before barren functions in mitosis.

Though carry over of S phase defects in the form of chromatin bridging is common (Su et al., 1999; Ji et al., 2004; Xu and Du, 2003; Sibon et al., 1999, 2000; Frenz and Glover, 1996), it is possible that the chromatin bridges occur independently from the
S phase acceleration, or that improper chromosome segregation results in S phase acceleration. Interestingly, the expressivity of the chromatin bridge phenotype was not strongly correlated with the duration of S phase (data not shown). The rate of DNA replication in early cell cycles in *Xenopus* is dependent on a preceding mitotic step in which a TopoII-dependent shortening of origin spacing (Lemaitre et al., 2005). However, if loss of PhP causes loss of TopoII from chromatin, as suggested by their association at the PRE (Lupo et al., 2001), a delay in S phase, rather than an acceleration, would be expected. Though it can not be ruled out that problems with chromosome segregation cause problems during subsequent S phases, the fact that the timing of mitosis does not change in these mutants strongly suggests that *php* plays a role in S phase and not mitosis (Fig. 2-4).

**2.3.2 *php* cell cycle phenotypes are due to abrogation of the DNA damage checkpoint response**

I suggest that the mitotic defects of *ph* mutants arise due to loss of the DNA damage checkpoint response. Such a role for *php* is strongly suggested by the lack of cell cycle arrest in *ph*<sup>410</sup> mutants following irradiation (Fig. 2-6B). A role for *php* in the DNA damage checkpoint could explain the sporadic nature of the bridges in *php* mutants as well as their ability to resolve, if they are the result of paired sisters within damaged regions in nuclei that fail to arrest before entry into mitosis. The presence of S phase acceleration accompanied by chromosome bridging supports a role for *php* in the DNA damage checkpoint process (Fig. 1-1; 2-4).
It has not been conclusively demonstrated that the role of PhP in the DNA damage checkpoint is direct. The cell cycle defects in S phase length, chromosome segregation and cell cycle arrest may arise indirectly as a consequence of misregulation of PcG target genes. The occasional overlap of PhP with regions undergoing repair could be explained by damage to regions of PhP binding, and does not necessarily implicate PhP in this process.

Much evidence supports the idea that genes important in cell cycle regulation are PcG targets (Oktaba et al. 2008; Martinez et al., 2006; Jacobs et al., 1999a,b). However PcG regulation of genes required for the DNA damage response is unlikely to explain the loss of checkpoint response observed. Loss of function mutations in \(\text{php}\) should lead to derepression (i.e. gain of function) of genes important for the DNA damage response. As loss of function mutations in checkpoint genes have the same phenotype as \(\text{ph}\) mutations, it is very unlikely that PhP (or PRC1) directly regulates genes required for the DNA damage response, but an indirect effect is possible. If loss of PhP indirectly resulted in a loss of one the checkpoint genes tested, a strong genetic interaction with \(\text{ph}^{410}\) would be the expected result, though none was observed (Fig. 2-5).

Very little transcription occurs during cell cycles 10-13, so the association of PhP with chromatin at this time is unlikely to be related to its silencing function. This raises the possibility that PhP is involved directly in the DNA damage response. This role has been suggested for other PcG proteins (Chen et al., 2009; Hong et al., 2008; Bergink et al., 2006). Most notably, in mammals, a member of the PRC1 complex ubiquitinates histone variant H2A.z in response to damage (Bergink et al., 2006). However the modification appears to occur during a late repair step, as there is no accumulation of
ubiquitin in cells deficient in repair (Bergink et al., 2006), and any direct role of PhP in the checkpoint response most come early, before initiation of cell cycle arrest and repair. There was no genetic interaction with known checkpoint genes (Fig. 2-5), so the specific role of PhP in the checkpoint remains unknown.

PhP is present at some damaged regions but not all, therefore its role (if any) in the response to damage must be limited either spatially or temporally (Fig. 2-7). PhP could play a direct role only when damage occurs at specific loci. The obvious candidates for these loci are PcG targets. Similar to DNA replication, the epigenetic states of target genes must be maintained during repair of damaged regions. When damage occurs in regions of the genome occupied by PcG proteins, PhP could function in a locus specific checkpoint that promotes cell cycle arrest and repair of damaged DNA using a mechanism that promotes faithful propagation of both genetic and epigenetic information.

2.3.3 A role for *php* in the DNA damage checkpoint response does not rule out a role in other S phase processes

The acceleration of S phase which occurs in *ph*410 mutant embryos is likely due to loss of the damage checkpoint, but it is possible that *php* plays an additional roles during S phase, and that the cell cycle phenotypes in embryos are the result of cumulative defects. Some factors involved in DNA synthesis cause S phase acceleration, chromatin bridges, or both, when mutated (Prasanth et al., 2003; Lee et al., 2006; Park et al., 2007). Although no acceleration of DNA synthesis was observed in discs, it could be argued that PhP plays a role in DNA synthesis that is unique to embryos. However, chromatin
bridges were also observed in larval brains (O’Dor and Brock, unpublished), suggesting that the cell cycle role of Ph is not unique to embryos.

Crammed colocalizes with PCNA during S phase (Yamamoto et al., 1997) and PhP and PCNA colocalize during S phase (O’Dor and Brock, unpublished). Further, a mouse Ph homologue co-immunoprecipitates with the origin licensing factor Geminin, and tethering of Pc to a replication origin suppressed firing (Aggarwal and Calvi, 2004; Luo et al., 2004). However the S phase and mitotic phenotypes are unlikely to be due to interaction with Geminin or PCNA, since no genetic interaction of either with php were observed (Fig. 2-5). The primary function of PhP in S phase is in the DNA damage response, and a role in DNA synthesis is unlikely, however a third possibility that PhP functions in chromatin assembly has not been ruled out.

If PhP is involved in chromatin assembly following S phase, and mutants skip this assembly stage, this could explain the cell cycle as well as the silencing defects in php mutants. Mutations in the chromatin assembly factor acf1 also cause accelerations of syncytial S phase 13, but no change in the length mitosis, resulting in an overall shortening of the cell cycle (Fyodorov et al., 2004). Also similar to php mutants, mammalian ACF1 dissociates from chromosomes during mitosis (Fig. 2-2; Bozhenok et al., 2002). In Drosophila, Acf1 together with ISWI, forms the ATP-utilizing chromatin assembly and remodeling factor (ACF), which in vitro promotes assembly of nucleosomal arrays (Ito et al., 1997, 1999). Although acf1 has no homeotic defects on its own, double mutants of acf1 and ISWI or acf1 and the histone chaperone nap1 and have A5 to A4 anterior transformations (Fyodorov et al., 2004).
Given the roles of ACF and PhP in chromatin structure, S phase acceleration observed in mutants could be due to improper chromatin assembly following DNA synthesis. This could cause S phase acceleration simply by skipping the assembly step, or by increased accessibility of replication factors and origin use in subsequent cycles. Origin use is unregulated in embryonic cycles (Shinomiya and Ina, 1991; Sasaki et al., 1999), and tighter restrictions in later cycles might mask any effect of defects in chromatin structure on origin use. This effect could be generalized or restricted to PcG target genes.

This study has shown that the mitotic defects observed in syncytial embryos from mothers with mutations in \textit{php} are caused by an acceleration of S phase. Mutations in \textit{php} cause a loss of ability to arrest the cell cycle in response to DNA damage in larval discs, and this points to a direct role for PhP in the DNA damage checkpoint response, though an indirect effect has not been ruled out. The chromatin bridge and S phase phenotypes in embryos are likely due to loss of DNA damage checkpoints, although it is possible that defects in chromatin assembly following DNA synthesis could contribute.
CHAPTER 3  

Pds5 indirectly suppresses the cell cycle phenotypes of php and is required for PhP-dependent homeotic gene silencing
3.1 Introduction

The cell cycle phenotypes of php are consistent with a role for PhP in S phase, and a loss of checkpoints in these mutants. The role of PhP in the cell cycle would likely be mediated by interaction with cell cycle proteins. PhP colocalizes with PCNA during S phase (Yamamoto et al., 1997), a mouse Ph homolog Co-IPs with Geminin (Luo et al., 2004), however neither interact genetically with php, in regards to the cell cycle phenotype (Fig. 2-5).

Like PcG proteins, many cell cycle proteins have dual roles in transcription and the cell cycle (Zhang et al., 2000; Shareef et al., 2003; Hallson et al., 2008; Inoue et al., 2008; Dorsett et al., 2005; Rollins et al., 2004), so it is also possible that the interactions between PhP and PCNA or PhP and Geminin are important for the effects on PhP on transcription, but not for its cell cycle of inheritance role. This is likely true for Geminin, as two geminin alleles suppress the homeotic defects observed in php mutants (O’Dor and Brock, unpublished).

In a study to identify potential interactors of PhP, our lab previously reported the stable association of a 120 kDa protein in a complex with FLAG- HA- tagged PhP (Wang and Brock, 2003). The 120 KDa protein was sequenced, and found to correspond to an uncharacterized gene, CG17509 (Wang and Brock, unpublished). CG17509 has since been identified as the Drosophila homologue of the cohesin Pds5 (Dorsett et al., 2005). This is the first identification of a complex containing only one member of the cohesin complex. Cohesins have a well established role in mediating sister chromatid cohesion, but they also have less well-defined roles in transcription in many organisms. This
chapter will dissect the role of the cohesin *Pds5* in both the cell cycle and homeotic phenotypes of *php*.

