THE ROLE OF POLYCOMB GROUP GENES IN MITOSIS IN DROSOPHILA MELANOGASTER

by

ESTER O'DOR

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ABSTRACT

In Drosophila, the Polycomb Group (PcG) of epigenetic regulators have classically been studied as chromatin regulatory proteins which maintain the repressed state of Hox genes during development, thereby maintaining the correct patterning of the anteroposterior axis. Though the mechanism of PcG-mediated inheritance is not yet known, it likely involves co-ordination with cell cycle regulators to ensure faithful transmission of repressed Hox states during cell division, and thus maintenance of the body plan. So far, very little evidence has linked the PcG to cell cycle progression. Recently, Lupo et al. (2001) demonstrated that a PcG mutant, polyhomeotic, exhibits chromatin bridges at anaphase, and an isoform of Polyhomeotic protein co-immunoprecipitates with cell cycle regulators Barren and Topoisomerase II. I have characterized mitotic defects in early embryos of a subset of PcG mutants and demonstrate that segregation defects and other cell cycle progression defects are common to many PcG mutants. This suggests that the PcG as a whole has a role in cell cycle progression. Characterization of the segregation defects focused on mutants of the polyhomeotic proximal gene (ph-p) and revealed that the chromatin bridges are restricted to chromatid arms, and likely arise during S-phase. PH-P protein partially co-localizes with the S-phase regulators Geminin and PCNA, further suggesting an S-phase role for PH-P. In early embryos, the chromatin bridges present a physical barrier to PH-P oscillation during the cell cycle, such that normal PH-P reassociation with chromatin following anaphase is delayed. To assess whether cell cycle progression affects PcG-mediated silencing during later development, I performed a number of genetic interaction assays between PcG mutants and mutants of known cell cycle regulators. All cell cycle mutants which themselves exhibit segregation defects enhance the adult homeotic phenotypes of PcG mutants. I propose a model which describes an S-phase role for PH-P, and suggests that cell cycle progression is a crucial component of epigenetic inheritance.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CID</td>
<td>Centromere Identifier (Centromere-specific Histone H3 variant)</td>
</tr>
<tr>
<td>CMM</td>
<td>Cellular Memory Module</td>
</tr>
<tr>
<td>CtBP</td>
<td>Carboxyl-terminus Binding Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole (DNA stain)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>E(z)</td>
<td>Enhancer of Zeste protein (<em>Drosophila</em>)</td>
</tr>
<tr>
<td>EPC</td>
<td>Enhancer of Polycomb</td>
</tr>
<tr>
<td>ETP</td>
<td>Enhancer of trithorax and Polycomb</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain Of Function</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HKMT</td>
<td>Histone Lysine Methyltransferase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone Methyltransferase</td>
</tr>
<tr>
<td>Hox</td>
<td>Homeobox cluster</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss Of Function</td>
</tr>
<tr>
<td>ME</td>
<td>Maintenance Element</td>
</tr>
<tr>
<td>MP</td>
<td>Maintenance Protein</td>
</tr>
<tr>
<td>PC</td>
<td>Polycomb protein (<em>Drosophila</em>)</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb Group</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen protein (<em>Drosophila</em>)</td>
</tr>
<tr>
<td>ph</td>
<td>polyhomeotic locus</td>
</tr>
<tr>
<td>PH-D</td>
<td>Polyhomeotic-Distal protein (<em>Drosophila</em>)</td>
</tr>
<tr>
<td>PH-P</td>
<td>Polyhomeotic-Proximal protein (<em>Drosophila</em>)</td>
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<tr>
<td>PRC1</td>
<td>Polycomb Repressive Complex 1</td>
</tr>
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<td>PRC2</td>
<td>Polycomb Repressive Complex 2</td>
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<tr>
<td>PRE</td>
<td>Polycomb Response Element</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-Association Motif</td>
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<tr>
<td>SET</td>
<td>SU(VAR)3-9, ENHANCER OF ZESTE, and TRITHORAX protein domain</td>
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<tr>
<td>TAF</td>
<td>TBP-Associated Factor</td>
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<td>TRE</td>
<td>Trithorax Response Element</td>
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<tr>
<td>TrxG</td>
<td>trithorax Group</td>
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CHAPTER 1 Introduction

I. Development and epigenetic regulation

All cells within a multicellular organism are isogenic, but vary enormously in size, morphology and function. Two fundamental questions which have been the basis for many developmental studies are: 1) how do cells acquire unique fates? and 2) how do cells with a determined fate maintain their developmental program following cell division? The processes of determination and differentiation during the development of the organism are programs that define distinct cellular identities by directing unique patterns of gene expression in response to external and internal stimuli. This highly complex process of temporal and spatial regulation of gene expression and repression drives the restriction of cell fate from the totipotency of the zygotic cell to the pluripotency of embryonic cells and stem cells to terminally differentiated adult tissues.

When determined or differentiating cells divide, their daughter cells must inherit the specific gene expression patterns that defined the mother cell’s fate and function, in order to faithfully continue the developmental program. This process called maintenance or cellular memory (Jacobs and van Lohuizen, 2002) ensures that gene expression patterns are faithfully copied to daughter cells such that the daughter cells “remember” the fate or identity that was determined in the mother cell. The process of maintenance is epigenetic because it is not the inheritance of sequence information that is key, but rather the inheritance of expression states of genes. Therefore, epigenetic inheritance is not dependent on Mendelian assortment and inheritance of genetic code (Cavalli, 2002).

Epigenetic maintenance has two distinct features depending on intra- or inter-cell cycle
function. Inheritance must be stable to mitosis such that epigenetic information is maintained (Ringrose and Paro, 2004). However, between cell divisions it must also be responsive to the external and internal stimuli of determination and differentiation programs that alter gene expression and thus change or narrow developmental fate (Brock and Fisher, 2005). The mechanisms behind this dichotomy of plasticity within a cell cycle and stability across cell division are key areas of the study of development.

II. Epigenetic maintenance of Hox gene patterning during development

During development, many differentiation programs are layered to build an adult morphology from a rudimentary body plan. One set of genes called the Hox (Homeotic, Homeobox) genes are keystone components for patterning the anteroposterior axis of all metazoans (Lewis, 1978; Krumlauf, 1994). Hox gene expression elaborates on the segmentation of the Drosophila embryo body plan previously defined by the segmentation genes (Martinez-Arias and Lawrence, 1985) and effectively assigns a fate for each embryonic segment. Discovered in Drosophila, Hox genes are evolutionarily conserved from sponges to mammals (Degnan et al., 1995; Ferrier and Minguillon, 2003). Hox genes are themselves transcription factors characterized by a DNA-binding region called a homeobox (McGinnis et al., 1984) and initiate a cascade of gene expression (Wolpert, 1994). The expression of Hox genes is spatially and temporally restricted and a combination of Hox genes expressed in a body segment defines the downstream transcription factors and genes that morphologically and functionally define a body segment. Hox genes are thus thought of as master switches or selector genes for their ability to select a fate for a given body segment. Mutations in Hox genes result in
homeosis, where one body part assumes the identity and morphology of another, therefore the epigenetic maintenance of specific patterns of \textit{Hox} gene expression is vital to maintain the body plan.

In \textit{Drosophila}, there are eight \textit{Hox} genes on chromosome 3 (called the \textit{HOM-C} complex) clustered into two linkage groups, termed the \textit{Antennapedia Complex (ANT-C}, containing the homeotic genes \textit{labial, proboscipedia, Deformed, Sex combs reduced} and \textit{Antennapedia}) and \textit{Bithorax Complex (BX-C)} containing the homeotic genes \textit{Ultrabithorax, abdominal-A} and \textit{Abdominal-B} (Lewis 1978; McGinnis and Krumlauf, 1992). The order of \textit{Hox} genes on the chromosome is co-linear with their expression patterns along the anteroposterior axis in flies and vertebrates (Kmita and Duboule, 2003). The initial pattern of \textit{Hox} gene expression is defined by transient transcription factors called the \textit{gap} and \textit{pair rule} genes, which serve to express and repress subsets of \textit{Hox} genes along the anteroposterior axis during early development (Martinez-Arias and Lawrence, 1985). This pattern of \textit{Hox} expression and repression must be maintained once the transient factors disappear in order to maintain the segmental identities already established (Wolpert, 1994). Furthermore, the patterns must be maintained for several cell divisions. The maintenance of \textit{Hox} gene expression patterns is a widely studied model system for epigenetic maintenance.

Other than anteroposterior patterning, \textit{Hox} genes also function in morphogenesis (Hombria and Lovegrove, 2003), patterning of limbs (Li and Cao, 2003), gonads (Estrada \textit{et al.}, 2003), and internal organs in animals (Lo and Frasch, 2003; Patterson and Potter, 2003), regulate hematopoietic differentiation in mammals (Owens and Hawley, 2002), and regulate flowering in plants (Ferrario \textit{et al.}, 2004). In addition, \textit{Hox} genes play an
important role in oncogenesis of a variety of cancers (Maroulakou and Spyropoulos, 2003).

III. The Polycomb Group and trithorax Group of epigenetic maintenance proteins

In Drosophila, there are two classes of genes that are epigenetic regulators of Hox expression. The Polycomb Group (PcG) and trithorax Group (trxG) of maintenance proteins maintain the repressed and active states (respectively) of Hox genes previously specified by the transient gap and pair-rule proteins (Simon, 1995; Kennison, 1995). PcG and trxG genes were first identified in Drosophila (Slifer, 1942; Garcia-Bellido, 1977; Jurgens, 1985; Lewis, 1947; Lewis, 1978) and homologues have since been identified in plants, nematodes, amphibians, fish, mammals (including humans) and other vertebrates (Brock and Fisher, 2005, and references therein). Mutations in Drosophila PcG and trxG genes do not initially affect Hox expression pattern along the anteroposterior axis since gap and pair-rule proteins set up Hox expression patterns in early embryogenesis. It is only after these transient factors disappear do PcG and trxG phenotypes appear (Simon, 1995). Once the gap and pair-rule proteins disappear, Hox genes become derepressed in PcG mutants and this results in anterior to posterior homeosis, similar to a GOF (gain-of-function) Hox mutation (McKeon and Brock 1991; Simon et al, 1992). Conversely, Hox genes fail to maintain active expression in trxG mutants also resulting in posterior to anterior homeosis, similar to a LOF (loss-of-function) Hox mutation (Kennison, 1995).

Most PcG mutants enhance each other’s posterior homeotic phenotypes, and suppress the anterior homeotic phenotypes of trxG mutants (Jurgens, 1985). Similarly, most trxG mutations enhance each other’s anterior homeotic phenotypes and suppress the
posterior homeotic phenotypes of PcG mutations (Shearn, 1989). However, genetic and molecular analysis of PcG and trxG shows that this initial classification is oversimplified. Mutants of a subset of genes formerly classified as PcG members exhibit both anterior and posterior homeotic transformations, suggesting they are required for maintenance of activation and repression of Hox genes (Gildea et al., 2000). The genes in this subset are called ETPs (Enhancers of Trithorax and Polycomb) and include PcG members Additional sex combs, Enhancer of zeste, Posterior sex combs, Suppressor 2 of zeste, Enhancer of Polycomb and Sex combs on midleg. However, it has been recently suggested that the ETP category be reclassified based on molecular interactions to avoid possible indirect effects by genetic classification alone (Brock and Fisher, 2005).

PcG and trxG proteins are collectively called Maintenance Proteins (MPs). In Drosophila, there are an estimated 30-40 PcG genes (Jurgens 1985, Landecker et al., 1994), 20 of which have been cloned and characterized, and of the trithorax Group, 15 have been cloned and characterized (Brock and Fisher, 2005 and references therein). Collectively, MPs have been studied as chromatin modifiers due to conserved protein domains and features that are shared with chromatin proteins. These include chromodomains and bromodomains which recognize methylated and acetylated histone tails, respectively (Kennison, 1995, Muller, 1995; Min et al., 2003, de la Cruz et al., 2005) protein-protein interaction domains such as SAM/SPM (also known as PNT or HLH domains, Kim et al., 2002; Kyba and Brock, 1998) and WD40 repeats which serve as protein complex assembly platforms (Simon et al., 1995). These features have been instrumental in pursuing MPs as chromatin modifiers that act in multimeric complexes (see below). Interestingly, the molecular function of most MPs is still unknown and only
a few MPs have been shown to possess enzymatic activity. The SET domains of Enhancer of zeste and the trxG proteins trithorax (trx) and Abnormal, small, and homeotic 1 (Ash1) are histone lysine methyltransferases (HKMTs) (Marmorstein, 2003). Many MPs also possess RING domains, which in other proteins have been shown to be E3 ubiquitin ligase domains in vitro (Aravind et al., 2003). The PcG protein Ring2 as well as a Ring2-containing PcG complex from human cells has Histone H2a-specific E3 activity in vitro, dependent on the RING domain of Ring2 protein (Wang et al., 2004). It remains to be seen whether other MP RING domains have E3 activity in vitro or in vivo.

Some PcG proteins also interact with Small Ubiquitin Related Modifier (SUMO)–conjugating enzyme Ubc9. Human PcG protein Polycomb2 and C. elegans PcG protein SOP2 each interact with Ubc9 and are required for the sumoylation of CtBP (Carboxyl-terminus Binding Protein) and SOP-2, respectively (Kagey et al., 2003; Wang et al., 2004).

IV. Maintenance Protein complexes and Maintenance Elements

Overall, the molecular or enzymatic functions of many MPs remain to be determined. However, MPs are generally members of large multimeric complexes, of which the various subunits further elucidate how MPs maintain the expressed or repressed state of their targets (discussed below). Further discussion of MPs in this thesis will primarily focus on PcG maintenance proteins.

Though PcG proteins have long been thought to be members of multimeric complexes as evidenced by genetic and physical interactions and shared binding sites on Drosophila polytene chromosomes (Jurgens, 1985; Kennison, 1995; Franke et al., 1992;
Kyba et al., 1998), purification of PcG-containing complexes has only recently intensified. The PRC1 complex (Polycomb Repressive Complex 1) in Drosophila was the first to be purified (Shao et al., 1999) and contains PcG members Polyhomeotic (PH), Polycomb (PC), Posterior sex combs (PSC) and Ring (also known as Sex combs extra) as well as trxG protein Zeste and non-MPs including basal transcription apparatus proteins TBP (TATA-Binding Protein) and TAFs (TBP-Associated Factors). In vitro studies have shown that PRC1 as well as the core complex containing only the PcG components as well as PSC by itself inhibits nucleosome remodeling by the human SWI-SNF complex (Francis et al., 2001). SWI-SNF complexes stably disrupt nucleosomes to allow transcription factors access to DNA template (Mohrmann and Verrijzer, 2005). A complex similar to PRC1 called CHRASCH (Chromatin-Associated Silencing Complex for Homeotics) was also purified from Drosophila, and its most notable differences from PRC1 include the histone deacetylase HDAC1 and a DNA-binding protein Pipsqueak (Huang and Chang, 2004). Mammalian PRC1-like complexes have also been identified and contain the mammalian homologues of the Drosophila PcG members, but do not contain TBP and TAFs (Levine et al., 2002; Lavigne et al., 2004; Wang et al., 2004).

Another PcG complex, PRC2 contains Enhancer of zeste, (E[z]), Extra sex combs (ESC) and Suppressor 12 of Zeste, as well as Polycomblike in a PRC2 variant (Czermin et al., 2002; Muller et al., 2002). The E(z) component of PRC2 is necessary for methylating histone H3 at lysine 27 via its SET domain (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). The Polycomb protein of PRC1 recognizes and binds to this modification, resulting in a model where PRC2 recruits PRC1 in a stepwise manner (Wang et al., 2004). In fact, PRC1 and PRC2 interact in
early pre-blastoderm Drosophila embryos (Poux et al., 2001a). However, there is evidence for the converse hierarchy, namely that PRC1 components are required for targeting of PRC2 components, and that PRC2 components are required continuously for silencing, not just for recruitment of PRC1 (Poux et al., 2001a; Poux et al., 2001b). The PRC2 component ESC is required for maintenance of silencing even if PC is already tethered to its target. In addition, tethered PC recruits ESC. PC also targets E(z) to promoters, and E(z) is also required continuously for maintenance of repression (Breiling et al., 2004). Therefore, a stepwise recruitment model is too simple to explain the crosstalk between PRC1 and PRC2. It is likely that components of either complex have additional roles and interactions with other proteins, which are not limited to a membership in a PRC1 or PRC2 complex. Of the roughly 20 Drosophila PcG genes cloned and characterized, only four (PH, PC, PSC and Ring) are members of PRC1 and three (E[z], ESC and Su[z]12) are members of PRC2, suggesting the existence of other PcG complexes. Alternatively, there may be many transient interactions between the PRCs and other PcG proteins that have not been identified by purifying soluble complexes. In addition, even the composition of PRC1 and PRC2 is not fixed, rather, membership appears to change according to purification protocols, cell source, species, developmental stage or tissue type (Otte and Kwaks, 2003). For example, the PcG protein Pleiohomeotic (PHO, a homologue of the mammalian transcription factor Yin-Yang 1 [YY1]) was shown to interact with both PRC 1 (in early 0-3 hour embryos) and PRC2 in both early and older (24 hour) embryos (Poux et al., 2002). While other reports also claim an interaction between PRC1 components and PHO (Mohd-Sarip et al., 2002)), others report YY1 in the human PRC1 complex but no PHO in Drosophila PRC1
(Saurin et al., 2001). In summary, not all PcG proteins can be categorized into a PRC complex and PRC complexes themselves appear to be more diverse and dynamic than reports suggest. Nevertheless, it is accepted that PcG proteins function as members of large multimeric protein complexes.

In mammals, PRC complexes have additional roles as well. Murine PRC1 and PRC2 are involved with X-inactivation and mouse PRC2 proteins transiently accumulate on the inactive X and methylate histone H3 at K27 (Erhardt et al., 2003; Plath et al., 2003; Silva et al., 2003). PRC1 members localize to and accumulate on the inactive X during initiation and maintenance phases of X-inactivation in mouse embryonic cells (Plath et al., 2004). Interestingly, the localization of PRC1 members is dependent on Xist, but not completely correlated with H3K27 methylation, again suggesting that a stepwise recruitment of PRC1 by PRC2-dependent methylation of H3K27 is an oversimplified model of maintenance. In humans, PRC2 (which is required for H3K27 methylation) and PRC3 (which contains a different isoform of EED, the mammalian ESC homologue, and methylates H1K26) have also been described (Kuzmichev et al., 2004).