Cohesins function to maintain sister chromatid cohesion from the time of their synthesis in S phase until their dissolution at anaphase. The cohesin complex contains Smc1, Smc3, Rad21 and Stromalin, and may form a ring that prevents separation of sister chromatids (Michaelis et al., 1997; Losada et al., 1998; Losada et al., 2000; Sumara et al., 2000; Vass et al., 2003). Nipped-B and Scc4 are required for the deposition of the cohesin complex onto chromatin prior to DNA synthesis (Ciosk et al., 2000; Arumugam et al., 2003; Gillespie and Hirano 2004; Seitan et al., 2006). During DNA synthesis, cohesion establishment requires the acetyl-transferase Eco (Uhlmann and Naysmith 1998; Skibbens et al., 1999; Toth et al., 1999; Milutinovich et al., 2007) possibly via interaction with the sliding clamp protein Proliferating Cell Nuclear Antigen (PCNA; Moldovan et al., 2006). This process could also depend on Pds5, as both *Pds5* and *Eco* mutants have a lost sister chromatid cohesion but not cohesin binding to chromosomes (Noble et al., 2006; Hartman et al., 2000; Tanaka et al., 2001; Dorsett et al., 2005; Losada et al., 2005; Guacci, 2007). In metaphase, the cohesin complex must be removed by cleavage of Rad21 by separase (Tomonaga et al., 2000; Uhlmann et al., 1999, 2000).

*Pds5* is required for sister chromatid cohesion in *Aspergillus nidulans*, *Sordaria*, budding yeast, fission yeast, humans, and *Drosophila* (van Heemst et al., 1999; 2001; Dorsett et al., 2005; Losada et al., 2005; Zhang et al., 2005). *Pds5* has been implicated in many things with respect to cohesin activity, most of which point to a role in regulating opening and closing of the cohesin ring. *Pds5* binds to the cohesin complex to the hinge
region, where it could mediate both stabilization and destabilization of ring architecture (McIntyre et al., 2007; Yoshimura et al., 2002, Sakai et al., 2003).

*Pds5* affects establishment of sister chromatid cohesion during DNA synthesis (Guacci et al., 2007; Rowland et al., 2009; Sutani et al., 2009; Noble et al., 2006). Cohesins are loaded onto DNA prior to replication but establishment of cohesion between sister chromatids occurs during or after DNA synthesis. In *Xenopus*, Pds5 is not required for cohesin binding to chromosomes, and depletion of Pds5 actually causes an increase in the amount of cohesin associated with chromosomes, but reduced cohesion between sister chromatids (Losada et al., 2005). A similar defect, “cohesin without cohesion”, occurs in *Eco* mutants (Guacci et al., 2007). The phenotypes of *Eco* and *Pds5* mutants, which show decreased cohesion without reduced association of the cohesin complex with chromosomes indicates that establishment of sister chromatid cohesion does not occur passively by replication fork progression through the cohesin ring. However, in the absence of both *Pds5* and *Eco*, cohesion, albeit diminished, still occurs (Tanaka et al., 2001; Rowland et al., 2009). Pds5 could function to prevent ring opening until it is required during or after replication fork progression. Eco, traveling with the replication fork, could antagonize Pds5, possibly via acetylation of core members of the cohesin complex, to actively establish cohesion (Rowland et al., 2009; Sutani et al., 2009; Moldovan et al., 2006).

Consistent with a role in regulating cohesin ring integrity, Pds5 has also been implicated in maintenance of sister chromatid cohesion prior to cleavage of Rad21 by separase, which causes sister chromatid segregation at anaphase. In yeast, Pds5 is sumoylated after mitotic entry by the SUMO isopeptidase Smt4. Stead et al (2003) have
proposed a model whereby Pds5 protects the cohesin complex from cleavage before anaphase, and sumoylation of Pds5 causes its dissociation, facilitating separase access and separation of sister chromatids.

Sister chromatid cohesion mediated by the cohesin complex is also required for double strand break repair (Sjogren and Naysmith, 2001). It is likely that association of sister chromatids in damaged regions facilitates repair by homologous recombination (Strom and Sjogren, 2007). The formation of double strand breaks promotes de novo assembly of cohesin complexes at the break site, as well as sister chromatid cohesion at the site which is stable through to anaphase (Strom et al., 2007; Unal et al., 2004).

Cohesins, including Eco and Pds5 are hypersensitive to DNA damaging agents, consistent with an inability to repair damage (Birkenbihl and Sabramani, 1992; Wang et al., 2002; Watrin and Peters, 2006).

Besides their role in sister chromatid cohesion and DNA damage repair, cohesins also function in transcription. In yeast, the cohesin complex also functions in gene silencing, and this is related to their function within the cell cycle (Huang and Moazed, 2006). In *Drosophila*, however, it appears that cohesins function in transcriptional activation. The cohesin loading factors nipped-B and Stromalin, and the core cohesin Smc1, are localized to the coding regions of active genes and overlap with each other, as well as with RNA Polymerase II (Misulovin et al., 2007). We identified the core cohesin Rad21 as trxG member vtd (Hallson et al., 2008). Although most studies have identified multiple cohesin subunits affecting or binding to common targets (Dorsett et al., 2005; Rollins et al., 2004), the effects of cohesins on transcription in *Drosophila* is likely separate from their cell cycle role because Smc1 and Stromalin are required for
expression of the *Ecdysone receptor* in non-dividing cells (Schuldiner et al., 2008; Misulovin et al., 2007). Whether cohesins function to regulate gene expression as a cohesin complex is unknown.

Because cohesins, as well as *polyhomeotic-proximal (php)*, function in both S phase and transcriptional regulation, the goal of this chapter will be to determine whether the unique complex containing both *php* and *Pds5* functions in the regulation of the cell cycle or in regulation of transcription. The two proteins directly associate, and therefore a synergistic genetic interaction between the two would suggest that they cooperate within a given function. There are two *Pds5* alleles available in *Drosophila* with which to test this hypothesis. The *Drosophila Pds5[E6]* allele is an N-terminal truncation of Pds5 in which cohesin binding to polytene chromosomes is dramatically depleted (Dorsett et al., 2005). The *Pds5[E3]* allele is a deletion of the entire *Pds5* coding region, but it does not affect cohesin binding (Dorsett et al., 2005). This difference could be explained by the loss of a regulatory domain in the truncation mutant, and is consistent with the model that Pds5 regulates cohesin ring stabilization. No other cohesins were identified in complex with PhP and Pds5 (Wang and Brock, 2003). The different effect of the two alleles, as well as the availability of other *Drosophila* cohesin mutants allows for the determination of whether the function of PhP in this complex is unique, or involves other members of the cohesin complex.
3.2 Results

3.2.1 Pds5 associates with endogenous PhP

In order to verify and determine the significance of the observation that PhP interacts with Pds5, an antibody against Pds5 was made and purified. A western blot on SL2 cell nuclear extracts with pre-immune serum, crude serum and purified IgG is shown in Fig. 3-1A. A band of the expected size of 120 kDa (Wang and Brock, 2003) is present in the lane probed with crude serum, and IgG, but not in the lane probed with pre-immune serum (Figure 1A; Wang and Brock, 2003). Purification of the IgG component of the rabbit serum improves the signal dramatically (Fig. 3-1A).

To determine whether endogenous PhP associates with Pds5, nuclear and cytoplasmic extracts were prepared from Drosophila SL2 tissue culture cells, and 50 µg of each were immuno-precipitated with an antibody that recognizes only the proximal form of Polyhomeotic (Hodgson et al., 1997). The material eluted from the beads was separated electrophoretically on a 7.5% SDS-acrylamide gel, transferred to nitrocellulose and probed with an antibody raised against Pds5 (Fig. 3-1). A band of the predicted and previously observed size of 120 kDa (Wang an Brock, 2003) was observed from the nuclear extract, confirming that endogenous as well as FLAG- HA- tagged PhP (Wang and Brock, 2003) associates with Pds5 in vivo.
Figure 3-1. Pds5 co-immunoprecipitates with endogenous PhP. Nuclear extracts were made from *Drosophila SL2* cells using the Pierce Nuclear Extract kit. (A) 75 µg of extract was run on a 7.5% SDS gel. Proteins were transferred to nitrocellulose membranes and probed with pre-immune or crude antisera, or purified IgG at a dilution of 1/1000. IgG purification improves the signal from a band that runs at approximately 120 kDa, the predicted molecular weight of PDS5 (asterisk). There is no band running at this level in the blot probed with pre-immune antisera. (B) 50µg of nuclear extract was immunoprecipitated with an antibody specific to PhP, washed, and run on a 7.5% SDS gel. Proteins were transferred to a nitrocellulose membrane and probed with purified IgG at a dilution of 1/100. A band of the expected size is shown (asterisk).


3.2.2 Different cohesins have different embryonic phenotypes during cleavage divisions

Pds5 was the only cohesin identified as a member of the complex containing PhP (Wang and Brock, 2003), and if this complex has a unique cell cycle function during S phase checkpoints that is different from the function of the cohesin complex, Pds5 mutants should have unique cell cycle phenotypes from the rest of the cohesins.

Embryos from mothers mutant for the core cohesin and trxG member Rad21 have embryonic defects consistent with problems in sister chromatid cohesion (Fig. 3-2A). Mitosis occurs as a wave beginning at each pole, so that more central nuclei are at an earlier mitotic stage than are nuclei at the poles. In embryos derived from wild-type mothers, most nuclei exhibit tight alignment of chromosomes at the metaphase plate in mitosis 13. Close to the poles, a small number of nuclei in the metaphase-anaphase transition can be recognized because of their looser alignment. Nuclei at the poles are in anaphase or telophase, as shown by the separated chromosomes. In embryos derived from mothers carrying the Rad21 γ-26–6 allele, there is an increase in the number of nuclei exhibiting the loose alignment of chromosomes seen at the metaphase to anaphase transition, and the chromosomes appear to be less condensed (Fig. 3-2A). This phenotype was never observed in wild-type embryos in this or in previous studies (O’Dor et al., 2006). No mitotic abnormalities were observed before mitosis 11, and these embryos subsequently achieve normal metaphase figures with proper condensation and alignment (data not shown).
Figure 3-2. Cohesins have chromatin bridges at anaphase and telophase, but segregation defects are unique to Rad21. (A) Embryos are shown in which nuclei closer to the pole have entered anaphase, but nuclei closer to the center are in metaphase. In embryos from mothers carrying the γ-26-6 allele of Rad21, a loosened stage, resembling a stage between metaphase and anaphase, is observed that is not present in Wild Type (OR) or other strains. (B) Examples of anaphase and telophase bridges in embryos of the indicated genotypes. TM3 is a third chromosome balancer, SM6 and CyO are second chromosome balancers.
Other than the segregation defects of *Rad21* mutants, all cohesin mutants examined, including *Pds5*, have chromatin bridges at anaphase and telophase. Both of the core cohesins examined, *Rad21* and *Smc1*, displayed chromatin bridges at anaphase and telophase (Fig. 3-2B). No cell cycle phenotypes were observed in *Smc1* mutants other than chromatin bridges at anaphase and telophase indicating the segregation defect is not common to the core cohesins (Fig. 3-2B). Bridges were also observed in mutants for the cohesin loading factor *Nipped-B* (Fig. 3-2B). The phenotype of *Pds5* mutants in syncytial stages is not unique, and does not rule out an S phase role for Pds5.

### 3.2.3 *Pds5* suppresses the chromatin bridge phenotype of *php*

Since Pds5 plays a role in sister chromatid dissolution during mitosis, but also may play a role in cohesin establishment during S phase, as well as DNA damage repair, the chromatin bridge phenotype of *ph410* mutants could be the result of the interaction between PhP and Pds5, which would be indicated by a synergistic genetic interaction between *php* and *Pds5*.

First it was necessary to determine the penetrance of the chromatin bridge phenotype in *Pds5* mutants during syncytial cycles. In order to determine this, I examined embryos derived from *Pds5* mutant mothers and calculated the frequency of severe chromatin bridges in these embryos. In embryos from single heterozygous *Pds5[E3]* and *Pds5[E6]* mutant mothers, 100% and 50% of embryos, respectively, have severe chromatin bridges (>25% of nuclei bridged) at telophase (Fig. 3-3), similar to the
Figure 3-3. \textit{Pds5} suppresses the chromatin bridge phenotypes of \textit{php} and \textit{Pc}. 0-2.5 hr embryos from mothers of the specified genotype were collected and stained with DAPI to visualize DNA. Embryos at anaphase or telophase were scored with regard to bridges between sister chromatids. The number of embryos in which greater than 25% of nuclei were bridged out of the total population of embryos (n=10-12) was then determined for each genotype. No embryos in which greater than 25% of nuclei were bridged were observed from mothers of genotypes \textit{ph410},w/+; \textit{Pds5}[E3]/+, or \textit{ph410},w/+; \textit{Pds5}[E6]/+, \textit{Pc15}/+, \textit{Pc15}/+, compared with 40% in balancer siblings. Similarly, no embryos in which greater than 25% of nuclei were bridged were observed from mothers of genotypes \textit{Pds5}[E3]/+; \textit{Pc15}/+ compared with 20% in balancer (SM6) siblings. See Materials and Methods for crossing schemes.
phenotype of the *php* mutant allele *ph*\(^{410}\), in which 40% of embryos from mutant mothers have severe chromatin bridges (Fig. 3-3).

In order to determine whether the mitotic phenotypes of *ph*\(^{410}\) are related to the interaction between PhP and Pds5, I looked at embryos derived from double heterozygous mothers. If PhP and Pds5 function together, a synergistic interaction would be expected. Surprisingly, in embryos from mothers of the genotypes *ph*\(^{410}\)/yw; *Pds5*[E3]/+ and *ph*\(^{410}\)/yw; *Pds5*[E6]/+, no telophase nuclei were observed to have severe chromatin bridging (Fig. 3-3). Both *Pds5* alleles completely suppress the chromatin bridge phenotype of *php* (Fig. 3-3).

In order to determine whether suppression of the cell cycle phenotype was common to any other members of the complex containing PhP and Pds5, I determined the frequency of chromatin bridging in double mutants of *Pc* and *Pds5*. In embryos derived from double heterozygous mutant mothers of *Pc* and *Pds5*, 0% of embryos at telophase had severe chromatin bridges, compared with 20% of balancer siblings (Fig. 3-3). *Pds5* also suppresses the chromatin bridge phenotypes of *Pc*.

**3.2.4 S phase delay during cycle 13 occurs in Pds5 mutants, but is suppressed by php**

The chromatin bridge phenotype of *php* is due to acceleration of S phase caused by loss of DNA damage checkpoints. If the complex containing Pds5 and PhP functions in the cell cycle during S phase, *Pds5* mutants should also have defects in S phase. I
therefore examined the cell cycle timing of Pds5 mutants to look for defect during S phase. Interestingly, in 2 out of 5 Pds5 mutant embryos, S phase 13 was extended to 24 minutes (Figure 3-4). An S phase duration of greater than 17 minutes was never observed in embryos of any other genotype (data not shown), and this resulted in an average timing for interphase 13 that is longer in Pds5 mutants than in any other genotype (Fig. 3-5). S phase delay in syncytial embryos is consistent with DNA damage repair, and could indicate either an increase in DNA damage, or a decrease in DNA damage repair in these mutants.

Interestingly, in double mutants of php and Pds5, no embryos had an interphase 13 duration of greater than 17 minutes (data not shown), and the average duration of this stage was comparable to the average duration in control embryos (Fig 3-5). This indicates that without php, the lack of Pds5 does not cause a delay in mitotic entry. This is consistent with a role for php in DNA damage checkpoint activation, as increased DNA damage or lack of damage repair would be ignored in checkpoint mutants.

3.2.5 S phase phenotype of php is suppressed by mutation in Pds5

Because Pds5 suppresses the mitotic phenotype of php, and this phenotype is likely due to an acceleration during S phase, I wondered if Pds5 mutations would also suppress the S phase acceleration phenotype of php. Live images were collected of embryos double heterozygous mothers using the H2A.z-GFP to visualize cell cycle progression. Cell cycle timing was determined as described previously. In double heterozygotes, S phase timing is restored to the wild type duration (Fig 3-5). Mitotic
Figure 3-4. *Pds5* mutant embryos have a delay at S phase 13. Cell cycle timing for the 13th embryonic S phase in Control (w/w; +/+ ) and *Pds5[E3]* heterozygotes. Two out of six *Pds5* mutant embryos had significant delays at S phase 13 such that this stage lasted longer than 20 minutes.
**Figure 3-5. S phase acceleration is suppressed by Pds5.** Confocal images of syncytial cell divisions in *Drosophila* embryos were taken every 60 seconds using a fly strain expressing histone H2A.Z fused to GFP, and cell cycle staging was determined by chromatin condensation. Cell cycle timing was determined from 5 nuclei per embryo, and 5 embryos per genotype were used to determine the mean. Error bars represent standard error of the mean. For simplification, genotypes do not include the H2A.z-GFP transgene. The durations of S phase and mitosis at all stages in embryos from mothers of genotype *ph410,w/w; Pds5[E3]/+* (blue bars) are identical to control embryos (black bars). Checkpoint activation extends the duration of S phase 13 in embryos from mothers of genotype *w/w; Pds5[E3]* (red bars), but delay at S phase 13 does not occur in double heterozygotes (blue bars).
timing also remained similar to the wild type duration (Fig 3-5). This data indicates that, in addition to the mitotic phenotype, *Pds5* mutations suppress the S phase phenotype of *ph*\(^{410}\), but it does not indicate whether the suppression is direct or indirect.

**3.2.6 Mitotic phenotypes of *php* are suppressed by the cohesin loading factor *Eco* but not by core cohesin subunits**

Because the suppression of the chromatin bridge phenotype could be direct or indirect, I wondered if it was unique to *Pds5*, or if other cohesins not identified as part of the complex containing PhP and Pc would also suppress the chromatin bridge phenotype.