Members of the trithorax Group are also members of large multiprotein complexes (Papoulas et al., 1998; Kennison, 2004). In Drosophila, the trxG protein Brahma (along with Osa and Moira) is a member of the BAP (Brahma Associated Protein) complex and the related PBAP complexes. Brahma is similar to the ATPase subunits of the yeast SWI/SNF and RSC chromatin remodeling complexes and thus the brahma-containing complexes of Drosophila are thought to modify chromatin to facilitate transcription (Mohrmann et al., 2004). Another complex, TAC1, contains trithorax and has histone acetyltransferase activity (Petruk et al., 2001; Petruk et al., 2004). In addition,
TAC1 is recruited to the hsp70 heat shock locus to facilitate rapid transcription in response to heat stress likely via modulating transcriptional elongation (Smith et al., 2004). Other complexes include trxG proteins ASH1 and ASH2 (Papoulas et al., 1998). In humans, the trithorax homologs MLL and MLL2 are components of distinct histone methyltransferase complexes related to the COMPASS (Complex Proteins Associated with Set1) complex of yeast (Miller et al., 2001; Hughes et al., 2004). MLL is also a component of a large supercomplex, which contains subunits from multiple transcription complexes and has the capability to acetylate, deacetylate, methylate and remodel nucleosomes (Nakamura et al., 2002).

V. Maintenance Protein targets and mechanisms of function

Maintenance Proteins bind to complex cis-regulatory elements of their target genes called Maintenance Elements (MEs) or Cellular Memory Modules (CMMs) (Brock and van Lohuizen, 2001; Cavalli and Paro, 1999). The best-characterized MEs regulate the Drosophila Hox genes. Hox MEs are situated within very large regulatory elements, which span hundreds of kilobases (Busturia and Bienz, 1993; Simon et al., 1993). The large region contains functional units that regulate expression of specific genes of a Hox cluster in a pattern specified both temporally and spatially. Within these functional units, enhancers, silencers and MEs regulate the initiation and maintenance of Hox expression (Simon et al., 1993; Chan et al., 1994). MEs themselves are composed of modules of interspersed and overlapping but distinct units called Polycomb Response Elements (PREs), which bind PcG proteins and Trithorax Response Elements (TREs), which bind trxG proteins and cooperate to maintain proper patterns of Hox gene silencing and
expression (Tillib et al., 1999). The subsets of complexes that bind these elements are specified spatially and temporally in the developmental program to dictate Hox expression and repression patterns. MEs are required continuously for maintenance since deletion of ME components even during later stages of development results in homeosis as maintenance of proper Hox gene expression is lost (Sengupta et al., 2004). In Drosophila, MEs have been identified at only a few other loci including the transcription factor engrailed, the morphogen hedgehog, and the PcG gene polyhomeotic (Kassis, 1994; Fauvarque and Dura, 1993; Maurange and Paro, 2002).

Further dissection of ME modules uncovers conserved DNA sequences which serve as binding sites for MPs including the only DNA-binding PcG proteins Pleiohomeotic (PHO) and PHO-like (Brown et al., 1998; Brown et al., 2003; Mihaly et al., 1998), the trxG proteins GAGA factor (GAF, the product of the trithoraxlike gene) (Strutt et al., 1997; Ringrose et al., 2003) and Zeste (Saurin et al., 2001). In addition, a DNA-binding factor called Pipsqueak recognizes the same GAGAG consensus motifs as GAGA factor and has been shown to bind to MEs (Hodgson et al., 2001). These DNA sequences are necessary for recruiting some MPs or for maintenance of expression or repression, but alone are not sufficient to recapitulate ME function. Given the complexity of ME organization, it is likely that other factors are required to recruit MPs. For example, the GAGAG consensus-binding motif of Pipsqueak is found in a larger family of proteins (Siegmund and Lehmann, 2002) suggesting that other Pipsqueak family members may recruit MPs to MEs. In addition, DNA-binding factors for additional repeated sequence motifs found in MEs (Tillib et al., 1999; Hodgson et al., 2001) have not yet been identified. So far, MEs have not been found in mammals,
though binding sites for human PcG proteins SU(Z)12 and YY1 (the PHO homologue) have been identified near Hoxa9 (Cao and Zhang, 2004).

Other than at MEs, MPs bind the promoters of their target genes in *Drosophila* (Orlando *et al.*, 1998; Lyko and Paro, 1999) and mammals (Milne *et al.*, 2002; Nakamura *et al.*, 2002; Kirmizis *et al.*, 2004) suggesting that MPs affect transcription. A genome-wide prediction of MEs using an algorithm based on PHO, Zeste and GAGA-factor binding sites highlighted many potential MEs near promoters in *Drosophila* (Ringrose *et al.*, 2003). In addition, TBP and TAFs co-purify with PRC1 components (Shao *et al.*, 1999; Saurin *et al.*, 2001; Wang and Brock, 2003) further linking PcG proteins and transcriptional repression. Also, transcription factors are not excluded from the promoters of silenced PcG targets suggesting that PcG-mediated silencing does not silence by smothering access to other factors (Breiling *et al.*, 2001). In fact, silencing of the *hsp26* heat shock promoter by a PRE construct does not preclude binding of TBP, RNA Polymerase or Heat shock factor to the promoter, but prevents DNA melting and initiation of transcription (Dellino *et al.*, 2004).

Interestingly, trxG proteins are also involved at endogenous heat shock loci and appear to affect transcriptional initiation and elongation. The trxG protein GAGA-factor is involved in the establishment of an initiated, paused polymerase complex, which is able to respond to heat induction for rapid heat shock protein transcription (Leibovitch *et al.*, 2002). The Trithorax protein is recruited to *hsp70* heat shock promoter as part of the TAC1 complex and is required for full heat shock response. TAC1 binds just downstream of the transcriptional start and methylates and acetylates histones. The positioning within the coding region suggests a role in elongation. PcG proteins PC and
E(z) also bind regions downstream of the promoter of the Hox gene Ubx, and in the case of E(z), methylation of H3K27 downstream of the promoter ensues (Wang et al., 2004). In addition, TBP, RNAPolII and elongation factors bind active and repressed Hox Abd-B promoters in Drosophila cell lines, however, the repressed Abd-B promoter was enriched for PRC components and correlated with methylated H3K9 and H3K27 at the promoters (Breiling et al., 2004)

As discussed, the biochemical functions of only a few MPs are known and mostly involve post-translational modification of histone tails. Therefore, localized chromatin modifications and alteration of the histone code have been proposed to be integral to MP-mediated maintenance. The histone code hypothesis proposes that patterns of post-translational modifications of histones provide a code that specifies active or repressed chromatin states (Jenuwein and Allis, 2001). As discussed previously, E(z) methylates H3K27 and possibly H3K9 as a subunit of PRC2 complexes. PcG proteins can recruit histone deacetylase (HDAC) co-repressors to silence targets (Chang et al., 2001; Tie et al., 2001). The SET domains of Trithorax, Trithorax-related and Ash1 (and mammalian homologues) are H3K4 histone methyltransferases Milne et al., 2002; Nakamura et al., 2002; Byrd and Shearn, 2003; Sedkov et al., 2003, Smith et al., 2004). Some trxG proteins interact with histone acetyltransferase (HAT) coactivators (Bantignies et al., 2000). Acetylated lysine residues of histones are recognized by bromodomains (present in trxG proteins Trx and Brahma) and are thought to recruit chromatin regulators, thereby reading and translating the histone code (Kanno et al., 2004). All trxG members so far described belong to chromatin modifying and/or histone modifying complexes. In
addition, the binding of MPs to MEs or promoters is often correlated with modification of histone tails, making localized chromatin alterations the favoured model for MP function.

The presence of MPs at target promoters supports an earlier model for MP function. MP binding to both MEs and promoter regions suggests an interaction between MEs and promoters. Models involving the looping of intervening DNA (which would restrict enhancer-promoter interactions and inhibit transcription) have been proposed long ago (Pirotta, 1995). Though there is no direct evidence for ME-promoter interaction, it was recently shown that MEs interact with each other in vivo, in addition, and GAGA-factor (trxG protein) is involved in enhancer-promoter interactions suggesting that ME-promoter interactions may be possible (Lavigne et al., 2004). Recently, techniques such as Chromosome Conformation Capture (3C) demonstrated that expression of mammalian globin cluster genes is regulated by multiple interactions between cis-regulatory elements of the locus control region of the globin cluster with the promoters of the active genes, while inactive genes were looped out from the interaction center (called the active chromatin hub) (Palstra et al., 2003; Patrinos et al., 2004). It is likely that similar techniques will be applied to determine whether MEs and promoters assemble into a comparable 3-dimensional structure to regulate MP-target expression.

Other models for ME and MP function are based on compaction or alteration of chromatin structure beyond localized histone modifications or chromatin remodeling. The crystal structure of Polycomb protein bound to methylated H3K27 reveals Polycomb as a dimer, suggesting that PC protein may link nucleosomes to form a repressive chromatin structure (Min et al., 2003). Recently it was shown that the core complex of PRC1 (which contains the four PcG subunits Ph, Pc, Psc and Ring) compacts
nucleosomal arrays in vitro (Francis et al., 2004). Interestingly, this activity required the presence of intact nucleosomes, but not histone tails. These data seem to contradict those above that indicate PcG silencing is via inhibition of the early stages of transcription and does not involve restricted access of DNA to transcription factors. In addition, epigenetic control of gene expression must still be responsive to developmental cues and potentially alter expression to follow a developmental program, suggesting that extreme or permanent chromatin alterations akin to heterochromatinization may be unlikely during development. However, it is possible that MPs employ distinct mechanisms at different targets, during different times of development, or throughout different stages of epigenetic maintenance.

VI. Mechanisms of epigenetic inheritance

As previously mentioned, epigenetic maintenance involves two main features. Namely, the transcriptional profile that defines a cell's fate and identity must be sustained within each cell (with the potential to change as dictated by internal and external developmental cues), and must also be transmitted to daughter cells upon cell division to continue the developmental program. Upon inheritance of the transcriptional profile, the daughter cells must then each sustain the expression patterns and respond to developmental changes, and in turn transmit their expression patterns to their daughter cells. The mechanisms of MP function described above do not address potential differences between these phases of epigenetic maintenance. For PcG silencing, it is clear that the proposed models suggest mechanisms for maintaining transcriptional profiles within the confines of interphase. Furthermore, other models which suggest
transcriptional repression occurs via the inhibition of initiation or elongation (but not the exclusion of transcription factor binding) provides the capability to respond to developmental changes that are required during development. However, none of the models above address the mechanism of inheritance of transcriptional profiles through cell division.

Models which address inheritance propose that the transcriptional status of MP targets is conveyed as an “epigenetic mark” which is read and interpreted following mitosis to re-establish that state (Cavalli and Paro, 1998; Pirrotta et al., 2003). MPs may be involved in establishing and interpreting the mark. Histone modifications have been popularized as the mechanism by which MPs function to maintain transcriptional states, and in support of this model, several PcG and trxG proteins have been found to modify histone tails (see previous sections). Therefore, histone tail modification has been proposed to be the epigenetic mark for inheritance. In particular, histone tail methylation was thought to be a likely candidate since no enzymes which reverse methylated histones have been discovered until recently (Lachner et al., 2003, Shi et al., 2004). The potential permanence of methylation coupled with a proposed semi-conservative inheritance of histones during DNA replication (Korber and Horz, 2004; Tagami et al., 2004) made methylation an attractive model. The trx and Ash1 trxG proteins have HMT (histone methyltransferase) activity as does the PRC2 complex, and loss of HMT activity correlates with loss of maintenance of active or repressed states by these MPs (Muller et al., 2002; Klymenko and Muller, 2004). However, arguments against semi-conservative histone deposition (Henikoff, 2004) reveal that over time, a histone mark would be diluted, then lost, making dependence on histone modifications as the sole epigenetic
mark problematic. In addition, a histone lysine demethylase conserved from yeast to humans was recently discovered, arguing that methylation is not necessarily a permanent modification (Shi et al., 2004). A possible alternative to histone modification is the use of histone variants as the epigenetic mark. Histone variants H3.3 and H2A.Z appear to mark active loci and are deposited using a replication-independent mechanism by novel deposition factors (Ahmad and Henikoff, 2002b; Henikoff et al., 2004). Histone variant MacroH2A is deposited on the inactive X in mammals (Chadwick and Willard, 2001) and may prevent transcription by preventing SWI-SNF nucleosome remodeling and transcription factor binding at promoters (Angelov et al., 2003).

Irrespective of the nature of the epigenetic mark that transmits transcriptional status, MPs must be involved in the inheritance and interpretation of the epigenetic mark rather than just its establishment. First, MPs are required continuously throughout development. The downregulation of PRC1 components in cultured Drosophila cells results in loss of silencing at the abd-A Hox gene, and loss of E(z) binding at the promoter is correlated with loss of H3K9 and H3K27 methylation after several cell divisions (Breiling et al., 2004). This argues that an epigenetic mark cannot continuously self-propagate without MPs and thus by itself is not sufficient for epigenetic inheritance. In addition, ME sequences themselves must be required to interpret the mark and re-establish transcriptional status during the next interphase as deletion of PRE sequences results in loss of silencing immediately in the next cell cycle (Sengupta et al., 2004). Though it is clear that MPs are required for the inheritance process, it is not known whether MPs employ a mechanism for epigenetic inheritance, which is distinct from the transcriptional activation, and repression models discussed.
VII. Maintenance Proteins and cell cycle progression

It is possible epigenetic inheritance has distinct mechanisms for interpreting transcriptional status to establish epigenetic marks, transmitting the epigenetic marks across a cell division, and interpreting the epigenetic marks in the daughter cells to re-establish correct transcriptional status. Though there is little evidence for the mechanism of these events, MPs are likely involved at all phases. Since these phases occur during the many biochemical events that accompany mitosis, it is likely that there is significant co-ordination between cell division and epigenetic inheritance. So far, proposed mechanisms do not consider the role that cell cycle progression may have on epigenetic inheritance, or vice versa, whether the inheritance mechanism involves cell cycle regulation. Passing on epigenetic information must involve some form of co-ordination with the cell cycle, yet links between cell cycle progression and the Polycomb group have not been fully explored.

Prior to this study, very little evidence indicated that there are associations between cell cycle regulators and the PcG. In *Drosophila*, the gene *ccf* (*centrosomal and chromosomal factor*, now known as corto) is required for proper mitotic progression and cell division (Kodjabachian *et al.*, 1998). Adult flies mutant for *ccf* show homeotic transformations and also enhance the homeotic phenotypes of flies mutant for the PcG genes *Polycomb*, *polyhomeotic* and *Enhancer of zeste*. A link between the PcG and DNA replication was made when PCNA, required for polymerase processivity during DNA replication, was shown to co-localize with the PcG protein Cramped on S-phase chromosomes (Yamamoto *et al.*, 1997). Mutations in the gene encoding *Drosophila* PCNA also enhance cramped homeotic phenotypes. Reduced mitotic frequencies and
proliferation defects have also been described for mutants of *Enhancer of zeste* (Phillips and Shearn, 1990), which was previously identified in a screen for essential cell cycle genes (Gatti and Baker, 1989).

PcG proteins were also observed to associate with chromatin in a cell cycle-dependent manner. In *Drosophila* embryos, Polyhomeotic (PH), Polycomb (PC), and Posterior sex combs (PSC) proteins are observed associated with chromatin at S phase, almost completely dissociate by metaphase and reassociate at telophase (Buchenau *et al.*, 1998). Similarly, BMI1, the human homologue of *Drosophila* PSC shows the same pattern in primary and tumour cell lines (Voncken *et al.*, 1999). Incidentally, murine Bmil, originally identified as an oncogene, is also required for regulating lymphoid cell proliferation via repression of the *ink4a* tumour suppressors locus (Jacobs *et al.*, 1999). Mel18, another mammalian Psc homologue, was originally identified as a tumour suppressor and was shown to inhibit cell cycle progression likely via repression of c-myc, leading to downregulation of cyclins and CDKs (Tetsu *et al.*, 1998). Two *Drosophila* PcG members also implicated in controlling cell proliferation were Multi sex combs (MXC), which behaved as a tumour suppressor (Santamaria and Randsholt, 1995) and Sex comb on midleg (SCM), which has multiple domains with homology to the lethal(3) malignant brain tumour (l(3)mbt) tumour suppressor protein (Bornemann *et al.*, 1996). Very recently, mammalian PcG proteins were shown to interact and co-localize with Geminin, a replication-licensing factor (Luo *et al.*, 2004).
VIII. Polyhomeotic and cell cycle progression

The most intriguing link between the PcG and the cell cycle was that early embryos mutant for the PcG genes polyhomeotic (ph) exhibited chromatin bridges at anaphase resulting from sister chromatids failing to properly segregate (Lupo et al., 2001). Chromatin bridges are chromatin that has failed to segregate with the rest of the chromosomes and therefore appear to join, or bridge, a pair of daughter nuclei. Furthermore, it was shown that one PH isoform physically interacts with the cell cycle proteins Barren and Topoisomerase II in vitro. Barren is a Drosophila protein homologous to a cofactor of the condensin complex, which in yeast is required for proper chromosome condensation after DNA replication (Lavoie et al., 2000), and in Drosophila is required for sister chromosome segregation (Bhat et al., 1996b). Topoisomerase II is a major component of the chromosome scaffold and is an enzyme that decatenates intertwined DNA strands (reviewed in Swedlow and Hirano, 2003).

In Drosophila, polyhomeotic is a complex locus with a tandem duplication encoding two distinct genes, polyhomeotic-proximal (ph-p) and polyhomeotic-distal (ph-d) (Dura et al., 1987; Hodgson et al., 1997). Mutations in either gene lead to very similar posterior homeotic transformations similar to LOF Hox phenotypes (Dura et al., 1985; Dura et al., 1987; McKeon and Brock, 1991).