Embryos from mothers with mutations in core cohesin members *Smc1* fail to suppress the chromatin bridge phenotype of *php*, but slightly enhance the phenotype to approximately double that observed in *ph*\(^{410}\) heterozygotes (Fig 3-6). The suppression of the chromatin bridge phenotype by *Pds5* is not related to its global role within the cohesin complex because the core cohesin *Smc1* does not suppress *php*. The frequency of severe chromatin bridging in embryos with mutations in *Smc1* is identical to the frequency observed in embryos with mutations in *ph*\(^{410}\) (Fig. 3-6). A doubling of the chromatin bridge phenotype of *php* due to *Smc1* is observed. Therefore the enhancement of *ph*\(^{410}\) by *Smc1* is additive (Fig 3-6). Both *Pds5* and *Smc1* have chromatin bridges at anaphase and telophase, but interact differently with *php*. Therefore, there is no correlation with the mitotic phenotypes of cohesins and their suppression of *php*.

Mutations in separase also have no effect on the chromatin bridge phenotype of *ph*\(^{410}\), indicating the suppression effect is independent from the mitotic role of *Pds5* (Fig.
Figure 3-6. Other cohesins do not suppress the chromosome bridge phenotype of ph<sup>p</sup> with the exception of the cohesin establishment factor eco. 0.25 hr embryos from mothers of the specified genotype were collected and stained with DAPI to visualize DNA. Embryos at anaphase or telophase were scored with regard to bridges between sister chromatids. The number of embryos in which greater than 25% of nuclei were bridged out of the total population of embryos (n=10-12) was then determined for each genotype. Only embryos from mothers of genotype ph410<sub>W</sub>+/+; eco/+ did not have high severity chromatin bridging. TM3 is a third chromosome balancer. See Materials and Methods for crossing schemes.
3-6). Mutations in the cohesin loading factor *nipped-B* also do not synergistically affect the chromatin bridge phenotypes of *php*, as the frequency of bridging is only about double that seen in *ph*<sup>410</sup> heterozygotes (Fig. 3-6). Pds5 antagonizes the cohesion establishment function of Eco (Tanaka et al., 2001; Sutani et al., 2009; Rowland et al., 2009), and interestingly, double mutants of *php* and *eco* do not have chromosome bridges at telophase (Fig. 3-6). *Pds5* and *eco*, but not other cohesins, suppress the cell cycle phenotype of *php*, which indicates that suppression is not unique to members of the complex containing PhP and Pds5, and therefore could be indirect effect of the S phase role of Pds5 and Eco.

### 3.2.7 Loss of *Pds5* in imaginal discs delays mitotic entry following DNA damage.

If the suppression of the cell cycle phenotypes of *php* by *Pds5* is direct, Pds5 must function to antagonize DNA damage checkpoint activation by PhP, and *Pds5* mutants should have an increased checkpoint response in response to DNA damage. In order to determine the effect of DNA damage on *Pds5* mutants, *Pds5* mutant larvae were exposed to 5 Gy of radiation, and after 2 hrs wing discs were dissected and stained with an antibody against the mitotic marker phosphorylated serine 28 of histone H3.

Irradiation of wild-type larvae results in checkpoint activation and cell cycle delay (Laurencon et al., 2003). Loss of *Pds5* results in an increase in the number of delayed cells, resulting in none or very few mitotic cells in these discs following irradiation (Fig. 3-7A). This indicates that *Pds5* mutants have an increase in the duration of cell cycle arrest following DNA damage. This could be caused by an increase in the DNA damage
Figure 3-7. *Pds5* mutants have increased cell cycle arrest following DNA damage. (A) The number of mitotic nuclei was determined by staining with an antibody specific to mitotic marker phosphorylated serine 28 of Histone H3 (AbCam) after irradiation by a 60Co source. Following irradiation, Discs from *Pds5[E3]* mutants have more cells arrested before entry into mitosis than do discs from OR. Error bars represent Standard Deviation (SD). (B) Increase in arrest following DNA damage could be due to antagonism of the DNA damage checkpoint by Pds5, which would suggest that it against directly with PhP. (C) Alternatively, Pds5 could function downstream of Ph to mediate damage repair.
checkpoint response, consistent with a role for Pds5 in directly inhibiting checkpoint activation by PhP (Fig. 3-7B), however the known role of cohesins in DNA damage repair would cause the same phenotype (Fig. 3-7C).

3.2.8 Alleviation of the checkpoint response in discs from php mutants is epistatic to the radiation sensitivity in discs from Pds5 mutants

If Pds5 functions to prevent DNA damage checkpoint activation, Pds5 mutations should suppress the lack of damage checkpoint activation that occurs in php mutants (Fig. 3-6B). However, if Pds5 functions in DNA damage repair, downstream of checkpoint activation by PhP, php mutations will be epistatic to Pds5 (Fig. 3-7C). In order to determine whether Pds5 functions directly to antagonize PhP, or functions indirectly downstream of PhP, I examined the response to DNA damage in php Pds5 double mutants. Double mutant larvae were exposed to 5 Gy of radiation, and the number of mitotic cells two hours later was determined as previously described. Surprisingly, Pds5 mutations do not suppress the loss of checkpoints observed in php mutants. Discs from php mutants are indistinguishable from discs of double mutant flies (Fig 3-8). This indicates that in terms of the cellular DNA damage response, php is epistatic to Pds5 (Fig. 3-8). The increase in checkpoint delay in Pds5 mutants is masked by the loss of the delay due to mutation of php (Fig. 3-8), and Pds5 functions downstream of PhP in the cell cycle (Fig. 3-7C).
Figure 3-8. *php* is epistatic to *Pds5*. The number of mitotic nuclei was determined by staining with an antibody specific to mitotic maker phosphorylated serine 28 of Histone H3 (AbCam) after irradiation by a 60Co source. The number of mitotic cells in double mutant discs is very similar to the number of mitotic cells in single mutants of *php* (compare columns two and four).
3.2.9 *Pds5* is required for *php* dependent silencing

An indirect genetic interaction between *php* and *Pds5* in the cell cycle means that the complex containing the two gene products does not play a role in the cell cycle, but it could function in transcriptional silencing. Posterior transformations result from de-repression of *Hox* genes in anterior segments. In order to determine whether *Pds5* mutations cause de-repression of *Hox* genes, heterozygous mutant flies were examined for the presence of posterior transformations. No posterior transformations were observed in heterozygous flies with either allele of *Pds5* (data not shown), however both mutations are homozygous lethal (Dorsett et al., 2005), and therefore it could not be determined whether posterior transformations were present in null mutant flies.

Flies mutant singly for *php* in the genetic background of *Pds5* mutants (balancer siblings) have posterior transformations at a penetrance of less than 20% (Fig. 3-9). To determine whether *Pds5* plays a role in PhP mediated silencing of *Hox* genes, the expressivity of posterior transformations were calculated in adult males doubly mutant for *php* and each of the two alleles of *Pds5*. Both *Pds5* alleles strongly enhance the posterior transformations caused by *php* mutations, as close to 100% of double mutant males had posterior transformations (Fig. 3-9). This synergistic interaction suggests that *Pds5* cooperates with PhP to maintain silencing of *Hox* genes.

If the complex containing PhP and *Pds5* is required for silencing, mutations in other cohesins should not enhance the homeotic defects seen in *php* mutants. Consistent with a unique role in silencing, *Pds5* is the only cohesin to strongly enhance the homeotic
Figure 3-9. *Pds5* enhances the homeotic defects of *php*. Abdominal pigmentation was used to indicate posterior transformation of abdominal segment 4 (A4) to abdominal segment 5 (A5) in male flies. Posterior transformations occur in double mutants for *php* and both alleles of *Pds5* at much higher penetrance than in balancer siblings (first four columns). Other cohesins do not significantly enhance the penetrance of posterior transformations. Double mutants of *Rad21* and *php* have a lower penetrance of posterior transformation compared with balancer siblings (last two columns).
defects of *php* (Fig. 3-9). Consistent with the trxG role in activation of *Hox* genes, *Rad21* suppresses the homeotic phenotypes of *php* mutants (fig. 3-9).

### 3.2.10 Pds5 binds to regulatory regions and the promoter of the *Hox* gene *Ubx*

Ivana Komljenovic performed the experiments described in this section.

If Pds5 directly cooperates with PhP to maintain *Hox* gene silencing, it should bind directly to *Hox* genes. ChIP experiments were performed using primers to the promoter and PcG Response Elements (PREs), which mediate PcG dependent silencing, of the *Hox* gene *Ubx* (Fig. 3-10A). Pds5 was significantly enriched at some sites within the PRE, but not all sites, indicating binding to these regions is selective (Fig. 3-10B). Further, Pds5 binds to specific regions of the promoter (Fig. 3-10B), which has also been observed for PcG proteins, including PhP (Hodgson and Brock, unpublished). Therefore, Pds5 is directly localized to the *Hox* gene *Ubx* in the region of the *bxd* maintenance element and promoter.
Figure 3-10. Pds5 localizes to regulatory elements and the promoter of Ubx. (A) Primer map showing the locations of the primers used in B. Primers L1 through L8 are within the *bxd* Maintenance Element. Their locations are shown in detail in the lower figure. U1 through U4 are upstream of or within the promoter of *Ubx*. (B) Results of ChIP with an antibody specific to Pds5. Pds5 is specifically enriched over the IgG control in the L3, L5, and L7 regions of the *bxd* maintenance element, and U1 and U4 regions of the promoter.
3.3 Discussion

3.3.1 *Pds5* does not have unique cell cycle phenotypes during cleavage divisions

*Rad21* mutants are the only cohesin mutants examined that have a mitotic phenotype consistent with defects in sister chromatin cohesins (Hallson et al., 2008; Fig. 3-2). The absence of this phenotype in other cohesin mutants is somewhat surprising given that the initial characterization of cohesins was based on loss of sister chromatid cohesion (Michaelis et al., 1997). Loss of *Pds5* results in premature dissolution of sister chromatids in larval brains (Dorsett et al., 2005). All embryos examined were heterozygous for the mutations indicated, and therefore presumably had enough protein product present for proper cohesion, and complete loss of sister chromatid cohesion would be lethal. Severe depletion of *Rad21* in embryos injected with Drad21 dsRNA show multiple severe defects such as polyploidy, aneuploidy and chromosome breakage (Vass et al., 2003).

The segregation defect observed in *Rad21* heterozygotes may not be present in other cohesin mutants because of the unique role *Rad21* plays in the cohesin complex. In yeast, *Xenopus*, and human, *Rad21* homologues are cleaved to relieve sister chromatid cohesion at anaphase (Uhlmann et al., 1999; Tomonaga et al., 2000; Hauf et al., 2001). The *Rad21* allele that gives rise to the segregation phenotype in embryos contains a mutated splice acceptor site: this is predicted to give an altered mRNA, resulting in a truncated rad21 protein, which would be unable to interact with its N-terminus to form a cohesin ring structure (Hallson et al., 2008). This could result in the assembly of unstable cohesin rings or fewer functional rings, which fail to maintain sister chromatid cohesion.
under the tension of the mitotic spindle. These more severe defects are likely due to cumulative mitotic failures resulting from depletion of Drad21/Vtd below levels present in embryos derived from heterozygous Rad21 mothers.

Mutations in Pds5 lead to severe mitotic defects. (Fig. 3-2, 3-3). These phenotypes are also observed in embryos with mutations in other cohesin subunits (Fig. 3-2). The appearance of chromosome bridging at anaphase and telophase in a variety of cohesin mutants including Pds5 in a variety of organisms is not uncommon upon close inspection of the literature (Tomonaga et al., 2000; Toyoda et al., 2006; Tatebayashi et al., 1998; Losada et al., 2005). These phenotypes could be to disruption of events occurring downstream of cohesion, such as chromosome condensation (Hartman et al., 2000). This phenotype could also be indicative of other as yet unidentified cell cycle roles for individual cohesins or the cohesin complex. Chromatin bridges are also consistent with the role of cohesins in DNA damage repair. The unresolved chromatid arms could be sisters associated in regions still undergoing damage repair. The presence of this phenotype in all cohesins examined indicates that Pds5 does not have a unique role during these cell cycles (Fig. 3-2).

3.3.2 Suppression of the embryonic cell cycle phenotypes of php by Pds5 is indirect

Mutations in Pds5 suppress both of the embryonic cell cycle phenotypes of php, and the association of the two proteins suggests that this could be due to direct antagonism of the same cell cycle process (Fig. 3-1; 3-3; 3-5). However, the epistatic relationship between the two in the DNA damage response in imaginal discs supports the
demonstration in chapter 2 that \emph{pds5} is required for the DNA damage response and does not function in DNA damage checkpoints. These data, as well as the suppression of the chromatin bridge phenotype by \emph{Eco}, which is not a member of the complex containing PhP, indicates that the suppression in indirect (Fig. 3-6).

Loss of DNA damage checkpoints can cause accelerations in the S phase rates in syncytial cycles as well as chromatin bridges at anaphase and telophase (Sibon et al., 1999; Fogarty et al., 1997; Stumpff et al., 2004), and, as discussed in Chapter 2, the loss of checkpoints in \emph{php} mutants likely contributes to the presence of both phenotypes. Depletion of \emph{Pds5} could slow progression through certain processes during S phase to allow for DNA damage checkpoint activation, and therefore indirectly suppress both the S phase acceleration and chromatin bridge phenotypes of \emph{php}. The fact that both \emph{Eco} and \emph{Pds5} suppresses the chromatin bridge phenotype is consistent with suppression being indirect because \emph{Eco} functions in the same process of S phase as \emph{Pds5} (Tanaka et al., 2001; Rowland et al., 2009; Sutani et al., 2009). Furthermore, the suppression of both of the embryonic phenotypes of \emph{php} supports the primary conclusion of Chapter 2, namely that the primary role of PhP is in S phase, and defects during S phase give rise to the chromatin bridges observed during mitosis.

\subsection*{3.3.3 The epistatic relationship between \emph{php} and \emph{Pds5} in the DNA damage response supports a role for \emph{php} in the DNA damage checkpoint}

The decrease in the number of mitotic cells in imaginal discs from \emph{Pds5} mutants following DNA damage is consistent with the role of cohesins in repair (Fig. 3-7).
Damaged nuclei delay entry into mitosis until repair can be completed, and less Pds5 will result in a reduced ability to repair damage and therefore an increase in cell cycle delay.

Significantly, double mutants of php and Pds5 do not arrest before entry into mitosis, and php is epistatic to Pds5 (Fig. 3-8). This supports a role for PhP in the DNA damage checkpoint response because abrogation of the checkpoint results in loss of cell cycle delay, but an inability to initiate repair of damaged regions. This would have the effect of skipping the stage at which Pds5 is required, and masking of the cell cycle delay phenotype. This occurs at the expense of not repairing damaged DNA, which would predictably result in increased lethality in these mutants following exposure to damage agents.

A loss of checkpoints preventing delay in repair due to loss of Pds5 is also observed in syncytial cycles during S phase. Single Pds5 mutants have a delay during S phase 13, consistent with the presence of DNA damage, but this delay is lost in double mutants (Fig. 3-4, 3-5). This supports a role for Pds5 in the repair of DNA damage, and a role for php upstream of that role, because in double mutants the lack of checkpoint activation allows for DNA damage to be overlooked. Cell cycle delay in Pds5 syncytial cycles occurs during the last syncytial S phase (Fig. 3-4, 3-5). This is likely due to damage accumulating, due to things such as unresolved bridges during prior divisions, such that a significant delay is only observed during the later cycle. In the earlier syncytial cycles in the absence of DNA damage, the S phase role of Pds5 is more prominent than its role in repair, and it suppresses the phenotypes of php.
3.3.4 The complex containing PhP and Pds5 is involved in stable silencing of Hox genes

Other proteins identified as part of the complex containing PhP, Pc, and Pds5 include the chaperones Hsc70-4 and Droj2 (Wang and Brock 2003). Mutations in both of the genes encoding these proteins result in enhancement of the homeotic phenotypes of both Ph and Pc, supporting a role for this complex in PcG silencing of Hox genes (Wang and Brock, 2003). The fact the mutations in Pds5 strongly enhance the homeotic defects of php further supports this idea (Fig. 3-9). Further, Pds5 directly localizes to the Ubx promoter and regulatory regions, supporting a direct role in silencing (Fig. 3-10).

No other cohesins strongly enhanced the homeotic phenotypes of php, or were identified as members of this complex (Fig. 3-9; Wang and Brock, 2003). This is the first demonstration of a possible role for Pds5 outside of its interaction with cohesins that I am aware of. In human cells, only 20-30% of cellular cohesins are associated with Pds5 and just over half of cellular Pds5 is associated with cohesins (Sumara et al., 2000; Losada et al., 2005), therefore association of Pds5 with other protein complexes could occur.

This is also the first demonstration of a direct role for a cohesin in Homeotic gene silencing in Drosophila. Most evidence, including our identification of Rad21 as a trxG gene, points to a role for cohesins in activation of gene expression in Drosophila (Hallson et al., 2008; Misulovin et al., 2007; Schuldiner et al., 2008). There is some evidence for cohesins functioning directly in gene silencing in Drosophila. The core cohesin and trxG protein Rad21 as well as the core cohesin Smc1 and Stromalin bind to regulatory elements and the coding region of the cut gene, and reductions in them result in increased
cut expression (Dorsett et al., 2005). Loss of the other loading factor Nipped –B, which also binds to the coding region and regulatory elements of the cut gene results in activation of cut gene expression (Rollins et al., 2004).

The silencing role of cohesins at the cut locus is likely separate from the silencing role of Pds5 at Ubx because many cohesin complex members are involved in cut silencing. The two Pds5 alleles behave differently with regards to cut gene expression. The Pds6[E6] allele, which encodes an N-terminal truncated protein causes a loss of cohesin binding to polytene chromosomes, and an increase in cut gene expression (Dorsett et al., 2005). However the null mutant Pds5[E3] causes a small decrease in cut gene expression (Dorsett et al., 2005). This difference is likely due to the role of other cohesins in cut gene expression. Consistent with the fact that the effects of Pds5 on Hox gene expression are separate from its role as a cohesin, the two Pds5 alleles are virtually identical in their enhancement of homeotic defects (Fig. 3-9).

It is unknown how the cohesin complex, or how Pds5 in complex with PhP and Pc, exert their effects on transcription. Nipped-B mutations increase the inhibitory effect of a gypsy transposon inserted in the Ubx gene, which acts as an insulator, blocking activation of Ubx by remote enhancer. This suggests that Nipped-B normally facilitates long-range chromosomal interactions (Rollins et al., 1999; Gause et al., 2008). In mammals, cohesins are recruited to boundary elements (Parelho et al., 2008; Bowers et al., 2009; Mishiro et al., 2009). Both boundary elements and long-range enhancer-promoter interactions are important for PcG mediated silencing (Gellon and McGnnis, 1998). Recently it has been shown in yeast that methylation of H3K9 is lost during S phase, but that the cohesin recruitment caused by the methylation mark is stable to DNA
synthesis (Gullerova and Proudfoot, 2008). Cohesins could function at boundary elements, facilitate long-range chromosomal interactions, or even function as an epigenetic mark.