It has long been assumed that the high degree of similarity of the phenotypes of ph-proximal and -distal mutants argues that the two genes are equivalent (Dura et al., 1987). However, Hodgson et al. (1997) revealed important sequence, regulation and functional differences between Ph-Proximal and Distal. Though the two proteins are indeed very similar with high levels of amino acid identity and similarity, there are two
unique regions in PH-P that differ markedly from PH-D. In addition, there are two isoforms of the PH-P protein, a larger 170kDa isoform (PH-P170), and a smaller 140kDa isoform (PH-P140), which originates from translation at an internal methionine. There are also differences in the regulation of transcription of the proximal and distal units. \textit{ph-proximal} has two distinct transcript sizes detected in early embryos. The abundance of each appears to be regulated during embryogenesis, in addition, sex-specific differences in adult flies were also observed. The abundance of the \textit{ph-distal} transcript differs from the \textit{ph-proximal} transcripts, and appears to be regulated differently during embryogenesis. Functional studies using a transgene containing the \textit{Ubx Hox} gene promoter with a PRE fragment and a LacZ reporter gene demonstrated that ectopic expression of the reporter has a different pattern in \textit{ph-proximal} mutants than for \textit{ph-distal} mutants. These data collectively support a functional difference between the genes.

As discussed, PH is a subunit of PRC1, a protein complex that is thought to prevent transcription by selectively blocking access to chromatin remodelers and transcription factors (Shao \textit{et al}., 1999; Francis \textit{et al}., 2001; King \textit{et al}., 2002). Interestingly, PRC1 contains both proximal and distal PH proteins (Shao \textit{et al}., 1999). A separate study identified a unique complex containing only PH-P isoforms in conjunction with heat shock cognate 4 (Hsc4), a novel chaperone (Droj2), TBP and some TAFs (Wang and Brock, 2003). Therefore, membership in different complexes is potentially another functional difference between PH-P and PH-D. In addition, it was only the 140kDa PH-P isoform which co-immunoprecipitated with Barren and Topoisomerase II (Lupo \textit{et al}, 2001). These differences all suggest unique functions for \textit{proximal} and \textit{distal} genes, and for Proximal protein isoforms.
IX. Early Drosophila embryogenesis as a model system for the study of mitotic progression

The novel cell cycle phenotype of ph mutants and the association of PH-P140 protein with the cell cycle regulators Barren and Topoisomerase II (discussed above) is the most intriguing evidence for a link between epigenetic maintenance and cell cycle progression. The focus of this thesis is to further explore this link between PcG-mediated inheritance and mitosis, with a focus on PH-P as a representative PcG member.

A convenient system to study mitotic progression is in early Drosophila embryos. The cell cycles of early embryos differ from those in later embryogenesis (reviewed in Tram and Sullivan, 2002). The first thirteen mitotic divisions are very rapid ranging from 10 to 25 minutes due to very abbreviated G1 and G2 phases. In essence, these early mitoses consist of alternating DNA replication and segregation phases, with no intervening gap phases. The rapidity of these divisions is made possible because they take place in a syncytium with no accompanying cytokinesis. Also, maternal proteins and RNA deposited during oogenesis dictate all early embryonic processes for the first three hours of development, making zygotic transcription to supply factors for mitotic processes unnecessary. The first thirteen mitotic cycles are also synchronous, allowing for simultaneous assessment of many nuclei at the same mitotic stage.

The first seven mitotic cycles occur in the interior of the embryo, followed by a migration of the majority of the nuclei to the outer cortex just below the plasma membrane, where they continue to divide in a monolayer until the fourteenth mitotic cycle. These cortical, or somatic nuclei are then partitioned into individual cells to become the cellular blastoderm. Cellularization, mitotic asynchrony and zygotic
transcription begin during the fourteenth mitotic cycle, at approximately 3 hours into development, immediately followed by gastrulation. Therefore, 0-3 hour embryos provide a convenient system to study large numbers of nuclei and assess any mitotic abnormalities in PcG mutants.

The lack of intervening gap phases between S and M phases of mitosis in the early syncytial divisions together with the mechanism that keeps nuclei dividing synchronously means that the cell cycle checkpoints of later divisions do not operate in the early embryos. Delaying the cell cycle to fix abnormalities would compromise the synchrony of the nuclear divisions (Tram et al., 2003). However, delays in cell cycle progression of individual nuclei do trigger a mechanism called nuclear fallout that internalizes the faulty nuclei to the embryo’s center, removing them from the cortical population and preventing them from becoming somatic nuclei (Sullivan et al., 1993). This system is efficient at keeping the nuclear divisions synchronous and rapid. Occurrence of nuclear fallout is a reliable readout of problems with mitotic progression during early embryonic cell cycles. Therefore, early Drosophila mitotic cycles provide a convenient assay system for assessing potential mitotic roles in the PcG.

X. Thesis Aims

The role of PcG proteins in the inheritance of transcriptional status of their targets through cell division is not known. Though it is likely that any such role involves coordination with cell cycle progression, there is very little data to support such a hypothesis. This thesis initiates the study of the potential role the PcG may have in cell cycle progression and vice versa, the role which cell cycle regulatory processes may have
in PcG-mediated maintenance. Chapter 2 is a thorough examination of mitotic phenotypes of a variety of PcG mutants with a focus on \textit{ph-proximal}. Chapter 3 attempts to characterize the cell cycle stage at which Ph-Proximal may act. Chapter 4 explores the possible contribution of cell cycle regulators to epigenetic maintenance. The discussion in Chapter 5 explores the contribution of this thesis and recent reports on the mechanism of epigenetic inheritance by the PcG and provides a model and a novel perspective of the co-ordination between maintenance and mitosis.
CHAPTER 2 Characterization of mitotic phenotypes in Polycomb group mutants

I. Introduction

Previous evidence linking the Polycomb Group with cell cycle regulators has been limited (see Introduction). In addition, the data are not consistent and there is no mechanistic link between the PcG and cell cycle progression. The most intriguing evidence is that early embryos of polyhomeotic mutants exhibit chromatin bridges between anaphase nuclei (Lupo et al., 2001). This data highlighted a novel phenotype for polyhomeotic and a possible new role for a gene classically studied as an epigenetic regulator. In addition, the co-immunoprecipitation of the 140kDa isoform of Ph-Proximal with Barren and Topoisomerase II (Lupo et al., 2001) suggested a possible mechanism via known cell cycle regulators involved in chromatin condensation. This data was persuasive, but was only one of a disparate collection of reports that linked the PcG to the cell cycle. Given this, two major questions emerged which form the basis of this study. First, do the PcG as a whole have a role in mitosis and cell cycle progression? Second, is regulation of cell cycle progression part of the epigenetic inheritance process?

To begin to answer the first question, the simple approach taken was to ask whether the chromatin bridge phenotype described for polyhomeotic was unique, or whether other PcG mutants exhibit segregation defects as well. The strategy was to examine early embryos of other PcG mutants for the segregation phenotypes described for polyhomeotic. However, since there are many dozens of alleles of the approximately 15 PcG members cloned (Fisher and Brock, 2005; Orlando, 2003), a select group of PcG mutants were chosen based on known broad classifications of PcG members. The PcG
mutants chosen included *polyhomeotic*, *Polycomb* and *Posterior sex combs* as representative members of the PRC1 PcG complex (Shao et al., 1999), and *Enhancer of zeste* as the representative of the PRC2 complex (Kuzmichev et al., 2002). At the time this study was initiated, PRC2 was known as the Esc-E(z) complex (Ng et al., 2000; Tie et al., 2001). Two representative PcG members which were not found in either complex, *cramped* and *Additional sex combs* were also chosen. Of this group of six PcG members, *Additional sex combs, Enhancer of zeste* and *Posterior sex combs* are also classified as ETP group genes as they enhance the homeotic phenotypes of both PcG and trxG genes, whereas *polyhomeotic* and *Polycomb* are PcG group genes which enhance homeotic phenotypes of PcG genes and suppress those of trxG genes (Gildea et al., 2000) as outlined in Chapter 1. It is currently unknown whether *cramped* enhances or suppresses trxG homeotic phenotypes, therefore it has not been classified as either PcG or ETP. To determine if any of these six PcG mutants exhibited chromatin bridges at anaphase, the DNA of 0-3 hour embryos of laboratory stocks were stained and examined for segregation defects using confocal microscopy.

Though *polyhomeotic* was previously shown to have an anaphase bridge phenotype, Lupo et al., (2001) only provided one image in support of this observation, with no report on the penetrance or expressivity of the phenotype compared to wild-type embryos. In addition, there was no systematic examination of embryos for other mitotic phenotypes. This limited assessment of the embryos in the absence of negative controls restricts the conclusions that can be drawn from the report and demands a comprehensive re-examination of the segregation defects in *polyhomeotic* mutants.

Given the rapid nuclear divisions and lack of DNA repair mechanisms during
early embryogenesis (Tram and Sullivan, 2002), it is reasonable to expect a certain basal frequency of mitotic defects to occur even in wild-type embryos. Therefore, a thorough evaluation of the penetrance and expressivity of segregation phenotypes is required for both PcG mutant and wild-type embryos to ensure any mitotic defects encountered in PcG mutants are significantly above wild-type levels. In this study, both embryo collections and documentation of the mitotic defects were standardized (See Materials and Methods) to allow comparison of the penetrance and expressivity of segregation defects to wild-type levels and also between different PcG mutations and alleles.

Standardized collection and fixation procedures with a negative control also circumvent the skewing of results due to the response of *Drosophila* to hypoxic conditions. Hypoxia and anoxia result in chromatin bridges and other mitotic defects (DiGregorio *et al.*, 2001; Douglas *et al.*, 2001). Therefore, each collection of PcG mutant embryos and was accompanied by a simultaneous collection of control wild-type embryos. Having a control group for each collection ensures that the frequency of mitotic defects scored for PcG mutants is not an artifact of the collection or fixation procedures.

The occasional defective nuclei resulting from the rapid mitoses in early embryos are removed from the nuclear population by the nuclear fallout mechanism (Tram *et al.*, 2003). Therefore, if PcG has a role in mitotic progression, any delays in the mitotic program due to the PcG mutation may trigger the nuclear fallout mechanism at a much higher frequency than the occasional mitotic defect in wild-type embryos. This prediction was tested by examining the sub-cortical space of PcG mutant and wild-type embryos for evidence of fallout nuclei. Since nuclear fallout is only visible after nuclear migration to the cortex following mitotic cycle 8, PcG and wild-type embryos between
mitotic divisions 9-13 (corresponding to Bownes' stage 4) were examined for nuclear fallout.

The anaphase bridges described for *polyhomeotic* are identical to those of *barren* mutant embryos (Bhat *et al.*, 1996b). The shared mitotic phenotype and the co-immunoprecipitation of Polyhomeotic with Barren and Topoisomerase II suggested that the role of Polyhomeotic in proper sister-chromatid segregation is dependent on chromatin condensation and DNA decatenation (Lupo *et al.*, 2001). Though there is no experimental evidence, this hypothesis implies that problems with chromatin condensation and sister chromatid decatenation leads to the segregation failures in *barren* and *polyhomeotic* embryos. Alternatively, the anaphase bridges may simply be abnormally long chromosomes due to incomplete condensation (Lavoie *et al.*, 2000).

Since mitotic defects of *polyhomeotic* were not fully described, it is impossible to infer the cause of chromatin bridges without thorough phenotypic analysis. If chromatin bridges are due to defects in chromosome condensation, then in addition to segregation defects, condensation phenotypes may be visible in *polyhomeotic* and other PcG mutant embryos. The most direct approach to test this prediction was to examine the nuclei of PcG mutant embryos at metaphase at which time chromosomes are in their most compact state, and look for evidence of defects in chromatin condensation.

If mitotic defects are common to many PcG mutants, then there may be a link between epigenetic regulation by the PcG and cell cycle progression. Furthermore, regulation of the cell cycle may be a necessary component of the epigenetic inheritance process. In order to focus efforts on answering these questions, one representative PcG member was chosen to further study a possible role in cell cycle progression. Our
laboratory has published extensive data on *polyhomeotic* as an epigenetic regulator. Because *polyhomeotic* mutants were previously shown to have a mitotic phenotype, all further experiments will focus on *ph* mutants.

However, as discussed in Chapter 1, the *polyhomeotic* locus is a tandem duplication with a proximal and distal gene (Dura *et al.*, 1987). The *ph*$_{504}$ deletion used in this study and that of Lupo *et al.*, (2001) is a large deletion encompassing both genes at the *ph* locus. Though the two genes are nearly identical, there are two unique regions in the ph-proximal protein that differ markedly from ph-distal, in addition, there are differences reported in the expression and regulation of the two genes during embryonic development (Hodgson *et al.*, 1997). Since *ph*$_{504}$ deletes both the proximal and distal loci, it was necessary to determine whether the mitotic phenotype was due to a deficiency of *ph*-proximal, *ph*-distal, or was an effect of having both loci deleted. The approach to answering this question was to examine proximal- or distal-specific mutant embryos for mitotic defects and compare penetrance and expressivity to wild-type and *ph*$_{504}$ mutants. Given that an isoform of Ph-Proximal co-immunoprecipitated with Barren and TopoII, the prediction is that only the *ph*-proximal mutation will exhibit mitotic defects. If this hypothesis is true, it would be the first major phenotypic difference described and represent a key functional difference between the proximal and distal genes.

As discussed in Chapter 1, early embryonic cell cycles are unique among embryonic mitotic divisions due to the rapid and synchronous nuclear divisions within a syncytium. Given these unique circumstances, it is possible that any role of Polyhomeotic in cell cycle progression is limited to these early embryonic nuclear divisions, and that the later developmental role for Polyhomeotic is restricted to
epigenetic regulation. To address this question, brains from third-instar wandering stage larvae of polyhomeotic mutants were dissected and examined for mitotic phenotypes. Larval brains are an ideal tissue to use in this assay because PH is expressed (Fauvarque et al., 1995). In addition, since a subset of brain cells is mitotically active, brains have been classically employed for studying zygotic cell cycle defects (Gatti and Baker, 1989). The presence of mitotic defects in larval brains would suggest that the cell cycle role of polyhomeotic potentially encompasses all mitotic divisions, and that early embryos are an ideal model system to further study the cell cycle role of the PcG.

II. Results

i. Mitotic defects are not restricted to polyhomeotic mutants

To determine whether the segregation phenotype described for polyhomeotic was unique or shared by other PcG members, early embryos of six PcG mutants were chosen to examine for mitotic phenotypes. For each PcG mutant, 0 to 3 hour-old embryos were collected, fixed and stained with the nucleic acid dye TOTO-3 (Molecular Probes) as described in Materials and Methods. Each embryo up to mitotic stage 13 (end of Bownes’ stage 4) was examined for mitotic defects. Mitotic stage 14 embryos are undergoing the switch to zygotic transcription and were therefore not included in this analysis. Cycle 14 embryos are easily identified because they possess approximately 6000 very small nuclei, which are beginning to divide asynchronously (Tram et al., 2003).

The most striking mitotic phenotype observed in PcG mutant embryos was chromatin bridges at anaphase and telophase indicating a failure of sister chromatids to
properly segregate (Figure 2-1 and 2-2). Chromatin bridges appear as brightly staining DNA bridging two sets of chromosomes that have passed the metaphase to anaphase transition and have attempted to segregate to their respective poles. Completely segregated anaphase nuclei appear as independent entities with no intervening chromatin bridging the space between them (Figure 2-1A). In PcG mutant embryos, chromatin bridges at anaphase were frequently detected. Examination at a high magnification revealed late anaphase nuclei connected by chromatin bridges of varying thicknesses (Figure 2-1 B-D). The bridges may be extensive and thick as in Figure 2-1B giving the joined nuclei an irregular shape such that they are almost unrecognizable as anaphase nuclei, or the bridges may be thin and spindly as in Figure 2-1C and D, appearing as thin chromatin connections between otherwise normally-segregated sets of sister chromatids. To eliminate false positives, sister chromatids in the early stages of anaphase were not scrutinized for presence of chromatin bridges. At early anaphase, sister chromatids are normally in very close proximity (Figure 2-1E) therefore any connections observed between sister chromatids at the early stages of anaphase are likely part of the normal segregation process. Fully segregated late anaphase nuclei are separated from each other by a distance of approximately 2μm (Figure 2-1C), therefore any chromatin observed to be joining anaphase nuclei separated by this distance was scored as an anaphase bridge. This criterion was applied to all anaphase figures. It is possible that a portion of the thin chromatin bridges at anaphase (see Figure 2-1D) is due to the tendency of the X chromosome to segregate behind the autosomes. It was not possible to distinguish this event from a segregation defect therefore a proportion of thin anaphase bridges may be false positives. However, most chromatin bridges were very thick chromatin joining
anaphase nuclei as in Figure 2-1A. In addition, since the same scoring criteria was also applied to wild-type embryos, any false positives due to latent X-chromosome segregation will be negated by the negative controls. Figure 2-1F illustrates how scanning of embryos at low power allows easy identification of anaphase bridges since the nuclei appeared as very long and oval-shaped rather than the rounded fully separated nuclei.

Chromatin bridges are also found at telophase (Figure 2-2). Telophase nuclei have decondensed chromatin and are preparing to enter the next mitotic cycle. In wild-type embryos, telophase nuclei appear as completely separate round entities with no intervening bridging chromatin (Figure 2-2 A). In contrast, chromatin bridges often joined telophase nuclei in PcG mutant embryos. As in anaphase bridges, there is brightly staining chromatin joining two nuclei that have attempted to segregate. Telophase nuclei joined by bridges are easily identified by their “dumbbell” shape (Figure 2-2 B-D). Telophase bridges were highly penetrant and expressive where often more than 50% of nuclei pairs were joined (see Figure 2-2B as an example). The chromatin bridges were found at various thicknesses indicating that different amounts of chromatin were left behind at the midline, unable to segregate to opposite poles (Figure 2-2D).