These potential mechanisms could explain the effects of the cohesin complex on gene expression, however it is unknown how Pds5 functions outside of the complex to mediate silencing of Hox genes. The role of cohesins in gene expression is conserved from yeast to humans (Jessberger, 2003), and it is possible that individual members of the complex, such as Pds5, have diverged in their function, and their effects on transcription are independent from their effects on the cell cycle. The final chapter will discuss possible ways in which both the function of PhP in the DNA damage checkpoint and the role of cohesins in transcription could participate in epigenetic inheritance mediated by the Polycomb Group.
CHAPTER 4 Discussion
4.1 The Role of the DNA Damage Checkpoint in Epigenetic Inheritance

*Polyhomeotic* plays a role in the damage checkpoint that is likely direct. Cell cycle arrest is lost in response to DNA damage, suggesting a loss of checkpoints. For reasons discussed in Chapter 2, it is likely that this effect is direct, however PcG proteins do regulate cell cycle genes (Oktaba et al. 2008; Martinez et al., 2006; Jacobs et al., 1999a). Neither members of the ATM nor the ATR pathway affect the frequency of bridges in *php* mutants, suggesting PhP does not directly or indirectly function in those pathways. If the role of PhP in the checkpoint response is indirect, it will be interesting to determine the target genes misregulated in these mutants. However, as mentioned in Chapter 2, misregulation causing loss of a checkpoint protein would have to be indirectly mediated by the PcG, whose typical phenotype is derepression.

It is conceptually difficult to study the function of checkpoint genes in the absence of damage, because even in the absence of an external cause, there are hundreds of damage events in each cell cycle (Takada et al., 2007). The progressive developmental delay that occurs during cleavage cycles cumulates in the mid-blastula transition (MBT) after division 13, at which point zygotic transcription begins. Checkpoint mutants *grapes/chk1* and *mei-41/ATR* have accelerated S phases, and fail to initiate the MBT (Sibon et al., 1999; Sibon et al., 1997). Cell cycle delay itself is not necessary for the MBT or zygotic transcription, likely due to the presence of independent pathways that promote the degradation of maternal transcripts (Takada et al., 2007; Bashirullah et al., 1999). It has therefore been suggested that the function of the checkpoint genes in syncytial embryos is simply to respond to DNA damage, and that they are not required for unperturbed development. However, there is evidence from many organisms that
checkpoint mutations cause developmental defects and embryonic lethality (Conn et al., 2004; Kalogeropoulos et al., 2004; Liu et al., 2000; Takai et al., 2000; Petrus et al., 2004; Shimuta et al., 2002). The mammalian grapes homolog chk1 plays a role in normal cell cycles to delay the onset of mitosis via Cdk1/Cyclin B (Schmitt et al., 2006; Zachos and Gillespie, 2007). Therefore an additional role for checkpoint genes cannot be ruled out.

One major goal for the future is the elucidation of if and how this cell cycle role for PhP is related to its role in epigenetic inheritance. It is possible that during Drosophila syncytial cycles, the DNA damage checkpoint functions in the absence of damage, to induce a developmentally controlled delay in the cell cycle. This suggests that checkpoint proteins could play a role in normal development to coordinate proliferation with differentiation. As discussed in Chapter 1, preventing proliferation of differentiated cells could protect them from the challenges to maintenance that occur during S phase and mitosis.

Checkpoints could also play a very different role in epigenetic maintenance as effectors of an epigenetic checkpoint. Such a checkpoint, similar to the DNA damage checkpoint, would ensure that epigenetic information is faithfully propagated. Certain PcG proteins could function as detectors or as signals for loss of maintenance, possibly as a difference in gene expression patterns between homologs or sisters, with cell cycle arrest mediated by the DNA damage checkpoint. However, ph is not a good candidate for this role, as ph mutants failed to arrest cell cycles in response to DNA damage, suggesting that Ph functions within the DNA damage checkpoint itself, and doesn’t simply utilize it for arrest in response to loss of epigenetic silencing. The mouse PcG protein RYBP stabilizes p53, which leads to cell cycle arrest or apoptosis (Chen et al.,
2009). It has not been tested whether loss of RYBP causes an increased sensitivity to DNA damage, but a lack of sensitivity would be expected if RYBP functions as part of an epigenetic checkpoint.

An interesting example of the role of DNA damage in epigenetic inheritance is the *S. pombe* mat locus. Lagging strand synthesis through the region originating from a centromere proximal replication origin results in a double strand break, due to failure to remove an RNA primer. This double strand break acts as an imprint to induce mating type switching in one daughter cell (Dalgaard and Klar, 1999). A similar targeted replication induced break could function as an epigenetic mark of silent regions. Human RING1B, a member of PRC1, ubiquitinates histone H2A during damage repair (Bergink et al., 2006), and this modification has been shown to be required for stable repression of PcG targets (Fang et al., 2004; Wang et al., 2004a). During replication, certain PcG proteins could function to induce DNA damage by preventing primer excision, and the resulting lesion could function as a mechanism to promote the inheritance of H2A ubiquitination, as well as binding of PcG proteins to silent regions. The checkpoint function of Ph would therefore promote cell cycle arrest to ensure proper repair of the lesion, and at the same time ensure proper maintenance of silencing.

### 4.2 A Role for PcG Proteins in Chromatin Assembly After DNA Synthesis

PhP functions in the DNA damage checkpoint response, but this does not rule out an additional role in chromatin assembly after DNA synthesis. Because PcG proteins can bind to modified histones, and PcG proteins are histone modifiers themselves, the
persistence of either at the replication fork could function as an epigenetic mark. Recently, re-ChIP experiments first with antibodies against PCNA which binds the leading and lagging strand during DNA synthesis, and Fen1, which binds only the lagging strand, have shown that PcG proteins are present on newly replicated DNA of less than 200bp in length. Further, the histones that are present on newly replicated DNA are unmodified (Mazo and Brock, unpublished). This is strong evidence that binding of PcG proteins and not modified histones are the epigenetic mark that is stable to DNA synthesis. This also implies that following replication, PcG proteins reproduce the histone modifications that were present before synthesis. To determine whether PcG proteins direct histone modification after DNA synthesis requires a system in which DNA fragments corresponding to different distances from the replication fork. The presence of modifications on larger fragments but not smaller would confirm that modifications are not stable to DNA synthesis.

Following DNA synthesis, aspects of chromatin structure other than histone modifications could also be re-established by PcG proteins. The core members of PRC1, including Ph, have the ability to non-specifically condense a nucleosomal template in vitro (Francis et al., 2004). It is unknown if such higher order chromatin structures exist in vivo, or how they are related to PcG dependent silencing. It is likely that such structures would also be disrupted during DNA synthesis, and could be re-established behind the fork by PRC1. A more easily assayed higher order chromatin structure that is mediated by the PcG is looping between remote enhancers and promoters (Lanzuolo et al., 2007; Tiwari et al., 2008a, b). These loops may require the presence of PcG proteins to be re-established after DNA synthesis. Knockdown of EZH2 results in loss of both
H3K27 methylation within looping regions, as well as disruption of the loop structure (Tiwari et al., 2008b). It could be the case that PcG mediated histone modifications function to promote loop formation, and that re-establishment of histone modifications behind the replication fork indirectly results in re-establishment of the loop structure.

4.3 Cohesins and Homeotic Gene Regulation

Chapter 3 demonstrates that the cohesin Pds5 has a direct and novel role in Hox gene silencing, likely as a member of a unique complex containing the PcG protein Pc and PhP (Wang and Brock, 2003). Cohesins have been implicated in transcription in many organisms. In Drosophila most data points to a role for cohesins in activation of transcription. Cohesins and the loading factor Nipped-B localize to the 5’UTRs and introns of active but not silent genes (Misulovin et al., 2008). The Rad21 gene in Drosophila was initially identified as trxG member verthandi (vtd; Kennison and Tamkun, 1988; Hallson et al., 2008). As expected for a trxG gene, vtd has Suppressor of Polycomb [Su(Pc)] activity (Felsenfeld and Kennison, 1995; Schulze et al., 2001) and vtd mutations act as suppressors of Moonrat, a dominant gain-of-function allele of hedgehog (Schulze et al, 2001). The identification of Pds5 as a direct repressor of Ubx is the first demonstration of a role for cohesins in silencing of Hox genes. It will be interesting to determine how Pds5 functions to repress transcription.

A major question arising from the role of cohesins in transcription is whether or not this role is independent from their role in the cell cycle. In Drosophila, their transcriptional effects are likely separate from the role within the cell cycle because they
can affect transcription in cells that are not dividing. Both Smc1 and Scc3 are required for proper axon patterning, likely due to loss of expression of the ecdysone receptor gene (Schuldiner et al., 2008). This phenotype can be rescued by post-mitotic expression of SMC1 (Schuldiner et al., 2008). Further, by replacing Rad21 with a form that can be inducibly cleaved, Pauli et al (2008) were able to show that cleavage of Rad21 in non-dividing neurons caused significant defects. This, as well as an abundance of evidence that different cohesins affect the transcription of different genes suggests that their role in transcription is independent from their cell cycle role.