Often, bridged nuclei are clustered in clearly defined “zones” within the embryo. Figure 2-3 illustrates an extreme example where the nuclei that are joined by chromatin bridges are in a zone that extends approximately halfway along the length of the embryo. Beyond this mark are completely segregated telophase nuclei with no bridging chromatin.
Figure 2-1. (next page) Early embryos of PcG mutants have chromatin bridges at anaphase and telophase. Embryos were fixed and their DNA stained with nucleic acid dye TOTO-3 as described in Materials and Methods. The maternal genotype is indicated in the top left of each panel, and the scale bar at the lower left of each panel represents 10μm. (A) An example of completely normal anaphase from a wild-type embryo. All segregating nuclei (arrows) are separated from their former sister chromatids with no chromatin bridges visible in the space between them (arrowhead). (B) Late anaphase nuclei joined by thick chromatin bridges (arrow) from embryo of \( ph^{504}w/FM7C \) mutant mothers. In this field, all segregating nuclei are joined by very thick chromatin bridges and are almost unrecognizable as anaphase nuclei. (C) Examples of embryos with low-frequency anaphase bridges. In each panel, examples of bridged nuclei are highlighted with the arrow, while completely segregated normal-looking nuclei are shown with the arrowhead. In both panels, the arrow highlights late anaphase nuclei separated by approximately 2μm, but still joined by a chromatin bridge. This distance criterion was used to score connections between anaphase nuclei as a chromatin bridge. The expressivity was scored as low in these instances whereas in B, it was scored as high frequency. (E) Early anaphase nuclei are in such close proximity that it is difficult to distinguish between naturally lagging chromosomes and chromatin bridges. Chromatid arms appearing close together in early anaphase nuclei (arrows) were not scored as anaphase bridges. (F) Even at low magnification, bridged late anaphase nuclei are identifiable as oblong nuclei joined by chromatin (arrow) and easily distinguished from fully segregated nuclei (arrowhead).
Figure 2-1. See legend on previous page.
Figure 2-2. Early embryos of PcG mutants have severe chromatin bridges at telophase. The maternal genotype is indicated in the top left of each panel, and the scale bar at the lower left of each panel represents 10\(\mu m\). (A) An example of completely segregated telophase nuclei (arrows) from a wild-type embryo. (B, C) Examples of high-frequency telophase bridges where approximately 50% of nuclei pairs are joined by chromatin bridges. (D) Example of varying thicknesses of chromatin bridges. The chromatin bridges joining telophase nuclei may be very thick (arrow) or appear as thin, spindly connections (arrowhead). Still, other nuclei pairs have completely segregated and appear wild-type (chevron).
Figure 2-3. Nuclei joined by chromatin bridges are often clustered in zones. An early embryo of the PcG mutant *polyhomeotic* exhibits severe chromatin bridges joining telophase nuclei. In this case, the joined nuclei only occupy approximately the top half of the embryo's length, below which appear completely segregated telophase nuclei. A detail of the bridged telophase nuclei is shown in the inset. The scale bars represent 10μm.
ii. The penetrance and expressivity of segregation defects in PcG mutants is significantly above that found in wild-type embryos

In order to determine the significance of the segregation phenotypes and be able to compare between PcG mutants, the penetrance and expressivity of chromatin bridges were examined. Standardized embryo collection and fixation procedures were followed, and each PcG embryo collection was accompanied by a simultaneous collection of wild-type embryos. Each embryo in the collection was examined for mitotic stage and the presence of chromatin bridges at anaphase or telophase. It is important to note that since only one surface of the embryo was viewable under the microscope, all embryos were categorized based on the phenotype at their visible surface only. Also, embryos at mitotic stages 1 to 8, prior to nuclei migrating to the cortex, were never observed to have chromatin bridges, for any PcG mutant tested. Therefore, only embryos with anaphase or telophase nuclei residing in the cortical monolayer (mitotic stages 9-13) were included in this analysis. Nevertheless, all embryos at anaphase and telophase, regardless of mitotic stage were thoroughly examined for mitotic defects.

The frequency of anaphase and telophase stage embryos in PcG mutants did not differ markedly from those in the wild-type collections (Figure 2-4A) indicating no mitotic arrest. The penetrance of chromatin bridges within the anaphase and telophase embryos is significantly above that of wild-type embryos. The average for all PcG embryos examined was 84% of anaphase and telophase embryos exhibiting chromatin bridges, while the average for the wild-type collections was 28%. This demonstrates that the segregation defect previously described for polyhomeotic mutant embryos is shared by other PcG mutants and in all cases is significantly above wild-type levels. To further
assess differences between wild-type and PcG mutants and for comparison between PcG mutants themselves, the expressivity of the segregation defects was carefully examined as well. For each collection, all embryos at anaphase and telophase were categorized as having high-frequency, low frequency or no chromatin bridges (Figure 2-4B). The criteria for having high frequency bridges was that over 25% of visible nuclei pairs must be joined. Low frequency bridges were scored when less than 10% of visible nuclei pairs were joined. 10% is an arbitrary number. Generally, the low-frequency embryos had approximately 5% joined nuclei while the high frequency embryos were consistently at minimum 40% joined nuclei. The phenotype of joined nuclei was either very severe or mild, with no intermediate distribution.

All PcG mutants exhibited high expressivity with at least half of all embryos exhibiting chromatin bridges with at least 40% of nuclei joined (see Figure 2-2B as an example). Such high-frequency segregation defects were never observed in wild-type embryos. On average, 72% of the wild type embryos at anaphase or telophase appeared completely normal, with no visible chromatin bridges whereas only 15% of PcG derived embryos on average were phenotypically normal. The expressivity for polyhomeotic was the most severe out of all the PcG mutants tested. 8 of the 10 embryos at anaphase and telophase exhibited very severe segregation defects while the remaining 2 embryos exhibited low-frequency defects. No embryo at either anaphase or telophase was phenotypically normal. The result for polyhomeotic confirms earlier reports of anaphase bridges in $ph^{504}$ mutants (Lupo et al., 2001) and also demonstrates that the chromatin bridges persist to telophase. The frequency of severe chromatin bridges is very similar for each PcG mutant, though polyhomeotic appears to be the most severe. Moreover, the
Figure 2-4. (next page) Embryos from PcG mutant mothers have a high frequency of segregation defects. 0-3 hour embryos of the genotypes indicated were examined for penetrance and expressivity of anaphase and telophase bridges. Embryos from a single collection were fixed and stained as described in materials and methods, then mounted on a single slide. (A) Comparison of the frequency of anaphase and telophase embryos encountered in PcG mutant embryos (in bold) compared to wild-type. There is no great variation with the frequency of anaphase and telophase embryos between PcG mutants and wild-type embryos indicating no metaphase arrest. The average for all PcG collections was 7.8\% of all embryos and for Oregon-R was 7.12\% of all embryos examined. The penetrance of segregation defects in anaphase and telophase embryos is significantly greater in PcG embryos (average of 84\%) than wild-type (average of 28\%). (B) Examination of the expressivity of the segregation defects reveals further differences between wild-type and PcG embryos. All embryos at anaphase and telophase were categorized as having either high frequency of chromatin bridges (over 25\% of nuclei pairs were joined, red bars), low frequency (less than 10\% nuclei pairs joined, light blue bars) or no visible chromatin bridges (white bars). All PcG mutant embryos exhibited high penetrance and expressivity of chromatin bridges compared to control wild-type embryos. Though a proportion of wild-type embryos did exhibit chromatin bridges, the expressivity was very low with only one or few joined nuclei pairs per embryo, if any.
A  The penetrance of segregation defects in PcG mutants.

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>(n) Embryos surveyed</th>
<th>% at ana/telophase</th>
<th>% segregation defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>$ph^{504}$w/FM7C</td>
<td>142</td>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>209</td>
<td>7.7</td>
<td>25</td>
</tr>
<tr>
<td>$Pc^{XT109}$/TM6C</td>
<td>182</td>
<td>6.6</td>
<td>83</td>
</tr>
<tr>
<td>$Pc^{4}$/TM6C</td>
<td>180</td>
<td>8.8</td>
<td>62.5</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>155</td>
<td>7.1</td>
<td>20</td>
</tr>
<tr>
<td>$Psc^{1}$/CyO</td>
<td>164</td>
<td>9.1</td>
<td>80</td>
</tr>
<tr>
<td>$Psc^{15}$/CyO</td>
<td>109</td>
<td>8.3</td>
<td>37.5</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>148</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>$crm^{3}$/TM3Sb</td>
<td>169</td>
<td>7.1</td>
<td>83</td>
</tr>
<tr>
<td>$crm^{13}$/TM3Sb</td>
<td>126</td>
<td>7.1</td>
<td>89</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>175</td>
<td>7.4</td>
<td>38</td>
</tr>
<tr>
<td>Asx^{1}$/CyO</td>
<td>138</td>
<td>8.0</td>
<td>91</td>
</tr>
<tr>
<td>Df(trix)CyO</td>
<td>156</td>
<td>8.3</td>
<td>78</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>113</td>
<td>7.0</td>
<td>19</td>
</tr>
</tbody>
</table>

B  The expressivity of segregation defects in PcG mutants.

Figure 2-4. See legend on previous page.
wild-type control embryos exhibited consistently low penetrance and low expressivity
segregation defects in every collection, indicating that the segregation phenotypes
observed for the PcG mutants were significant, and not due to hypoxic conditions or
fixation artifacts.

iii. Segregation defects are first detected following nuclear migration to the cortex

All mitotic divisions under maternal control are potentially subject to mitotic
errors. However, not all early embryonic mitoses are equivalent. The first nine of the
thirteen synchronous embryonic mitoses occur within the interior of the embryo (Figure
2-5A) and are roughly 10-14 minutes long (Tram et al., 2002). During the 9th and 10th
mitotic divisions, the nuclei migrate to the embryo’s cortex and continue dividing in a
monolayer. After cortical migration, the mitotic cycles approach 25 minutes in length
until the 14th division, at which time nuclei begin dividing in asynchronous zones.
Segregation defects in all the PcG mutants tested were only found in mitosis 9 or later
(Figure 2-5B), when nuclei have moved to the cortex of the embryo. No segregation
errors, not even thin chromatin bridges between a single pair of anaphase or telophase
nuclei were observed prior to cortical migration, in any of the embryos surveyed.

iv. There are many mitotic phenotypes in addition to segregation defects

As discussed in Chapter 1, the nuclear fallout mechanism in early embryos
ensures the removal of faulty nuclei from the cortical population and internalizes them
into the yolk. Evidence of nuclear fallout is an excellent indicator of problems with
mitotic progression. Therefore, the embryos collected from each PcG mutant were
Figure 2.5. Mitotic defects appear following the onset of mitotic cycle 10 after the nuclei have migrated to the surface of the embryo. (A) An example of a PcG mutant embryo with normal nuclei prior to mitotic stage 10. The nuclei have not migrated to the cortex, and are still in the interior of the embryo (indicated by double arrows). The diagram at the top right of the panel indicates that the confocal images were taken from the middle of the embryo, where the nuclei reside at this stage. No mitotic defects were observed from any PcG mutant embryos at this stage. The large starburst-shaped set of chromosomes (arrow) are the remnants of the meiotic sisters of the female pronucleus, which is visible in every embryo until breakdown at mitotic stage 10 (Foe et al., 1993). (B) Mitotic stage 10 embryo from ph504 mothers, immediately after nuclear migration to the cortex. It is only at this stage or later that any evidence of mitotic defects are encountered (arrow). The diagram at the top right of the panel indicates that the confocal stack was taken of surface nuclei only. The scale bar represents 10µm in both panels.
Figure 2-5. See legend on previous page.
examined for the presence of fallout nuclei. Fallout nuclei are found in the sub-cortical space between the yolk and the surface nuclei in mitotic stage 9-13 embryos. Mitotic stage 14 embryos were not included in this assay because gastrulation begins shortly after this stage. This ensures that normal involuting and migrating cells are not mistaken for fallout nuclei.

In order to distinguish fallout nuclei from the normally-occurring internal yolk nuclei, nuclear fallout was only scored when nuclei were found at 2-20 μm below the cortex (Figure 2-6A, left panel, double arrow). Below this plane is the more dense internal region of the embryo where yolk nuclei and yolk protein resides (black area with arrow). Within 2-20 μm below the cortex, the fallout nuclei are still in the process of internalization, and were scored as such. In wild-type embryos, nuclear fallout appears at very low penetrance and expressivity, with most embryos having no fallout nuclei at 2-20 μm below the cortex (Figure 2-6 A, middle panel). In cases where nuclear fallout was detected, usually only one or a few nuclei have fallen out (right panel).

Embryos of PcG mutants have a severe nuclear fallout phenotype (Figure 2-6B-D). Nuclear fallout is visible as a “hole” within the regular pattern of nuclei at the cortex of the embryo, as if nuclei were missing from the population (Figure 2-6 B, left panel). Below the hole are the missing nuclei in the process of being internalized (right panel). In PcG mutants, fallout nuclei are generally in larger clusters or in pairs, with multiple fallout events in each embryo (Figure 2-6B). The fallout nuclei are most often round and appear similar to late telophase nuclei (Figure 2-6C).

The penetrance and expressivity of the nuclear fallout phenotype was determined for each PcG mutant tested (Figure 2-7) and is significantly above wild-type for each PcG
Figure 2-6. (next page) Nuclear fallout in early embryos of PcG mutants. Parental genotype is indicated at the top left of each panel. Scale bar represents 10μm in each panel. (A) Fallout nuclei are different than the internal yolk nuclei of embryos. The left panel illustrates the internal yolk nuclei found at the center of all embryos (arrow), approximately 30-50μm below the cortex. Fallout nuclei on their way to the center were scored at 5-25μm below the cortex (grey area, double-arrow) in order to distinguish fallout events from yolk nuclei. The cartoon at the lower right of the panel indicates the projection to generate this image was from a stack of confocal sections extending to the middle of the embryo, approximately 30μm. Wild-type embryos have very low penetrance of the fallout phenotype. Most wild-type embryos have either no fallout events (middle panel) or generally only one or two fallout nuclei per embryo (right panel, arrow). The center and right images were generated from a thin stack of confocal sections, approximately 5μm below the cortical surface. The entire sub-cortical space of each embryo was scanned for fallout nuclei.

(B) Evidence of nuclear fallout at the cortex of a polyhomeotic mutant embryo. Normally, the pattern of cortical nuclei is regular, covering the entire embryo with no obvious spaces. In the case of nuclear fallout, the cortex appears to have “holes” or breaks in the regular spacing of the nuclei (left panel, arrows). The holes are the result of nuclei falling out from the cortex into the interior of the embryo. Focusing approximately 5μm below the cortex, the fallout nuclei are visible directly below the area of the holes (right panel, arrows). (C) In PcG mutants, there are multiple fallout events in embryos. The fallout nuclei tend to be in clusters, as in (B), or in pairs as illustrated in (C). The left panel is the anterior end of the embryo, the right panel is the posterior. In this embryo, most fallout nuclei are in pairs and in close proximity, suggesting that the nuclei originated as sister chromatids. (D) In PcG mutants the morphology of the fallout nuclei most closely resembles late telophase, early S-phase nuclei (arrows).
Figure 2-6. See legend on previous page.
Figure 2-7. Embryos from PcG mutant mothers have a high penetrance and expressivity of nuclear fallout. 0-3 hour embryos of the genotypes indicated on the X-axis were examined for penetrance of high and low frequency fallout nuclei. Embryos from a single collection were fixed and stained as described in materials and methods, then mounted on a single slide. All mounted embryos with nuclei residing at the cortex (between mitotic stages 9 and 13) were surveyed for fallout nuclei. Each occurrence of nuclear fallout was categorized as either high frequency (red bars) or low frequency (light blue bars). The total number of embryos surveyed is the same as indicated in the Table of Figure 2-4A. Embryos from PcG mutants have a high penetrance of nuclear fallout when compared to wild type. There is some variability between the different PcG mutants, but nuclear fallout is consistently much more penetrant than in wild-type embryos.
mutant. Nuclear fallout was scored as being either low-frequency or high frequency.

Low frequency was scored as 5 fallout nuclei or less per embryo, and high frequency was any number of nuclei above that. Five fallout nuclei is again an arbitrary number. Most low-frequency fallout events had only a few fallout nuclei per embryo, similar to what is seen in wild-type embryos (See Figure 2-6A). High frequency fallout events were generally much more severe than 5 nuclei, with larger clusters of nuclei and multiple fallout events per embryo (See Figure 2-6B). Again, there was no intermediate distribution for the number of fallout nuclei. All PcG mutant alleles that showed severe segregation defects also showed a severe fallout phenotype.

Since all embryos collected and stained were carefully examined, a number of low-penetrant mitotic phenotypes were observed. Normally, early embryonic mitotic divisions are synchronous. A mitotic “wave” starts at one pole of the embryo and travels medially, resulting in the polar nuclei at a mitotic stage slightly ahead of the nuclei at the midline of the embryo (Figure 2-8A), a temporal difference which correlates with differences in Cyclin B distribution (Foe and Alberts, 1983; Ji et al., 2004). Nevertheless, usually only two mitotic stages are observed in a given embryo, with each mitotic stage in a clearly defined zone of similarly-staged nuclei (Figure 2-8A). In PcG mutants, some embryos exhibited mitotic asynchrony, with nuclei at all mitotic stages appearing within a single embryo, without clearly defined zones (Figure 2-8B).

Another low-penetrant phenotype was giant nuclei, often irregularly-shaped with multiple lobes or oval-shaped with an extremely large size (Figure 2-9A). Normally, telophase nuclei are 2-3μm in diameter whereas these large nuclei were 5-7μm in diameter at their widest point and may represent extreme segregation defects or polyploid
Figure 2-8. (next page) Embryos of PcG mutants experience mitotic asynchrony. (A) Mitotic division in wild type embryos is synchronous as the mitotic "wave" originates at the anterior and posterior poles of the embryo and travels towards the midline. Nuclei in the same neighbourhood thus tend to be at the same mitotic stage, denoted in the figure by striped lines. LA= late anaphase, EA= early anaphase, M=metaphase plate. Neighbouring nuclei in a given zone are synchronously dividing. (B) In PcG mutant embryos, synchronous nuclei appear as intermingled patches, rather than clearly defined zones. In this figure, metaphase nuclei are outlined with a white dotted line, early anaphase nuclei with a black dotted line, and late anaphase nuclei with a black dashed line. The scale bar represents 10μm in both panels.
Figure 2-8. See legend on previous page.
nuclei. A small proportion of embryos exhibited nuclei that were much smaller and more brightly staining than surrounding neighbouring nuclei (Figure 2-9B, arrows, arrowheads). These did not appear to be chromosome fragments since they stained very brightly, likely indicating a normal complement of DNA and a high degree of compaction. However, chromosome fragments were also observed, as very small fragments of DNA staining at levels similar to neighbouring nuclei (Figure 2-9C, arrows). Occasionally, some polyploid nuclei were observed (Figure 2-9D). Polyploid nuclei were only scored at the late prometaphase or metaphase stage since chromosome condensation facilitated easy counting of ploidy. These polyploids are likely the same species as the large bulbous nuclei seen in Figure 2-9A, but were observed at metaphase instead of telophase.

Other low-penetrant phenotypes included chromatin bridges that persisted to the prometaphase stage of the next cell cycle (Figure 2-10A). Normally, chromatin bridges were observed at only anaphase and telophase. In a few embryos, however, nuclei at prometaphase (identified by unevenly-staining chromatin in the process of condensing into individual chromosomes) were also bridged by chromatin.

Evidence of gaps or breaks within the chromatin bridges was also very infrequent. Only in a few embryos did small gaps appear within the bridging chromatin. These gaps were either found within very thin chromatin bridges (Figure 2-10B, both panels) or within thicker chromatin bridges revealing branched, or forked chromatin (Figure 2-10C). Together, all low-penetrant mitotic phenotypes accounted for 4-10% of total mitotic defects observed in the PcG embryos.
Figure 2-9. (next page) Embryos of PcG mutants have a variety of low-penetrant mitotic phenotypes. (A) Tri-lobed nuclei (arrow, left and center panels) and large oval nuclei (arrowheads, center and right panels) both appear to be unsuccessful attempts at segregation. (B) Small very brightly staining nuclei (arrows, all panels) appear in a field of more diffusely stained normal sized nuclei. These may be highly compacted in comparison to the wild-type surrounding nuclei. (C) Small chromosome fragments (arrows, all panels) usually appear adjacent to large nuclei (arrowheads, all panels) and may represent non-disjunction events resulting from unsuccessful segregation. Note the large nucleus in the right panel (arrowhead) has a clumpy appearance and resembles a cluster of chromosome fragments. The center panel also shows a highly compacted nucleus (chevron) resembling those described in (B). (D) A large polyploid nucleus at the metaphase stage (arrow) appears to have at least three times the 4N genomic complement normally found at metaphase in a wild-type nucleus (arrowhead). Scale bars represent 10 \( \mu \text{m} \) in all panels.
Figure 2-9. See legend on previous page.
Figure 2-10. (next page) More low-penetrant mitotic phenotypes in embryos of PcG mutants. (A) Occasionally, chromatin bridges were observed at prometaphase. In this embryo, the nuclei at the top portion appear to be at the late S-phase/prometaphase boundary as seen by punctate bright spots of chromatin within the nuclei indicating condensing chromatin. In contrast, previous figures have shown chromatin bridges between nuclei at late telophase/pre S-phase which appeared uniformly-staining indicating a uniformly decondensed state. Towards the bottom of the figure, nuclei have progressed to later prometaphase as chromatin appears to be more condensed and beginning to organize into distinct chromosomes. What appear to be chromatin bridges are observed joining these late prometaphase nuclei together (arrows). (B) Evidence of chromosome strand breakage is also at very low penetrance. In these panels, projections of chromatin appear between pairs of telophase nuclei, with a distinct space at the midline (arrowheads, both panels). These projections resemble thin chromatin bridges, and the space may be a breakage of the DNA strand. (C) Further evidence for DNA strand breakage. In this embryo, a pair of late anaphase nuclei also has a chromatin bridge which appears to be broken (arrowhead, left panel). Zooming in on the bridge reveals chromatin that appears to be branched, or forked, with the one half of the fork “broken” (arrow, right panel) while the other half is still connected (arrowhead, right panel). The scale bar represents 10µm in all panels.
Figure 2-10. See legend on previous page.
v. Chromatin condensation is normal in most PcG mutants

Since Polyhomeotic was shown to co-immunoprecipitate with Barren and Topoisomerase II, two cell cycle proteins involved in chromatin condensation, embryos of PcG mutants were examined for defects in chromosome condensation. At metaphase, sister chromatids are at their most condensed state and congress at the midline of the mitotic axis prior to segregation. All PcG embryos with nuclei at the metaphase plate were carefully examined for any puffs of under-condensed chromatin or uncondensed nuclei within a field of metaphase nuclei.

Embryos of PcG mutants that exhibited segregation defects did not have under-condensed chromatin phenotypes at metaphase (Figure 2-11 A, B). All metaphase figures appeared normal. In the rare occasions that puffy nuclei with uncondensed chromosomes did appear within a field of compact metaphase plate nuclei (Figure 2-11C), it was likely they were asynchronous nuclei lagging behind the rest of the field rather than a failure to condense or decondensation. These puffy nuclei appear similar to early prometaphase nuclei that are starting to condense some areas of chromatin.

Interestingly, one PcG mutant, Enhancer of zeste (E(z)) was uncovered many years ago in a screen to identify essential cell cycle genes in Drosophila (Gatti and Baker, 1989). As for other PcG mutants, early embryos of two alleles of E(z) mutants were collected, fixed and stained as described, and examined for both condensation defects and segregation defects. In wild-type embryos, metaphase chromosomes appear very highly compacted and are uniformly and brightly-staining (Figure 2-12A, left panel). In contrast, early embryos of E(z) mutants exhibited significantly under-condensed chromatin at metaphase, causing the metaphase plate width to increase significantly from
Figure 2-11. (next page) Embryos of PcG mutants have normal metaphase figures with no defects in chromosome condensation. (A) $ph^{504}$ embryos show tightly condensed chromosomes at metaphase with no puffs of decondensed chromatin. A wild type embryo is shown in the inset for comparison. (B) Normal metaphase in the early embryo from a Polycomb mutant. (C) Uncondensed nuclei (arrow) amidst metaphase nuclei may be a case of mitotic delay rather than defects with condensation. These nuclei appear uniformly undercondensed, appearing as wild-type prophase or early prometaphase nuclei. This field also shows nuclei at other stages of condensation (late prometaphase nuclei, arrowhead and metaphase nuclei, chevron), further arguing that the low frequency of nuclei that appear undercondensed in a group of metaphase nuclei are likely delayed behind the majority. The scale bar represents 10$\mu$m in all panels.
Figure 2-11. See legend on previous page.
a normal width of approximately 2μm to approximately 5μm (Figure 2-12A, both panels). Examining under-condensed chromatin at high magnification reveals lighter-staining fuzzy puffs of chromatin at the metaphase plate, punctuated by brightly staining chromatin (Figure 2-12B).

Another phenotype encountered at metaphase in embryos from E(z) mutants is chromosome misalignment at the metaphase plate. In wild-type embryos, the sister chromatid arms are at such a tight configuration at the metaphase plate that telomeric ends are not always readily distinguished. In addition, the width of both sets of aligned chromosomes is approximately 2-2.5 μm (Figure 2-13A). In E(z)-derived embryos, the chromatid arms of metaphase chromosomes fail to align properly at the metaphase plate and instead project outward from the metaphase plate (Figure 2-13B to D), giving some chromosomes a “spidery” appearance instead of the tightly-aligned “bar” configuration seen in wild-type embryos.

Contrary to all other PcG mutants surveyed, embryos of E(z) mutants did not exhibit segregation defects significantly above those seen in wild-type embryos (Figure 2-14). Most anaphase and telophase figures were completely normal (Figure 2-14A to C), and the rare occurrence of any chromatin bridges was of very low penetrance and expressivity with only one or two nuclei joined by a thin chromatin bridge (Figure 2-14D), similar to wild-type embryos. Nuclear fallout was also of low penetrance in E(z)-derived embryos, differing from the other PcG mutants examined. Interestingly, when fallout nuclei were observed, they were often at the metaphase stage (Figure 2-14E), which differs from the highly penetrant telophase-looking fallout nuclei from the other PcG mutants surveyed.
Figure 2-12. Embryos from $E(z)$ mothers have an undercondensed chromatin phenotype. (A) Fully compact chromosomes at the metaphase plate from wild-type embryos (left panel) have a uniform staining intensity and are approximately 2$\mu$m wide (dashed lines, arrow). In comparison, metaphase chromosomes in embryos from $E(z)$ mothers (right panel) stain unevenly, and are close to 5$\mu$m wide at their most compact. (B) Detail of unevenly stained and undercondensed metaphase chromosomes of $E(z)$ mutant embryos. The more lightly-staining diffuse chromatin (arrowheads) is punctuated by fully condensed brightly staining chromatin (arrows). Compare to the bright and evenly stained chromosomes of the wild-type embryo in (A). Scale bar represents 10$\mu$m in all panels.
Figure 2-13. Chromosome arms are misaligned at the metaphase plate in $E(z)$ embryos. (A) Normal alignment of chromosomes at the metaphase plate in wild-type embryos, with all chromosome arms aligned within $2.5\mu m$ (dashed lines). (B-D) Close-up view of metaphase chromosomes in $E(z)$ embryos shows chromosome arms which point away from the metaphase plate (arrows) giving the metaphase chromosomes a “spidery” appearance. Scale bar represents $5\mu m$ in all panels.
Figure 2-14. (next page) Embryos from *Enhancer of zeste* mutant mothers do not show segregation defects. (A) Completely normal late anaphase nuclei from $E(z)^{63}$ mothers. All segregated nuclei are completely independent and there are no chromatin bridges. (B) Normal telophase nuclei from $E(z)^{63}$ mothers. (C) $E(z)^{66}$ embryos also have normal anaphase figures. (D) Any chromatin bridges encountered in $E(z)$ embryos (arrow) are of very low penetrance and expressivity, similar to wild-type. (E) Nuclear fallout (arrow, both panels) is also of low penetrance, with fallout nuclei often at the metaphase stage (right panel). Scale bar represents $10 \mu m$ in all panels.
Figure 2-14. See legend on previous page.
A summary of the penetrance of all mitotic phenotypes observed in both alleles of $E(z)$ mutants surveyed is provided in Figure 2-15. Undercondensed chromatin and chromosome misalignment were the most penetrant phenotypes observed. Nuclear fallout was slightly more penetrant than in wild-type embryos, but was nowhere near the high penetrance seen in other PcG mutants (see Figure 2-6). $E(z)$ embryos did not exhibit segregation defects significantly above frequencies seen for wild-type embryos, and severe chromatin bridges were never observed.

**vi. A deficiency in polyhomeotic-proximal is necessary and sufficient for highly penetrant mitotic phenotypes**

Interestingly, most PcG mutants examined (with the exception of $E(z)$) had strikingly similar mitotic phenotypes, and similar penetrances. However, the PcG mutant *polyhomeotic* appeared to have the most penetrant and expressive segregation and nuclear fallout phenotypes (see Figures 2-4A and 2-7). In addition, the most severe segregation defects were observed in embryos from $ph^{504}$ mothers where nearly every pair of telophase nuclei were joined by thick chromatin bridges (Figure 2-16A, top right panel). This extreme expressivity was not observed in the other PcG mutants. Given the high penetrance and expressivity of mitotic defects in *polyhomeotic* embryos in conjunction with the interaction of a PH isoform with Barren and Topo II (Lupo *et al.*, 2001), $ph$ was chosen to further study the role of the PcG in mitotic progression. However, as discussed in the introduction, the mutation in $ph^{504}$ flies deletes both the *proximal* and *distal* genes of the $ph$ locus. Therefore, to determine whether the mitotic phenotypes of *polyhomeotic* were due to a deficiency in *proximal, distal* or both $ph$ genes, a collection of mutants
Chromatin Bridges (at ana/telophase)
Nuclear Fallout (total embryos)
Decondensation (at metaphase)
Metaphase Misalignment (at metaphase)
Other (total embryos)

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<th>E(z)66/TM6C n=202</th>
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<tr>
<td>Other</td>
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Figure 2-15. Summary of mitotic defects found in embryos from E(z) mutant mothers. The mitotic defects encountered are unique to E(z) among the other PcG mutants surveyed. The predominant mitotic phenotypes are undercondensed chromosomes and chromosome arm misalignment at the metaphase plate, both of which are not visible phenotypes in other PcG mutant embryos. The decondensed and misaligned chromosomes are represented here as a percentage of embryos at metaphase. The penetrance of chromatin bridges (expressed here as a percentage of total embryos at ana/telophase) is very similar to that in wild-type embryos, and while penetrance of nuclear fallout is higher in E(z) embryos than in wild-type, the frequency is much lower than found in other PcG mutants. Nuclear fallout is expressed as a percentage of total embryos surveyed.
specifically disrupting either proximal or distal or both were examined for mitotic defects.

One allele of a ph-proximal-specific deletion was used for this analysis. $ph^{409}$ is an inversion that disrupts ph-proximal and is kept as a heterozygote over an X-chromosome balancer (Dura et al., 1987). One ph-distal specific allele was available. $ph^{401}$ is a deletion within ph-distal only and is kept as a homozygous stock. A third ph allele, $ph^2$, is a deletion which results in an in-frame fusion of the N-terminus of ph-proximal to the C-terminus of ph-distal, was also examined (Dura et al., 1987). 0-3 hour embryos of these alleles were collected as well as embryos from the $ph^{504}$ stock, and a control wild-type stock. The embryos were collected, fixed, stained and surveyed for mitotic defects as before. Figure 2-16 illustrates typical anaphase and telophase figures for embryos from $ph^{504}$, $ph^{409}$ and $ph^{401}$ alleles. Interestingly, embryos from the $ph^{504}$ and $ph^{409}$ stocks which both have a ph-proximal deficiency exhibited severe segregation defects at both anaphase and telophase whereas embryos from the distal-specific $ph^{401}$ allele looked more like wild-type embryos.

Embryos from $ph^{409}$ mothers exhibited the most severe segregation phenotypes seen in any PcG mutant. On occasion, every pair of anaphase and telophase nuclei was joined by very thick chromatin bridges (Figure 2-16A middle row). All alleles with a ph-proximal deficiency exhibited the same segregation and nuclear fallout phenotypes characteristic of PcG mutant embryos. Embryos from ph-distal mothers had very normal looking anaphase and telophase figures (2-16A, bottom row) and any segregation defects were of very low penetrance and expressivity reminiscent of wild type embryos. However, unlike the $E(z)$ embryos (which also lacked a segregation phenotype), ph-distal
embryos always had normally condensed chromatin and normally aligned metaphase chromosomes (Figure 2-16B, right panel).

In order to quantify the contribution of *ph-proximal* and *ph-distal* to mitotic progression, the *ph* alleles were scored for mitotic defects in the same rigorous manner as the other PcG mutants. Comparing all *ph* alleles tested shows that a *ph-proximal* deficiency is both necessary and sufficient for segregation defects (Figure 2-17A) and nuclear fallout (Figure 2-17B). A deficiency for *ph-proximal* alone (*ph*409 allele) has very similar penetrance and expressivity of chromatin bridges and nuclear fallout as does a deletion of both *proximal* and *distal* genes (*ph*504 allele). In addition, a deletion of *ph-distal* alone showed a very low penetrance and expressivity of mitotic phenotypes, similar to what is seen in wild-type embryos. There is no contribution to mitotic phenotypes from *ph-distal*. In addition, embryos from *ph*2 mutant mothers showed extensive mitotic defects similar to *ph*409 and *ph*504 alleles. As mentioned, *ph*2 is a mutation resulting in a chimeric protein fusing the amino terminus of ph-proximal in frame with the carboxyl terminus of ph-distal. In effect, the C-terminal half of ph-proximal is missing, therefore the mitotic phenotypes observed in *ph*2 embryos suggest that the C-terminal unique region of Ph-Proximal may be required for mitotic progression. However, the penetrance and expressivity of *ph*2 mitotic phenotypes was slightly lower than for *ph*409 and *ph*504, indicating that the N-terminal unique region of Ph-Proximal may be involved in mitotic progression as well. A second *ph-proximal* allele, *ph*410 was also tested for mitotic phenotypes, and significant chromosome bridges and nuclear fallout was observed, confirming that *ph-proximal* is necessary and sufficient for proper mitotic progression. Embryos from *ph*410 mothers were only scanned for
Figure 2-16. Figure continued and legend on following page.

Telophase

Phased, wild-type

distal mutant

Phased, wild-type

Proximal mutant

Phased, wild-type

Proximal and distal mutant

Anaphase
Figure 2-16, continued. The highly penetrant mitotic defects in \( ph^{504} \) mutant embryos are due to a deficiency in \( ph\)-proximal only. (A) Anaphase (left column) and telophase (right column) nuclei in embryos deficient in \( ph\)-proximal and distal (top row), proximal alone (middle row) and distal alone (bottom row). Mitotic defects are dependent on a proximal deficiency since embryos from \( ph^{504} \) and \( ph^{409} \) mothers exhibited severe segregation defects. Anaphase and telophase nuclei from \( ph^{401} \) mothers appeared identical to wild-type. The same mitotic defects were encountered in \( ph^{409} \) embryos as in \( ph^{504} \) embryos, but severe segregation defects are shown here as an example. No severe chromatin bridges were encountered, only the occasional single joined nuclei (arrow, bottom left panel) which is also occasionally present in wild-type embryos. (B) Fallout nuclei (left panel, arrow) are also rarely encountered in \( ph\)-distal mutant embryos. Here, a small cluster of fallout nuclei are shown as an example. In contrast to \( E(z) \) embryos, metaphase figures are always normal, with no undercondensed or misaligned chromosomes encountered (right panel). Scale bar represents 10\( \mu \)m in all panels.
Figure 2-17. (next page) Summary of major phenotypes shows that a deficiency in ph-proximal is necessary and sufficient for defects in early embryonic mitotic cycles. (A) A survey of chromatin bridges at anaphase or telophase shows that embryos derived from females with both a ph-proximal and distal deletion (ph$^{504}$) or a proximal deletion only (ph$^{409}$) have a similar penetrance of high-severity chromatin bridges (red bars). Embryos with a distal deletion only (ph$^{401}$) have low-severity chromatin bridges (light blue bars) or no visible chromatin bridges (white bars), similar to wild-type. Embryos from ph$^2$ mothers (where the amino-terminus of PH-P protein is fused in frame to the carboxyl-terminus of PH-D protein) also have both high-severity chromatin bridge phenotypes, significantly higher than wild-type, but lower than a deficiency for full-length PH-P. (B) A survey of the same embryos as (A) for nuclear fallout shows a similar pattern. Embryos from mothers with a disruption in ph-p have similarly high penetrance of severe nuclear fallout events (red bars). Embryos from ph-distal mothers have only low-severity nuclear fallout events (light blue bars), at a frequency almost identical to wild-type. The number of embryos surveyed in both assays appears above bars in (A).
A Survey of chromatin bridges.