In humans, a role for cohesins independent from their role in sister chromatid cohesion has long been sought as an explanation for Cornelia de Lange Syndrome (CdLS), which is caused by heterozygous mutations in the human nipped-B homologue NIPBL, or occasionally by mutations in the core cohesin SMC1 human homologue SMC1L1 (Krantz et al., 2004; Tonkin et al., 2004; Musio et al., 2006). Developmental defects are observed in CdLS patients, though in cell lines only 40% of patients show mild cohesion defects (Kaur et al., 2005), suggesting that cohesion defects are unlikely to cause the disease (Dorsett and Krantz, 2009). In Drosophila Nipped-B heterozygous mutants, changes in gene expression but not problems in sister chromatid cohesion are observed, (Kaur et al., 2005; Rollins et al., 2004). Two other developmental syndromes, Roberts and SC phocomelia, have similar symptoms to CdLS. Both are caused by recessive mutations in the cohesin loading factor esco2/efo2, the human homologue of eco (Vega et al., 2005; Schule et al., 2005). Roles for different cohesins in gene regulation outside of their cell cycle function, such as that observed for Pds5, are therefore important to human disease progression. Key to this will be dissecting the roles
of different cohesins within different silencing or activating complexes at specific gene targets. Potential targets of cohesins could be identified as genes misregulated in patients with CdLS.

An interesting feature of Polycomb Response Elements (PREs), the DNA elements through which PcG proteins exert their function, is that they work in trans. A mini-white reporter gene flanked by PRE sequences is more repressed in the homozygous than heterozygous state, and this depends on somatic pairing (i.e. direct chromosomal interactions) between homologous and non-homologous chromosomes (Kassis et al., 1991; Fauvargue and Dura 1993; Bantignies et al., 2003). Somatic pairing was observed as long ago as 1916 in Dipteran species (cited in Cook, 1997). It also occurs in yeast, and to a lesser degree in more complex organisms (Comings, 1980; Tartof and Henikoff 1991). The same mechanisms governing homologous chromosome pairing during meiosis could explain pairing in somatic cells (Cook, 1997). Both pairing sensitive repression and the direct chromosomal interactions depend on PcG proteins (Bantignies et al., 2003), and it would be interesting to determine whether these interactions are mediated by cohesins by examining the association of homologs in the regions of PcG targets in cohesin mutants.

PREs are located thousands of base pairs from the promoters they control and a looping mechanism brings these elements into proximity of the promoter (Lanzuolo et al., 2007; Tiwari et al., 2008a, b). In fact, PcG proteins, as well as Pds5, ChIP to both the PREs and the promoter, which associate in vivo (Lanzuolo et al., 2007; Fig. 3-7). A role in facilitating such interactions has been previously suggested. Mutations in gene encoding the cohesin loading factor Nipped-B (Scc2) increase the inhibitory effect of a
gypsy transposon inserted in the *Ubx* gene, which acts as an insulator, blocking activation of *Ubx* by remote enhancer. This indicates that Nipped-B normally facilitates long-range chromosomal interactions (Rollins et al., 1999). If cohesins do facilitate intra-chromosomal interactions, they could do so by the formation of a cohesin ring complex encircling two chromosomal regions, rather than two sister chromatids. However, different cohesins have different effects in the *Ubx* insulator assay (Rollins et al., 2004), which could indicate that they do not function as an intact cohesin complex. Alternatively, these differences could be due to the different roles of different complex members in the regulation of complex binding. Only the Pds5[E6] allele, which abolishes cohesin complex binding to polytene chromosomes, and not the [E3] allele, which is a null but does not cause a loss of cohesin binding, had a strong effect on an insulator assay using the *cut* gene. Presumably, a mechanism would have to exist to allow for cohesin mediated intra-chromosomal interactions, which is normally prevented in favor of inter-chromosomal interactions.

Alternatively, cohesins could regulate enhancer-promoter interaction by regulating boundary elements. In mammalian cells, cohesin binding shows a preference for a sequence motif known to bind CTCF (Parelho et al., 2008). CTCF binds to insulator regions and blocks enhancer promoter interactions by organizing the genome into functional domains, and has been well characterized because of its role in controlling allele-specific expression at the imprinted locus Igf2/H19, and stage specific enhancer-promoter interactions in the β-globin locus (Filippova, 2008). It is possible that chromosomal looping mediated by CTCF could be due to cohesin mediating intra-chromosomal interactions. Depletion of Rad21 or SMC1 prevented the function of the β-
globin HS4 and imprinted locus H19 enhancer blocking insulator activity attributed to CTCF (Wendt et al., 2008). CTCF likely either directly or indirectly recruits cohesins to insulator regions, as knockdown of CTCF decreased cohesin association with CTCF binding sites, but did not affect sister chromatid cohesion. CTCF also binds to boundary elements in *Drosophila*, including those separating homeotic genes, and CTCF mutants have homeotic transformations (Holohan et al., 2007; Mohan et al., 2007). It would be interesting to determine whether binding of any cohesin complex members overlaps with CTCF binding sites in *Drosophila*, and if cohesins are recruited to boundary elements in CTCF mutants.

In yeast there is a strong relationship between silent chromatin and cohesin complex localization (Pidoux and Allshire, 2005). In both *S. cerevisiae* and *S. pombe*, high resolution mapping of cohesin complex members, including Pds5, indicates that cohesin complexes are primarily found in intergenic regions, especially regions of convergent transcription (Glynn et al., 2004; Lengronne et al., 2004). Further, transcriptional elongation into a region of cohesin binding through activation of an inducible promoter causes cohesin complex removal (Glynn et al., 2004). In *S. pombe*, cohesin associates with pericentric heterochromatin, and association of cohesin in this region but not along the chromosome arms requires the heterochromatin associated protein Swi6 (Bernard et al., 2001). Genome-wide ChIP has shown that the core cohesins and Nipped-B localize to transcriptional active regions, but it would be interesting to see if Pds5 has a different binding pattern more similar to the pattern of cohesins in yeast, which also act as silencers.
A recent groundbreaking study has shown that recruitment of cohesins to the region between convergent genes is necessary for transcription termination (Gullerova and Proudfoot, 2008). During G1, read-through transcription of convergent genes causes formation the formation of dsRNAs, which in turn activate the RNAi pathway and cause the formation of heterochromatin, including tri-methylation of H3K9 and recruitment of Swi6. Interestingly, these classic epigenetic marks are not retained through DNA synthesis. By G2, the cohesins Rad21 and Scc3 are recruited in a Swi6-dependent manner and the methylation mark is lost, concomitant with proper termination of the convergent transcripts so that overlapping transcripts are no longer made. Rad21 mutants are defective for transcriptional termination, and overlapping transcripts are still observed in G2. This can be tied to RNA mediated silencing of PcG targets in two different ways.

In Drosophila, the RNAi machinery mediates long-range interactions between PREs (Grimaud et al., 2006), and this could be facilitated by RNAi dependent recruitment of cohesins. However, this dependence on the RNAi machinery was not demonstrated to occur at endogenous loci, but only when a transgene was present in multiple copies. Silencing of transgenes without PREs that are present in multiple copies is also PcG and RNAi machinery-dependent. Importantly, mutations in components of the RNAi machinery do not have homeotic defects (Pal-Bhadra et al., 1997, 1999, 2002; Grimaud et al., 2006) so it is possible that transgene silencing and silencing of endogenous loci, though both mediated by the PcG, differ mechanistically.

Recruitment of cohesins to sites of overlapping transcription raises a more interesting possibility for their role in the regulation of Hox genes. Transcriptional interference from transcripts originating upstream of the Ubx promoter represses Ubx
transcription. Cohesins could function to terminate transcription from upstream promoters, thereby activating transcription of *Hox* genes. It would therefore be interesting to determine whether termination of transcripts upstream of *Ubx* is disrupted in *Pds5* mutants, leading to de-repression.

In yeast, the roles of cohesins in transcription seem to involve all members of the complex (Pidoux and Allshire, 2005), and appear to function in parallel to their cell cycle role. However this may not be the case in higher organisms, and perhaps the roles of individual complex members have diverged to function independently in transcription. The identification of complexes containing individual cohesins (Wang and Brock, 2003), or cohesin sub-complexes containing some members of the core complex will be an interesting first step to explaining how transcriptional regulation of certain genes seems to involve some members of the complex and not others and how different cohesins can effect either positive or negative regulation of the same target genes (Rollins et al., 2004; Dorsett et al., 2005).

This thesis has demonstrated a unique cell cycle role for a gene traditionally involved in transcriptional regulation, and a role in transcriptional regulation for a gene traditionally involved in the cell cycle. The functional links between stable silencing and proper cell cycle progression indicates that the two processes are tightly coordinated to promote the inheritance of not only genetic information, but epigenetic information as well.
CHAPTER 5 Material and methods
**Embryo Collection.** Embryos of standard laboratory stocks were collected at 25°C by placing approximately 200 flies of equal male: female ratio into an embryo collection chamber capped with nylon mesh at the top to ensure sufficient air supply. Eggs were deposited onto a 50mm Petri dish containing 5% sucrose, 2% agar and 2% apple cider vinegar, supplemented with live yeast paste. Flies were acclimatized to the laying chamber for three days before 0-2.5 hr embryos were collected for fixation, with frequent changing of laying plates.