![Bar chart showing chromatin bridges by maternal genotype.](image)

B Survey of nuclear fallout.

![Bar chart showing nuclear fallout by maternal genotype.](image)

Figure 2-17. See legend on previous page.
mitotic phenotypes and were not methodically surveyed as other PcG mutants were.

The most striking embryos from \( ph^{409} \) mothers exhibited completely disorganized chromosomes that appeared to have no regular pattern or normal ploidy (Figure 2-18). These embryos have very few and abnormally large nuclei. While normal nuclei are approximately 2\( \mu \)m in diameter, these large nuclei appear to have a 5-7 \( \mu \)m diameter. And while some nuclei have a normal spherical shape, others are more amorphous and very brightly-staining. There are also extensive chromosome fragments both near the nuclei and scattered throughout the embryo. Of the 169 embryos examined, three embryos of this phenotype were encountered, a penetrance of less than 2%. Though infrequent in \( ph^{409} \) embryos, this severe phenotype was not encountered in any other PcG mutant embryos, or in wild-type embryos.

**vii. Embryos from homozygous ph-proximal mothers do not uncover additional phenotypes**

Since the \( ph^{409} \) allele is maintained over an X-chromosome balancer, the females in the laboratory stocks are of mixed genotypes. Therefore, the \( ph^{409} \) embryos in the previous experiments were derived from a mixed population of females. Most females are heterozygous for the \( ph^{409} \) allele balanced by the \( FM7C \) balancer chromosome. Homozygous females are mostly lethal, but approximately 10% survive to adulthood as determined by counting the adults in a given laboratory stock bottle. No females homozygous for the balancer survive to adulthood. Therefore, nearly all of the embryos in the above experiments were derived from heterozygous females. Since early embryos do not express zygotic genes and are dependent on maternal deposits to get through early
Figure 2-18. Embryos from ph-proximal mutant mothers exhibit an extreme phenotype resulting from mitotic abnormalities. (A) Three examples of embryos which have failed to develop normally. Instead of an expanded nuclear population, they possess a few abnormally large nuclei and many chromosome fragments. Unlike previously-characterized mitotic defects, these abnormal nuclei also reside in the interior of the embryo, indicating defects occurred prior to mitotic stage 10. Note normally developed embryo with properly-sized nuclei at bottom of right panel (arrow). (B) Detail of chromosomal abnormalities in undeveloped embryos. Chromosomes are not organized into diploid nuclei, rather they are large amorphous collections of nuclear material (arrows, both panels). A large nucleus (chevron, left panel) resembling a late S-phase/prometaphase nucleus has a diameter close to $8\mu m$, indicating higher than 4N chromosomal complement. A normal 2N or 4N diameter is closer to $2\mu m$. Extensive chromosome fragments (arrowheads, both panels) are seen near nuclei and throughout embryo (see panels in A). Scale bar represents $10\mu m$ in all panels.
mitoses, the extent of their phenotype is a reflection of the maternal genotype. Therefore, most embryos from $ph^{409}$ lab stocks reflect a deficiency of one maternal $ph$-proximal gene. It is possible that embryos derived from a homogeneous stock of $ph^{409}$ homozygous mothers (therefore having a maternal deficiency of both $ph$-proximal genes) may uncover embryonic phenotypes not previously observed in embryos from the mostly heterozygous mothers.

0-3 hour embryos from homozygous $ph^{409}$ females were collected and their DNA stained with TOTO-3 nucleic acid dye. The embryos were surveyed for mitotic defects as before. Interestingly, no new phenotypes were seen indicating that a haploinsufficiency for $ph$-proximal is necessary and sufficient for defects in mitotic progression. Again, the most striking phenotype was embryos with giant nuclei and extensive chromosome fragmentation (Figure 2-19). These embryos were approximately 4% of the total population. And as expected, chromatin bridges of varying thicknesses at anaphase and telophase here were also observed (Figure 2-20A). Surprisingly there were also embryos that appeared normal, with no discernible mitotic defects as well as embryos with very low expressivity of mitotic phenotypes (Figure 2-20B). As in embryos derived from heterozygous $ph$-proximal females, all metaphase plate chromosomes appeared normal, with no under-condensed puffs of chromatin or misaligned chromosomes (Figure 2-20C).

viii. Cell cycle defects are not restricted to early embryonic nuclear divisions

Since early embryonic mitotic cycles are unique, it was possible that mitotic phenotypes for polyhomeotic-proximal (and thereby a mitotic role) was specific to these
Figure 2-19. (next page) Embryos from \emph{ph-proximal} homozygous mothers exhibit no new phenotypes, but have a higher penetrance of undeveloped embryos with large and fragmented nuclei. (A) Two examples of embryos with giant and fragmented nuclei. Embryos have some nuclei that appear normal (arrow, both panels) but also have several giant nuclei (arrowhead, both panels). (B) Detail of large amorphous nuclear material found in these embryos. Nuclei appear to be large collections of unorganized chromatin, with irregular shapes (arrows, all panels). Nuclei in right panel resemble nuclei that have not fully segregated, but are more irregularly shaped than the dumbbell-shaped nuclei previously encountered (see Figure 2-14B for example). (C) Detail of fragmented chromosomes. Chromosome fragmentation is extensive in these undeveloped embryos (arrows, all panels). Right panel shows two large nuclei joined by a thin chromatin bridge (arrowhead) indicating that these extremely abnormal nuclei were the result of repeated chromosome segregation failures without arrest. Scale bars represent 10\textmu m in all panels.
Figure 2-19. See legend on previous page.
Figure 2-20. (next page) Embryos from homozygous *ph-proximal* mothers have phenotypes identical to embryos from heterozygous mothers. (A) An example of chromatin bridges joining nuclei at telophase (left panel, arrows) and at anaphase (right panel, arrowheads). The chromatin bridges are of varying thicknesses, including thin and thick bridges, identical to what was seen in heterozygous *ph-p*-derived embryos. (B) Some embryos had very low expressivity of mitotic defects. In this embryo, most telophase nuclei are completely segregated (arrow, example). There are several nuclei that are larger and more brightly staining than the rest (arrowheads) and a “hole” in the cortex indicating a single fallout nucleus (chevron). (C) As in embryos derived from heterozygous *ph-p* mothers and from other PcG mutants (with the exception of *E(z)*), all metaphase figures appeared normal, with no evidence of undercondensed chromatin or misaligned metaphase chromosomes. Scale bars represent 10μm in all panels.
Figure 2-20.
See legend on previous page.
early mitoses. To determine if later cell divisions are also affected by a *ph-proximal*
deficiency, brains of third-instar wandering larvae were dissected, fixed and squashed prior to staining with DAPI. Some dissected larval brains were subject to colchicine treatment prior to fixing and staining. Colchicine arrests mitotically active nuclei at metaphase, therefore nuclei are present as either mitotic with paired metaphase chromosomes or as post-mitotic round nuclei. This allows identification of metaphase defects and any mitotic defects that persist past mitosis. Colchicine-treated brains from *ph*409 larvae show multiple chromosome fragments which appear as very small spots of nuclear material about 1/10th of the size of wild-type nuclei (Figure 2-21A). In any given field of view with approximately 200 nuclei, these fragments occur at an average of approximately 4%. These chromosome fragments were not observed in *ph*401 or wild-type brain preparations. As shown in Figures 2-9C, 2-18B and 2-19C, chromosome fragments also occur at a low frequency in *ph*409 derived early embryos. Chromatin bridges were not observed in colchicine-treated embryos.

The mitotically active nuclei of larval brains are present all stages of the cell cycle when not subject to colchicine treatment. In these preparations, the chromosomes and nuclei appear slightly more amorphous but are still distinguishable (Henderson, 2004). Figure 2-21B shows larval brain nuclei connected by chromatin bridges, exhibiting the characteristic dumbbell or oval shape also observed in embryonic nuclei with chromatin bridges. These bridged nuclei appear at close to 5% in a given field of approximately 200 nuclei. Again, no bridged nuclei were observed in brains of *ph*401 or wild-type nuclei.
Mitotic defects in *ph-proximal* mutants are not restricted to early embryonic mitoses. (A) Small chromosome fragments are found in larval brains. Brains of third-instar wandering larvae were dissected and stained with DAPI subsequent to colchicine treatment. Colchicine arrests mitosis at metaphase, so these cells are likely post-mitotic. This field shows that approximately 4% of the nuclei are very small fragments of chromatin (arrows) and may represent fragmented chromosomes, a phenotype also observed in early embryos. (B) Chromatin bridges join segregating nuclei. Larval brains without colchicine treatment were stained with DAPI and examined for segregation defects. Since colchicine is absent, mitotically active nuclei at all stages of mitosis are observed. Nuclei which appear to be at telophase are joined by chromatin bridges (arrows, all panels). The bridged nuclei resemble dumbbells (left and center panels) or are elongated ovals (right panel) both indicative of thick chromatin bridges, and both phenotypes which were observed in the embryos.
III. Discussion

i. The PcG has a role in cell cycle progression

The Polycomb Group was characterized as a group of genes required to maintain the proper repressed pattern of homeotic genes during development, and have classically been studied for their role as epigenetic regulators. In flies, the majority of investigations have focused on the mechanism behind PcG-mediated silencing and how this might regulate maintenance. This study has shown that the PcG may have an additional role in regulating the cell cycle. Early embryos of many PcG mutants exhibit highly penetrant and expressive phenotypes consistent with problems in cell cycle progression. These mutants include polyhomeotic-proximal (ph-p), Polycomb (Pc), Posterior sex combs (Psc), cramped (crm), Additional sex combs (Asx) and Enhancer of zeste (E(z)). A summary of all PcG genes and alleles tested for mitotic defects appears in Table 2-1. These PcG members were chosen because they are representatives of the different classifications of PcG members as discussed in the Chapter Introduction. Interestingly, there appears to be no correlation between the presence of mitotic defects and affiliation with any of the general classifications of PcG members. The three representative PRC1 members, polyhomeotic-proximal, Polycomb and Posterior sex combs shared identical chromosome segregation phenotypes to cramped and Additional sex combs, two PcG members not found in PRC1. The PRC2 representative Enhancer of zeste also exhibited mitotic phenotypes, but they were defects in chromatin condensation and metaphase alignment in contrast to the segregation defects of the other PcG genes tested. This phenotypic difference may reflect a different mitotic role for Enhancer of zeste than for the other PcG members tested. In addition, there was no correlation found between
Table 2-1. A summary of all PcG mutants surveyed for segregation defects at 0-3 hours of embryogenesis. Most PcG mutants were remarkably similar, with extensive chromatin bridges at anaphase and telophase. The exceptions are Enhancer of zeste and polyhomeotic-distal. While ph-distal had no mitotic defects significantly above wild-type embryos, E(z) did exhibit undercondensed chromatin and misaligned metaphase chromosomes (asterisk). Two alleles of barren, a member of the condensin complex, were also tested in the assays. This result confirms an earlier report where barren mutant embryos were shown to have segregation defects (Bhat et al., 1996).

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</tr>
<tr>
<td>Enhancer of zeste</td>
<td>E(z)^{66}/TM6C</td>
<td>✓ ∗</td>
</tr>
<tr>
<td>polyhomeotic distal</td>
<td>ph^{401} w/ph^{401} w</td>
<td>✓</td>
</tr>
<tr>
<td>barren</td>
<td>bar^{L105}/CyO</td>
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<td>barren</td>
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having mitotic defects and classification as a PcG or an ETP group member, since all six PcG genes exhibited significant mitotic phenotypes. Therefore, the broad classifications of PcG members based on genetic interactions and membership in purified complexes is not a predictor of having mitotic phenotypes. As other PcG and ETP group mutants are examined in future studies, it is likely they will also exhibit significant mitotic defects. In summary, these results demonstrate that the mitotic phenotype of polyhomeotic mutants previously reported (Lupo et al., 2001) is not unique and argues that the PcG as a whole may be involved in mitotic progression.

While the mitotic phenotypes uncovered in this study are significant, the presence of mitotic defects is not entirely informative. It is striking that the varied representatives of the PcG studied here have such a similar set of mitotic phenotypes at similar penetrance and expressivity. In parallel, the homeotic phenotypes of these and other PcG mutants also have a nearly identical set of characteristics. For example, mutations in 10 different PcG genes all result in similar ectopic expression patterns of the abdominal A and Abdominal B homeotic genes (Simon et al., 1992). While this is suggestive that the PcG have a common role in mitosis akin to having a common role in regulating Hox targets, the shared mitotic phenotype is not sufficient evidence to conclude whether PcG targets cell cycle regulators. Though there are potentially 80 targets for Polyhomeotic as judged by binding sites on polytene chromosomes (DeCamillis et al., 1992), there is so far no evidence that cell cycle genes are among the targets or that expression of cell cycle regulators is altered in polyhomeotic mutants. However, the possibility that the mitotic defects in PcG mutants are indirect due to regulation of cell cycle targets cannot be ruled out. Alternatively, PcG proteins may directly regulate cell cycle progression, possibly by
interacting with cell cycle regulators, as suggested by the co-immunoprecipitation of Polyhomeotic with Barren and Topoisomerase II, and the other interactions discussed in Chapter 1. Another possibility is that cell cycle regulation is itself a component of the epigenetic inheritance process and is thus governed by epigenetic maintenance proteins such as the PcG. These possibilities will be explored in the subsequent chapters of this thesis.

The chromatin bridges in PcG mutants signify a segregation defect. However, many different Drosophila cell cycle mutants from every stage of the cell cycle also have chromatin bridge phenotypes. Some examples include kinesin-like enzymes (Rogers et al., 2004), a variety of regulatory kinases such as polo kinase (Donaldson et al., 2001) and aurora-like kinases (Giet et al., 2001), replication checkpoint regulators such as grapes (Su et al., 1999; Ji et al., 2004), chk2 (Xu and Du, 2003), and mei-41 (Sibon et al., 1999), genes involved in sister chromatid segregation such as pimples and three rows (Stratmann and Lehner, 1996; Philp et al., 1994), among many others. The proteins of these genes act at many different points of the cell cycle, yet share a common mitotic phenotype with each other, and with PcG mutants. Even a mutation in Glutamine synthetase 1 (Gsl) which impairs the biosynthesis pathway of nucleotides result in incomplete DNA replication and chromatin bridges at anaphase and telophase (Frenz and Glover, 1996). Hypoxic conditions (DiGregorio et al., 2001; Douglas et al., 2001) and drugs which inhibit DNA replication (Sibon et al., 2000) also phenocopy the chromatin bridges of cell cycle mutants and PcG mutants. This argues that the common mitotic phenotype of the PcG mutants cannot be used as evidence of a common mitotic role. In addition, similarity of the mitotic phenotypes of the PcG mutants to known cell cycle regulators is
equally uninformative since defects in so many steps of the cell cycle lead to identical phenotypes. Therefore, the fact that polyhomeotic shares a common phenotype with barren is not supporting evidence for a mutual role in cell cycle progression. It suggests that interpretation of previous results were oversimplified (Lupo et al., 2001). The fact that most PcG genes tested share mitotic phenotypes identical to those of many cell cycle regulators does argue that the PcG as a whole has a role in cell cycle progression.

ii. Most chromatin bridges are likely resolved

The mitotic phenotypes described for PcG mutants is shared by many cell cycle regulators, therefore other assays need to be developed to further pursue the role of the PcG in mitotic progression. However, close examination of the mitotic phenotypes presented in this chapter allows a number of predictions to be made about the nature and consequences of the mitotic defects encountered in PcG mutant embryos.

Given the high penetrance and expressivity of the chromatin bridge phenotype in PcG mutant embryos (especially ph-proximal), one obvious question that surfaces is what becomes of the embryos with severe chromatin bridges, and more specifically, what happens to chromatin bridges themselves? The data presented in this chapter argue that chromatin bridges are likely resolved, allowing most embryos to continue development. Figures 2-4 and 2-19A show that every single ph-proximal-derived embryo (from ph^504 or ph^409 mutant mothers) with nuclei at anaphase or telophase exhibited chromatin bridges. Most had very high expressivity of the phenotype, where over 40% of nuclei pairs were joined by bridges. No embryo was encountered which had 100% fully segregated anaphase or telophase nuclei. However, anaphase and telophase embryos together were
7-9% of the total developed embryos, a proportion that is consistent with the short
duration of those mitotic phases (Ji et al., 2004). Normal anaphase and telophase
numbers argue that the embryos that exhibited severe chromatin bridges were not
developmentally arrested or dead. Also, with the exception of fallout nuclei, most
embryos from S-phase to metaphase appeared normal. Only a few embryos out of all
PcG alleles tested appeared to have bridged prometaphase nuclei, as appears in Figure 2-
10A. If chromatin bridges did not resolve, one would expect a higher proportion of these
prometaphase bridges, and possibly higher numbers of anaphase and telophase embryos.
Since every anaphase and telophase stage embryo was affected by chromatin bridges, but
embryos at subsequent mitotic phases appeared normal, chromatin bridges most likely
resolve. It is possible that the very thin chromatin bridges as shown in Figure 2-1D
(arrow) and Figure 2-2D (arrowhead) represent the resolution process such that the thin
chromatin connection between the nuclei is the remnant of what was more extensive
bridging chromatin.

If chromatin bridges did not resolve one may imagine that either the bridging
chromatin may break, or the bridged nuclei would remain connected and become one
larger polyploid nucleus. The very low penetrance of such phenotypes also argues for
resolution of chromatin bridges. If the bridges did not resolve and connected nuclei
persisted to the subsequent cell cycles, one would expect a much higher penetrance of
large or polyploid nuclei than were observed. Also, fragmented chromosomes and
evidence of chromosome breakage was also very infrequent. All low-penetrant mitotic
defects accounted for only 4-10% of the total mitotic defects observed in PcG embryos,
and as such only a few nuclei in each embryo were affected.
iii. Chromatin bridges likely introduce a delay in the mitotic cycle

Since the evidence argues that most bridged nuclei are resolved, it would appear that chromatin bridges do not have a detrimental effect on the cell cycle. However, approximately 40-48% of *ph-proximal*-derived embryos exhibited high penetrance and expressivity of nuclear fallout. This indicated that in nearly half of the embryos, clusters of nuclei were removed from the cortical population due to problems with cell cycle progression. It is likely that the nuclear fallout phenotype is a direct consequence of the chromatin bridge phenotype. Previously, *C(2)EN* embryos carrying an abnormally long second chromosome exhibited chromatin bridges between some nuclei since the extra-long chromosomes were not able to fully segregate in time (Sullivan et al., 1993). These bridged nuclei lagged behind neighbouring nuclei and were subsequently removed from the cortex by the fallout mechanism once they reached telophase. Therefore, the fallout nuclei observed in PcG mutants were likely previously-bridged nuclei not able to resolve in time to maintain overall mitotic synchrony. Interestingly, the fallout nuclei are never observed to be joined by chromatin bridges. This may be because the delay is only detected once the bridges are resolved, or the bridged nuclei "snap-back" and fuse with each other, as has been observed for bridged nuclei in embryos mutant for *grapes*, a checkpoint gene required at several cell cycle stages (Fogarty et al., 1994; Fogarty et al., 1997; Yu et al., 2000). Both scenarios are possible since fallout nuclei are generally detected as pairs of nuclei spaced at a similar distance as resolved telophase pairs (Figure 2-6C) or odd numbered clusters (Figure 2-6D). Occasionally the fallout mechanism may be unable to detect or remove delayed nuclei. If resolved, these nuclei appear as

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asynchronous to neighbouring nuclei, or, if bridged, they appear as prometaphase bridges, polyploidy, giant nuclei or chromosome breakage. These phenotypes are very low penetrant because nuclear fallout likely removes all significantly delayed nuclei.

In cases where embryos have 40-100% of nuclei pairs joined by very thick chromatin bridges (such as those depicted in Figures 2-2B and 2-16A respectively), it is likely that delays in the mitotic cycle are not reparable by the fallout mechanism due to the high proportion of joined nuclei. Over several cycles, these delays may accumulate into developmental delays or death. The embryos of ph-proximal mutants develop at a slower rate than those of wild-type flies as judged by the timed embryo collections undertaken here and unpublished observations by others in our laboratory. The slower developmental rate may reflect delays in the mitotic cycles due to segregation defects. In other cases, the most extreme segregation defects overwhelm the delay and fallout mechanisms and continue with the mitotic program until the segregation failures reach a critical point and the embryo dies. The embryos in Figures 2-18 and 2-19 likely represent this scenario. In these embryos, the cortex is completely disorganized with very large amorphous nuclei and extensive chromosome breakage. These embryos are probably dead and appear to be the result of cumulative effects of several rounds of segregation defects.

One way to test the effects of chromatin bridges on embryonic cell cycles and confirm the hypotheses presented in this discussion, would be to examine the mitotic cycle of early ph-proximal-derived embryos transgenic for a GFP-histone fusion (Clarkson and Saint, 1999) using live video microscopy. Initially, one would examine whether chromatin bridges are indeed resolved and whether unresolved bridges or

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delayed nuclei are removed by nuclear fallout. To determine if chromatin bridges introduce delays in the mitotic cycle, each stage of mitosis in an embryo demonstrating severe chromatin bridges would be timed and compared to GFP-histone transgenic embryos wild-type for ph-proximal. There should be a significant increase in the length of telophase or the following S-phase if resolving chromatin bridges does delay the mitotic cycle. Cell cycle delays in syncytial mitoses are already known for mutants in DNA replication checkpoint genes grapes and mei-41 and chk2 (Su et al., 1999; Ji et al., 2004; Sibon et al., 1999; Xu and Du, 2003). These genes require completion of DNA replication prior to mitotic entry in syncytial mitoses and exhibit segregation defects similar to those described for PcG mutants. Activation of the spindle checkpoint also delays mitotic progression (Logarinho et al., 2004) therefore it is likely that the mitotic defects of PcG embryos also delay the mitotic cycle.

This study has shown that mutations in PcG genes disrupt cell cycle progression, a new phenotype not previously described for PcG mutants. The phenotypic analysis in this chapter has established that the likely cause of cell cycle progression problems is a segregation defect. This segregation defect is partially resolved during early embryogenesis most likely by delaying the cell cycle and removing faulty nuclei. However, phenotypic analysis alone is not sufficient to pinpoint the exact cause of the chromatin bridges, whether PcG proteins directly interact with cell cycle regulators and what segregation defects may mean in the context of the classical role of the PcG, epigenetic silencing. The goals of the following chapters will be to answer these questions using polyhomeotic–proximal as the representative PcG member.
CHAPTER 3 Characterization of the mitotic role of Polyhomeotic-Proximal.

I. Introduction

Defects in mitotic progression are phenotypes shared by most PcG mutations tested suggesting that the regulation of the mitotic cycle is a shared role for PcG genes. Most PcG genes tested exhibited a remarkably similar set of phenotypes that were characterized by segregation defects and consequential delays in cell cycle progression. *polyhomeotic-proximal* exhibited very striking chromatin bridge phenotypes and was chosen for further study. Two major questions emerged from the detailed phenotypic analysis of PH-P mitotic defects. Firstly, what is the role of PH-P in the cell cycle progression, and secondly, how does this role relate to its established role in epigenetic maintenance?

To begin to understand the role of PH-P in mitotic progression, the nature of chromatin bridges resulting from *ph-p* mutations must first be understood. Specifically, what is cause of the bridging chromatin, and in which phase of the cell cycle do they arise? Chromatin bridges are a very common phenotype in many cell cycle mutants and may result from a deficiency of any one of the many components contributing to cell cycle progression, from any phase of the cell cycle. This presents a difficulty in the approach to answering the questions because phenotypic analysis alone is insufficient to determine the cause of chromatin bridges in *ph-p* mutants. One strategy to begin unraveling the cause of segregation defects in *ph-p* mutants is to narrow down the possibilities by assaying for easily identifiable characteristics of known causes of chromatin bridges. These include lagging chromosomes, unsegregated chromatid arms and telomere fusion. Lagging chromosomes are left at the midline of the metaphase plate.
during anaphase due to a lack of poleward movement (Sullivan et al., 1993) and may be
due to defects in spindle formation or maintenance, proper formation of kinetochores,
attachment of chromatid kinetochores to the spindle, spindle depolymerization and
chromosome movement. These steps are regulated by dozens of factors including
chromatin proteins, kinetochore proteins, cyclins, mitotic kinases, checkpoint proteins,
molecular motors and many others. Similarly, unsegregated chromatid arms may result
from telomere fusion, or defects in many steps including DNA replication, chromatin
condensation, and dissolution of sister chromatid cohesion. Two approaches to assay for
lagging chromosomes are to examine the morphology of the mitotic spindle at metaphase
in early embryos of PH-P mutants and assay for the presence of centromeres within
bridging chromatin. Chromatin bridges were also assayed for evidence of unsegregated
chromatin arms and fused telomeres. Though either scenario still presents a number of
mitotic steps that may be involved, these experiments provide an important first step in
understanding the nature of PH-P segregation defects.

Another strategy to establish at which stage chromatin bridges of PH-P mutants
arise is to determine at which mitotic stage PH-P may function by examining PH-P
distribution during the cell cycle. This provides a temporal map of PH-P association with
chromatin within the early embryonic mitotic cycles and allows predictions of which
stage a PH-P deficiency may result in segregation defects.

As described in Chapter 1, a previous study reported that an isoform of PH-P co-
immunoprecipitated with Barren and Topoisomerase II (Lupo et al., 2001) suggesting
that the mitotic role of PH-P was via these known cell-cycle regulators, and implicating
chromatin condensation as the mitotic stage at which PH-P is required. However the
discussion in Chapter 2 predicted that the mitotic role of PH-P may be independent of Barren and TopoII. To examine whether the reported interaction translates to a shared function in cell cycle progression, two approaches were taken. PH-P distribution in barren mutant embryos was examined to determine if a barren deficiency affects PH-P behaviour during the cell cycle. In addition, a genetic interaction assay was developed to examine whether barren enhanced the mitotic phenotype of PH-P.

The central strategy to pinpoint the cell cycle stage for the mitotic role of PH-P was to test for in vivo co-localization with known cell cycle regulators at different mitotic stages. To narrow the focus to a few possible candidates, cell cycle proteins previously reported to interact with PH or PH homologues were chosen. In addition to Barren, previous reports implicated the S-phase regulators PCNA and Geminin in interacting with PcG members. Murine Geminin co-immunoprecipitated and co-localized in cells with the mammalian PH homologue Rae28 (Luo et al., 2004). In flies, PCNA mutants enhance the homeotic phenotype of ph-p mutants (Hodgson and Brock, unpublished observations). To help determine if there is an in vivo interaction between PH-P and any of these cell cycle regulators, early embryos were labeled with antibodies against PH-P and these cell cycle regulators to assess for co-localization. Any positive result would not only help pinpoint a cell-cycle stage for PH-P, but highlight a possible functional interaction between PH-P and specific cell-cycle regulators in vivo.

The previous chapter also revealed that mitotic phenotypes are not found in ph-distal mutants. This intriguing result suggests that a mitotic role is a major functional difference between the two highly similar proteins. To assay for differences in PH-P and PH-D behaviour during the cell cycle, distribution differences were examined during
early embryonic mitoses and the degree of co-localization determined using antibodies directed against unique regions within the proteins.

II. Results

i. Chromatin bridges are due to unsegregated chromatid arms.

Chromatin bridges of ph-p mutants may result from chromosomes lagging at the metaphase plate during segregation. One possible cause of lagging chromosomes is defects in mitotic spindle formation and structure. To identify any defects in mitotic spindle morphology in PcG mutant embryos, both the DNA and spindle apparatus of 0-3 hour embryos from females mutant for the PcG genes ph-p and Asx were visualized and metaphase spindles were compared to those of wild-type embryos. Metaphase spindles of 0-3 hour embryos were collected from females from laboratory stocks of ph^409w/FM7C, Asx^CyRoi and Oregon-R flies, and fixed as outlined in Materials and Methods. The mitotic spindle was visualized by labeling embryos using an anti-tubulin antibody directly coupled to Alexa488 fluorophore (Molecular Probes), and DNA was stained with the nuclear dye TOTO-3 (Molecular Probes) as described in Materials and Methods. Early embryos provide the advantage of synchronous mitotic divisions such that dozens or hundreds of mitotic spindles may be examined in a single embryo allowing the identification of any low-expressing phenotypes.

Confocal sections of metaphase plate chromosomes reveal no visible differences in the morphology of metaphase spindles between those of wild-type and PcG mutant embryos (Fig 3-1). Approximately 150 embryos of each genotype were labeled, and of those, there were 5 and 7 ph-p and Asx-derived embryos (respectively) with nuclei at
Figure 3-1. Comparison of metaphase spindle morphology between wild-type and PcG mutant embryos. 0-3 hour embryos from laboratory stocks of Oregon-R, Asx^3/CyRoi and \( ph^{409}w/FM7C \) were collected and fixed as described in Materials and Methods. The spindle apparatus was visualized with an anti-tubulin antibody (Sigma) at 1:2000 dilution directly coupled to Alexa568 fluorophore via the Zenon Mouse IgG labeling kit (Molecular Probes). DNA was stained with 1\( \mu \)m TOTO-3 nucleic acid stain. There appear to be no major differences in spindle morphology between wild-type and PcG mutant embryos. In all cases, the mitotic spindle (green) appeared normal, as did metaphase chromosomes (red). The merged images (top row) reveal that the metaphase plate chromosomes are aligned normally at the midline of the metaphase plate between the spindle structures.
metaphase plate. The spindles of both ph-p and Asx embryos always appeared normal, with no evidence of defects in tubulin organization. Also, consistent with results in Chapter 2, all metaphase plate figures were always normal in both PcG mutants, with no evidence for chromosome misalignment at the metaphase plate or defects in anaphase onset and frequency.

The presence of centromeres near the midline of chromatin bridges of anaphase and telophase nuclei signal lagging chromosomes. To distinguish whether chromatin bridges contained unsegregated centromeric regions or were unsegregated chromatid arms, chromatin bridges joining anaphase and telophase nuclei were examined for the presence of centromeres in ph-p mutant embryos. Again, 0-3 hour embryos from ph\textsuperscript{406w/FM7C} females were collected and fixed. Centromeres were visualized using chicken anti-CID, an antibody which recognizes the centromere-specific histone H3 variant (Blower and Karpen, 2001). A secondary antibody coupled to Alexa488 fluorophore (Molecular Probes) was used to detect anti-CID. Of 13 embryos at the anaphase and telophase stages, 11 had severe chromatin bridges. The centromeres of these bridged anaphase or telophase nuclei were never found within the chromatin bridges joining the two sets of segregating chromosomes (Fig 3-2). The centromere signal was consistently found at the extreme opposite poles of the mitotic nuclei (Fig 3-2A) indicating that centromere segregation is normal. A single occurrence of a centromere found close to the midline is shown in Figure 3-2B (chevron), but is not contained within the chromatin bridge but rather within the fully segregated DNA. Even in cases where two nuclei were completely connected by a such a thick chromatin connection that the nucleus appeared oval, the centromeres were always situated at
opposite poles (Figure 3-2C) indicating that centromeres have successfully segregated during anaphase. This suggests that the bridging chromatin in \textit{ph-p} mutants are chromatid arms joining the two nuclei. Figure 3-2D illustrates that these images were obtained by scanning the entire nucleus from top to bottom to obtain a complete distribution map of the centromeres in bridged nuclei. The two experiments illustrated in Figures 3-1 and 3-2 indicate that the chromatin bridges in \textit{polyhomeotic} mutants are not due to lagging chromosomes left at the metaphase plate, rather it is likely that chromatid arm segregation is defective.

Failure of sister chromatid arms to segregate may result from incomplete removal of cohesin proteins that pair sister chromatids together at metaphase. To determine if cohesin persists on the chromatin bridges of anaphase and telophase chromosomes, early embryos of \textit{ph-p} mutants were stained for cohesin subunits. Early embryos of \textit{ph}^409\textit{w/FM7C} females were collected, fixed and their DNA stained as described in Materials and Methods. An antibody against the cohesin subunit Sccl (gift of M. Heck) was used to visualize cohesins within chromatin bridges. However, there was consistently no signal above background obtained with this antibody (data not shown). A variety of primary and secondary antibody titers, and labeling protocols were attempted but all techniques yielded the same result. Other antibodies against \textit{Drosophila} cohesin subunits were not available. Several attempts were made to label embryos using an antibody against a portion of human SMC1, another cohesin subunit, but there was no detectable signal indicating that the portion of human SMC1 protein against which the antibody was raised is not sufficiently conserved with \textit{Drosophila} SMC1.
Figure 3-2. Distribution of centromeres in bridged telophase chromosomes in embryos of *ph-p* mutants reveals that segregation was initiated normally. 0-3 hour embryos from *ph*<sup>409</sup><sub>W</sub>/FM7C laboratory stock were labeled with anti-Cid, an antibody recognizing the centromere-specific histone H3 variant at 1:50 dilution (gift of G. Karpen). Antibodies were detected with goat anti-chicken secondary antibody coupled to Alexa488 fluorophore (green, Molecular Probes). DNA was visualized with TOTO-3 nucleic acid dye (red). (A) Centromeres of sister chromatids in bridged telophase nuclei always appear at extreme opposite ends, near the poles of nuclei, regardless of whether chromatin bridges are thick or thin (arrows, arrowheads). (B) A single occurrence of a centromere appearing close to the midline of bridged nuclei (chevron) was encountered. Normally, all nuclei were at opposite poles of sister nuclei (arrows). (C) In cases where nuclei were joined by such thick chromatin bridges that the joined nuclei appeared oval, centromeres still mapped to opposite poles (arrows) indicating that segregation is correctly initiated in *ph-p* mutants. (D) Cartoon illustrating that images in A-C were generated using confocal sections scanning the entire bridged nucleus from top to bottom to ensure all centromere signals were detected and mapped. Scale bar represents 2.5μm in all panels.
Figure 3-2. See legend on previous page.
Defective chromatid arm segregation may also be due to telomeric fusion. Examination of chromosomes in early embryos does not reveal whether chromatin bridges are the result of fused telomeres. Fully condensed metaphase and anaphase chromosomes are too close in proximity to distinguish telomere ends and the decondensing chromatin of telophase chromosomes makes it impossible to discern individual chromosomes. To look for evidence of fused telomeres, metaphase chromosomes from brains of third-instar *ph-p* mutant larvae were dissected and examined. Larval brains were treated with colchicine to arrest mitotically active nuclei at metaphase, then swelled in hypotonic solution, fixed and squashed to obtain a monolayer of nuclei on a slide. The squashed brains were stained with DAPI and examined by confocal microscopy. Of the eight larval brains examined, all metaphase figures observed were well-spread and contained no evidence of telomeric fusion. Figure 3-3 shows an example of the condensed and paired chromosomes of a 4N metaphase nucleus. Chromosome 4 is very short and appears as small dots (arrows). All chromosome pairs are independent of one another indicating that telomere fusion does not occur in *ph-p* mutants.

**ii. Ph-Proximal protein distribution during cell cycle indicates an S-phase requirement in the nucleus.**

Defects may occur at any stage of the cell cycle and carry forward to appear as a chromatin bridge phenotype at anaphase and telophase. Therefore the chromatin bridge phenotype of *ph-p* mutant embryos themselves will not uncover the molecular role of PH-P protein during cell cycle progression. One strategy is therefore to examine PH-P
Figure 3-3. A spread of metaphase chromosomes from colchicine-treated larval brains of a \textit{ph-p} mutant. All 8 sets of paired sister chromatids are individual at metaphase, with no evidence of telomeric fusion. The fourth chromosome in \textit{Drosophila} is very short (arrow) and appears as a dot.
protein behaviour in embryos during different stages of the cell cycle to help narrow down the stage at which it may act in cell cycle progression.