**Embryo Fixation.** Embryos were dechorionated in 50% bleach then washed with Embryo Wash Buffer (120mM NaCl; 0.02% Triton X-100) and transferred into 5mL of 3.7% formaldehyde fixative in 1X Phosphate Buffered Saline. The fixative was overlayed with Heptane, which disrupts the embryos’ vitelline layer to allow fixative inside. Embryos were shaken gently for 20 seconds and then fixed at room temperature for 20 minutes on a rotator. After fixation, the formaldehyde layer was removed with a Pasteur pipette and 5mL room temperature methanol was added to dehydrate embryos. The embryos were then devitellinized by vigorous shaking in the methanol solution for 5 minutes and then washed three more times with fresh methanol. Embryos were stored at -20°C in methanol until staining.

**Embryo Rehydration.** Fixed embryos were rehydrated in 5mL of freshly prepared PBT (1X PBS, 1% Bovine Serum Albumin, 0.05% Triton X-100, pH 7.4) solution for 20 minutes at room temperature on a rotator, followed by several rinses in fresh PBT solution.
**Nucleic Acid Staining of Fixed Embryos.** Embryos were collected, fixed and rehydrated as described above. Embryos were stained with the nucleic acid dye DAPI at a concentration of 2.5 µg/mL for twenty minutes, followed by three washes for ten minutes each with PBT. PBT was removed and embryos were suspended in Vectashield mounting medium (Vector Laboratories).

**Immunostaining of Fixed Embryos.** Following rehydration, embryos were incubated in primary antibodies for 2 hours in PBT at dilutions of 1/500 for anti-PhP (Hodgson et al., 1997), 1/25 for anti-phosphorylated serine 28 (AbCam), 1/250 for anti-H2A.zser139p (AbCam). Following incubation with primary antibodies, embryos were washed six times for ten minutes each with PBT. Fluorescent coupled secondary antibodies were added to PBT as per the manufacturer’s directions (Molecular Probes), and embryos were incubated for sixty minutes, and then washed six times with fresh PBT for ten minutes each prior to nucleic acid staining and mounting in Vectashield mounting medium (Vector Laboratories).

**Confocal Microscopy and Image Rendering.** Images were obtained using a Zeiss Meta Confocal Microscope. All embryos or discs from a given collection were scanned. For scoring of cell cycle phenotypes, only severe chromatin bridges as described by O’Dor et al., 2006 were scored. Severe bridging is classified as a penetrant phenotype in which greater than 25% of the nuclei have bridges between sister chromatids. Less severe chromatin bridging (>25% of nuclei) is occasionally observed in Wild Type embryos, but severe bridging is not (O’Dor et al., 2006). Image analysis was done using ImageJ NIH software.
**Drosophila Stocks and Genetic crosses.** All crosses were performed at 25°C in vials containing standard cornmeal-agar medium supplemented with live yeast paste. For analysis of mitotic phenotypes, mothers of the indicated genotype were generated by crossing `ph410/w; +;+` virgin females with males of genotypes `yw; Pds5[E3]/SM6, yw;Pds5[E6]/SM6` (Kind gifts from D. Dorsett), `yw;Smc1/TM3, sb` (A kind gift from S. Page); `yw; P{w+y+}lark EY00297 eco EY00297` (Bloomington), `+; PBac{WH}Sse f03027` (Bloomington), or `+/+; nipped-B/CyO` (A kind gift from D. Sinclair). `Pc15/TM3,Sb` virgin females were also crossed with males from both `Pds5` mutant strains. Virgin females resulting from these crosses were genotyped based on the dominant marker present on the marker chromosome, and placed in cages for embryo collection along with an equal number of Oregon R (OR) males.

For live imaging, `w; +; H2A.Z-GFP` virgin females were used with an equal number of OR males in cages for embryo collection. `w; +; H2A.Z-GFP` virgin females were mated with `ph410/w; +` males, and all resulting virgin females were used with an equal number of OR males in cages for embryo collection. `w; +; H2A.Z-GFP` virgin females were mated with `w; Pds5[E3]/CyO` and resulting straight winged virgin females were used with an equal number of OR males in cages for embryo collection. For imaging of double heterozygotes, double homozygotes of `ph410/w; +; H2A.Z-GFP` were first generated, and virgin females were mated with `w; Pds5[E3]/CyO; +` males. Straight winged virgin females resulting from this cross were used with an equal number of OR males in cages for embryo collection.

**Live Imaging.** Embryos were prepared as described in Stumpff et al (2004). Images were
collected as \sim 7 \mu m stacks every 60 seconds using a Zeiss Meta Confocal Microscope. Embryos that did not survive to the end of mitosis 13 were not included in the data set to reduce artifacts produced by the imaging process. For statistical analysis the unpaired Student’s t test was used. Calculated t values were 0.983, 4.34, 0.922, 2.65 with seven degrees for freedom for mitosis 10, S phase 11, mitosis 11, and S phase 12 respectively, and 0.43, 2.04, 0.67 with nine degrees of freedom for mitosis 12, S phase 13, and mitosis 13.

**Thymidine Labelling.** Imaginal wing discs were dissected from third instar larvae with a total of four discs in each replicate. The discs were incubated in 5\mu l of a solution consisting of 30\mu l of Grace’s Insect Medium (Gibco) with 25\mu l of radioactively labeled thymidine, [methyl-\textsuperscript{3}H] (PerkinElmer) for 40 minutes. After the incubation the discs were immersed in 50\mu l of lysis buffer (396\mu l of 1X TE and 4\mu l of 1% SDS) for a minimum of 15 minutes and were then flash frozen in liquid nitrogen and kept at -20°C overnight. The replicates were thawed in a 37°C water bath and DNA was extracted. The extraction was performed by adding 100\mu l of phenol chloroform to each replicate already suspended in 50 \mu l of lysis buffer, vortexed and centrifuged for 10 minutes at 13000 RPM. The aqueous layer was removed and 25 \mu l was set aside for TCA precipitation via vacuum filtration. From the remaining portion of the aqueous layer 10 \mu l was removed from each replicate this 10 \mu l sample was combined with 10 \mu l of PICO green working solution (198 \mu l of 1X TE and 2 \mu l of Quant-iT PicoGreen dsDNA (Invitrogen)). This 1:2 solution was then further diluted with the PICO green working solution to a final dilution of 1:20. The DNA content of the 1:20 dilution was quantitated
with the Nanodrop ND-3300 Fluorospectrometer. Two measurements were taken for each replicate and the average was used for all calculations. The TCA precipitation commenced with adding 500 µl of cold 10% TCA solution and 2.5 µl of carrier DNA to the 25 µl DNA samples. The replicates were vortexed and then stored on ice for ten minutes. The chilled replicates were pipetted onto a Whatmann GF/C glass fibre filter pre-moistened with 250 µl of cold TCA resting on a Büchner flask. Each microcentrifuge tube was rinsed twice with 500 µl of cold TCA followed by a 500 µl rinse of 95% ethanol. The filter paper was then removed and placed in a scintillation vial and filled with Amersham’s BCS scintillation fluid. The samples were stored overnight in the dark. The DPM value of each sample was quantified using the Beckman LS 6000IC scintillation counter. Two scintillation counts were performed for each replicate and the average of the two counts was used for all calculations.

**DNA Damage Response.** Experiment was performed essentially as described in Laurencon et al (2003). Wandering 3rd instar larvae were irradiated with 5 Gy from a 60Co source, discs were dissected after 1.5-2 hrs, fixed in 3.7 % paraformaldehyde, and stored at -20 in methanol until staining was performed. Discs were stained with rabbit anti-phospho Histone H3 Ser28 (AbCam) at a dilution of 1/500 for ninety minutes in PBT. This was followed by six washes for ten minutes each in fresh PBT, and incubation with fluorescent coupled secondary antibody as per the manufacturer’s directions (Molecular Probes). Discs were then washed six times with fresh PBT for ten minutes each, followed by nucleic acid staining with DAPI at a concentration of 2.5 µg/mL. Discs were mounted in Vectashield (Vector Laboratories).
**Induction of Double Strand Breaks.** Following dechorionation, embryos were immediately immersed in 50μg/mL bleomycin in PBS, and overlayed with an equal volume of n-octane for permeabilization. Embryos were shaken briefly and then rotated for thirty minutes at room temperature. Embryos were then washed six times with fresh PBS before fixation and staining.

**Scoring of Homeotic Phenotypes.** Male flies resulting from crosses of *ph*\(^{410}\) virgin females to males mutant for cohesin subunits of the indicated alleles. Males of each resulting genotype were identified based on dominant markers on balancer chromosomes and the expressivity of posterior transformations was determined based on the number of flies with significant abdominal transformations from A4 to A5 (pigmentation of most of the abdominal segment) compared between balancer siblings to minimize the effects of the genetic background.
References


EMBO J 22, 3737-3748.


Cycle 3, 1196-1200.


The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol Cell Biol 22, 6070-6078.


binding domains. Proc Natl Acad Sci U S A 87, 2112-2116.


Ng, H. H., Jeppesen, P., and Bird, A. (2000). Active repression of methylated genes by the


Pal-Bhadra, M., Bhadra, U., and Birchler, J. A. (2002). RNAi related mechanisms affect both
transcriptional and posttranscriptional transgene silencing in Drosophila. Mol Cell 9, 315-327.