Wild-type 0-3 hour embryos were collected and fixed as described in Materials and Methods. To detect PH-P protein, an antibody directed against the N-terminal unique region of PH-P was used at 1:500 dilution and visualized with a secondary antibody coupled with the Alexa 488 fluorophore at 1:5000 (Molecular Probes). DNA was visualized by staining with the nucleic acid dye TOTO-3 and examined with confocal microscopy. PH-P protein has a regular pattern of association and dissociation with chromatin (Figure 3-4). PH-P appears to be most abundant on chromatin during the S-phase of the cell cycle (Figure 3-4A, top row). The PH-P signal is punctate, co-localizes with chromatin and is entirely contained within the nuclear boundary. As the cell cycle progresses into prometaphase and DNA is beginning to condense, nuclear PH-P now appears to be concentrated toward the nuclear periphery and there is significant PH-P detected outside of the nucleus (3-4A, middle row, arrowheads). By late prometaphase, most of the PH-P signal is outside the nucleus, with very little signal co-localizing with chromatin (bottom row). Figure 3-4B illustrates that the projection of the confocal sections of this figure were made from a relatively small set of sections (approximately 0.5 μm) toward the middle of the nucleus instead of including all confocal sections from the top to the bottom of the nucleus. This is due to the relatively high level of autofluorescence of embryonic tissues. If the complete stack was projected, then background signal intensifies and interferes with the perception of PH-P signal inside and outside the nucleus. Though a small set of sections was projected, the entire nucleus was always scanned in entirety to visualize PH-P colocalization with chromatin.
Figure 3-4. (next page) PH-P protein is removed from nuclei after S-phase. 0-3 hour wild-type embryos were labeled with a PH-P-specific antibody at 1:500 dilution and detected with 1:5000 dilution of goat anti-rabbit secondary antibody coupled to Alexa488. DNA was stained with TOTO-3 nucleic acid dye (red). (A) At S-phase (top row), all PH-P signal (green) is punctate and contained well within the nuclear periphery (outlined in white dotted line). As chromosomes begin to condense in prophase (middle row), less PH-P signal is detected within nuclei (white dotted line), and what remains is found close to the nuclear periphery. A significant portion of protein is outside of the nuclei in the common cytoplasm (arrowheads). By late prophase (bottom row) most PH-P is found outside of the nucleus (arrowhead). Scale bar represents 5$\mu$m in all panels. (B) The images presented in A were generated from a very thin (0.5$\mu$m) stack of confocal sections in order to reduce intensifying any background signal.
At the metaphase plate, most PH-P signal remains outside of the nucleus (Figure 3-5A, top panel, arrowheads). There is still very little nuclear PH-P signal at anaphase, but by late telophase, PH-P reappears within the nuclei and once again colocalizes with chromatin (middle and bottom rows, arrowheads). In summary, PH-P protein is abundant on chromatin during the stages of late telophase and DNA replication, and most protein appears to be removed from the nucleus during DNA condensation and segregation.

Interestingly, if the anti-PH-P antibody is used at a higher concentration, an additional signal is observed at the metaphase plate stage of the cell cycle. Anti-PH-P antibody used at a 1:100 dilution shows a PH-P signal directly adjacent to the metaphase plate chromosomes (Figure 3-6A). The weak signal appears at the location of the mitotic spindle (arrows). Embryos labeled with antibodies against PH-P and tubulin show a high degree of co-localization of PH-P and the spindle apparatus (Figure 3-6B). Figure 3-6C illustrates the set of sections that were used to project the confocal stack in 3-6B. The stack encompasses only the sections containing the spindle to avoid PH-P signal outside of the spindle planes to be projected as co-localization. Though these results were reproducible using a high antibody concentration, higher titers did not exhibit PH-P spindle colocalization possibly indicating that the antibody may be cross-reactive at higher concentrations.

iii. Maternal barren deficiency affects PH-P cycling.

Figures 3-4 and 3-5 illustrated that in the early mitotic cycles of wild-type embryos, PH-P cycles on and off chromatin. To determine whether PH-P cycling is dependent on its interaction with the condensin subunit Barren, PH-P distribution was
Figure 3-5. (next page) PH-P protein is absent from nuclei at metaphase and anaphase, but relocalizes to chromosomes by telophase. 0-3 hour wild-type embryos were labeled with a PH-P-specific antibody as previously described. DNA was stained with TOTO-3 nucleic acid dye (red). (A) At metaphase (top row), all PH-P signal (green) is punctate and almost entirely confined to the common syncytial cytoplasm (arrowheads) outside the nucleus (outlined in white dotted line). As chromosomes begin to segregate in anaphase (Middle row), PH-P signal (arrowheads) is still contained outside nuclei (white dotted line). By telophase (bottom row) most PH-P relocalized into the nucleus (arrowhead). Scale bar represents 10μm in all panels. (B) The images presented in A were generated from a very thin (0.5μm) stack of confocal sections in order to reduce intensifying any background signal.
Figure 3-5. See legend on previous page.
Figure 3-6. (next page) PH-P protein is detected at the metaphase spindle when higher concentrations of antibody are used. 0-3 hour wild-type embryos were labeled with 1:100 dilution of PH-P-specific antibody. DNA was stained with TOTO-3 nucleic acid dye and the spindle was visualized using anti-tubulin antibody as described for Figure 3-1. (A) Low magnification (200X) at metaphase (top row), reveals a weak PH-P signal which localizes near the metaphase chromosomes (arrows). (B) Higher magnification and zoom reveal some PH-P signal colocalizes to the spindle (bottom panels). Scale bars represent 5μm in all panels. (C) The images presented in A and B were generated from confocal sections encompassing only the sections containing the spindle apparatus to avoid PH-P signal outside of the spindle planes to be projected as colocalization.
Figure 3-6. See legend on previous page.
examined in embryos with a maternal barren deficiency. 0-3 hour embryos of barren\textsuperscript{1305}/CyO mutant mothers were fixed and stained with anti-PH-P as described in Materials and Methods. PH-P protein behaviour was again examined using confocal microscopy. Interestingly, PH-P protein cycling in barren embryos was nearly identical to that observed in wild-type embryos (Figure 3-7A, compare to 3-4 and 3-5). PH-P was most abundant within nuclei and on chromatin at telophase and S-phase (top row), and was removed from the nuclei during DNA condensation such that most PH-P signal was detected outside of the nuclear periphery by metaphase (bottom row). This is consistent with PH-P behaviour in wild-type embryos. The major difference is seen during S-phase, where there is significant PH-P signal outside of the nuclear periphery (3-7A, top row), compared to wild-type embryos where nearly 100% of the PH-P signal at the same mitotic phase is contained within the nucleus (Fig 3-4A, top panel). Both experiments used identical embryo collection, fixation and staining protocols as well as identical primary and secondary antibody titers and washes. In addition, the confocal iris size, laser power, gain and output settings were the same during image collections. Projection of the confocal stacks used to generate both sets of images used confocal sections at 0.1\mu m intervals for a total stack thickness of 0.5\mu m (Fig 3-7B). Therefore, the signal intensities and signal distribution of both sets of images can be directly compared. In summary, PH-P association with chromatin at S-phase is impaired in barren mutants.

\textit{iv. Chromatin bridges are a physical barrier to PH-P relocalization.}

Though embryos of barren mutant mothers exhibit less PH-P co-localized to chromatin at S-phase with significant PH-P remaining outside the nuclei, redistribution of
Figure 3-7. (next page) A maternal *barren* deficiency does not affect PH-P cycling off chromosomes. 0-3 hour embryos from *barren*<sup>1305</sup>/CyO mothers were labeled with a PH-P-specific antibody as described for Figure 3-4. DNA was stained with TOTO-3 nucleic acid dye (red). (A) At S-phase (top row), the majority of PH-P signal (green) is contained within the nuclear periphery (outlined in white dotted line). As chromosomes begin to condense in prophase (middle row), very little PH-P signal is detected within nuclei (white dotted line). Most protein is outside of the nuclei in the common cytoplasm. By metaphase (bottom row) nearly all PH-P is found outside of the nucleus. This pattern of PH-P removal from chromosomes is nearly identical to wild-type (compare to Figure 3-4), however with an increased cytoplasmic PH-P signal at late S-phase (top row). Scale bar represents 5μm in all panels. (B) The images presented in A were generated from confocal sections identical to those described for Figure 3-4 in order to allow direct comparison and to reduce intensifying any background signal.
Figure 3-7. See legend on previous page.
nuclear PH-P to the common cytoplasm during DNA condensation appeared normal. This suggests that only the S-phase and telophase association of chromatin is impaired in *barren* mutant embryos. It is not obvious whether this effect is a direct or indirect result of a *barren* deficiency. One possibility is that the maternal *barren* deficiency may impair molecular interactions required to redistribute PH-P protein to the chromatin after segregation is complete. Alternatively, since severe chromatin bridges at anaphase and telophase is the major mitotic phenotype in *barren* mutant embryos, the chromatin bridges themselves may act as a physical barrier to prevent relocalization of PH-P at telophase, independent of a PH-P-Barren interaction.

In order to determine whether chromatin bridges presented a physical barrier to relocalization, both bridged and segregated telophase nuclei from single 0-3 hour barren mutant embryos were examined for differences in PH-P-chromatin co-localization (Figure 3-8). Embryos in which the majority of telophase nuclei were segregated, the majority of PH-P signal was localized inside the nuclear periphery (Figure 3-8A, both rows). In these embryos, only a few telophase nuclei appeared bridged, joined by very thick chromatin bridges giving these nuclei an oval shape. Comparing PH-P signal between these oval (outlined in white dashed lines) and wild-type segregated nuclei (outlined in white dotted lines) reveal that only the segregated nuclei exhibit wild-type levels of PH-P signal colocalized to chromatin within the nuclei (arrowheads), while the bridged nuclei show that most PH-P remains outside the nuclear periphery (arrows). A small proportion of PH-P does co-localize to chromatin, even in these bridged nuclei. In cases where nearly every telophase nucleus was joined by thick chromatin bridges, most PH-P signal was found in the common cytoplasm surrounding the nuclear periphery.
(Figure 3-8B, arrows), similar to normal PH-P distribution at metaphase (compare to figure 3-5A top row and 3-7A bottom row).

For telophase nuclei joined by thick chromatin bridges, most PH-P does not relocalize to the nucleus. In cases where chromatin bridges were very thin, there appeared to be no difference in PH-P-chromatin co-localization between thin-bridged telophase nuclei (arrows) and segregated nuclei. (Figure 3-8C). There was similar levels of PH-P contained within segregated (outlined in white dotted line) and bridged (white dashed line) nuclei. Again, the experimental protocol and image collection was identical to Figure 3-5 and 3-7, and the confocal stack was projected from sections totaling 0.5μm (Figure 3-8D) to allow for comparisons between figures. PH-P relocalization is normal even in barren mutant embryos when telophase nuclei are fully segregated or joined only by very thin chromatin bridges. Both normal and inhibited PH-P relocalization can be observed in the same embryo, depending on the presence of thick chromatin bridges. Therefore, the dependence of PH-P relocalization on Barren is indirect, such that it is the resulting chromatin bridges of the maternal barren deficiency that affects PH-P behaviour.

v. barren does not enhance the mitotic phenotypes of ph-proximal mutations

PH-P and Barren were previously shown to co-immunoprecipitate in a complex with another cell cycle regulator Topoisomerase II. Though the previous experiment shows that a barren deficiency does not directly affect PH-P cycling during mitosis, a functional interaction is not ruled out. A genetic interaction assay was developed to test whether a barren mutation enhances the mitotic phenotypes of ph-p.
Figure 3-8. Figure legend and Figure continued on next page.
Figure 3-8, continued. Chromatin bridges are a physical barrier to PH-P relocating to nuclei following anaphase. 0-3 hour embryos from barren^{L305}/CyO mothers were labeled with a PH-P-specific antibody as described for Figure 3-4. DNA was stained with TOTO-3 nucleic acid dye. (A) At telophase, PH-P is only able to relocalize to fully segregated nuclei (both rows). In these embryos, the majority of nuclei are fully segregated (outlined in white dotted line) with only a few bridged nuclei (outlined in white dashed line). Only the segregated nuclei contain nearly wild-type levels of PH-P signal while bridged nuclei contain very little nuclear PH-P (compare arrows to arrowheads in right panels). (B) In embryos where the majority of nuclei are bridged, most PH-P signal at telophase remains outside the nuclear periphery (arrows). (C) When nuclei are joined by very thin chromatin bridges (outlined in white dashed line), the nuclear PH-P signal is nearly identical to that of fully segregated nuclei (outlined in white dotted line). (D) The images presented in A-C were generated from confocal sections identical to those described for Figure 3-4 in order to allow direct comparison and to reduce intensifying any background signal. Scale bar represents 10μm in all panels.
Since mitotic phenotypes of early embryos are due to maternal deficiencies, the "barren" and "ph-p" single and double heterozygotes were generated in females, and embryos resulting from matings of these mutant females were collected and examined. The generation of "barren-ph-p" double heterozygous females is outlined in Figure 3-9A. In these females both the FM7C and CyO balancer chromosomes that had previously balanced the "ph" and "barr" mutations respectively, were replaced by wild-type chromosomes to remove balancer effects from the assay. These females were mated with wild-type males and the resulting 0-3 hour embryos were collected and examined. Females with either "ph" or "barr" mutations alone over wild-type chromosomes were also generated and mated to wild-type males (Figure 3-9B, C). All three sets of females were collected as virgins and aged to ensure sibling matings had not occurred. All females were crossed to unmated wild-type males aged for 5 days after eclosure, and resulting 0-3 hour embryo progeny were collected, fixed and their DNA stained with TOTO-3. Though mitotic phenotypes of the resulting embryos are strictly a reflection of maternal genotype, wild-type males were used as mates to remove any possibility of problems with fertilization or other paternal effects. In addition, wild-type males and wild-type virgins from identical collection and aging protocols were also mated and resulting embryos collected as a negative control to ensure any mitotic defects encountered in the genetic enhancement assay were not due to hypoxia during mating, embryo collection or fixation procedures.

The embryos were examined for mitotic defects using confocal microscopy as before. All embryos within a single collection were examined and all mitotic defects were recorded. For embryos from the single "ph" or "barren" heterozygous mothers,
Figure 3-9. (next page) Crossing schemes for generating female flies to assay for genetic interaction of barren and ph-p. The standards for all crosses and embryos collections are described in Materials and Methods. (A) Crossing scheme to generate female flies doubly heterozygous for barren and ph-p. Balancer effects were removed by outcrossing to wild-type males, and the resultant females were again outcrossed to ensure parental effects in ensuing collected embryos were strictly maternal. (B) Generation of ph-p single heterozygote females. Again, females were outcrossed to remove balancer effects. (C) Generation of barren single heterozygous females.
Figure 3-9. See legend on previous page.
there were no new mitotic phenotypes encountered. All mitotic defects were identical to the PcG-type mitotic phenotypes described in detail in Chapter 2, including chromatin bridges, nuclear fallout, and others. Importantly, no embryos were encountered that were similar to those from homozygous *ph*409 mothers (see Figure 2-20) which exhibited very large and amorphous nuclei and severe chromosome breakage. In addition, the embryos from the barren-*ph-p* double mutant females all exhibited normal metaphase figures, with no evidence of decondensation or misalignment of chromatids at the metaphase plate.

Comparing the frequency of total mitotic defects resulting from each maternal genotype (Fig 3-10A), the highest proportion results from a maternal *ph-p* deficiency only, with 65% of all embryos exhibiting some mitotic phenotype. 35% of all embryos from a barren maternal deficiency only exhibit mitotic defects. Wild-type embryos have a very low penetrance (under 10%) of mitotic defects. Furthermore, there were no severe mitotic phenotypes encountered. All instances of chromatin bridges and nuclear fallout were of very low expressivity, which is consistent for all previous wild-type collections. Interestingly, embryos from females heterozygous for both *ph-p* and barren also did not exhibit any new phenotypes. Furthermore, the frequency of mitotic phenotypes encountered was nearly identical to that from the embryos of *ph-p* mothers, as if the contribution from barren was masked. This lack of additive or synergystic effects suggests that *ph-p* is epistatic to barren. Therefore, though both barren and *ph-p* are involved in mitotic progression, *ph-p* likely acts earlier in the mitotic cycle than barren.

Though the frequency of total mitotic defects suggests *ph-p* is epistatic to barren, examining individual categories of mitotic defects may uncover an additive or synergystic interaction between the two genes (Figure 3-10B). Mitotic defects were
Figure 3-10. (next page) barren does not interact genetically with ph-p. (A) A survey of total mitotic defects in embryos generated from the crosses described in 3-9. Embryos from double-heterozygous females (green column) exhibited a similar penetrance of mitotic defects as embryos from ph-p (yellow column) mothers (62% and 65% respectively). (B) Examination of individual categories of mitotic defects again reveals no additive or synergistic genetic interaction. In each category, the penetrance of mitotic defects were similar for the ph-p single heterozygotes and the barren-ph-p double heterozygotes. Graph Legend in A also applies to B.
A

maternal genotype of (n) embryos surveyed

<table>
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<tr>
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<th>Count</th>
</tr>
</thead>
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<tr>
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<td></td>
</tr>
<tr>
<td>$ph^{409}w$/+ (196)</td>
<td></td>
</tr>
<tr>
<td>$barr^{L305}$/+ (230)</td>
<td></td>
</tr>
<tr>
<td>$ph^{409}w$/+; $barr^{L305}$/+ (203)</td>
<td></td>
</tr>
</tbody>
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B

Comparison of categories of mitotic defects in embryos.

Figure 3-10. See legend on previous page.
separated into the categories of chromatin bridges, nuclear fallout, and other defects. The last category included every other phenotype such as polyploidy, mitotic asynchrony and other low-penetrant phenotypes described in detail in Chapter 2. Interestingly, for either chromatin bridge and nuclear fallout phenotypes, there were again no additive or synergistic effects, such that in both categories, the frequency of the mitotic phenotypes of embryos from the double heterozygous females was nearly identical to that of the embryos from \textit{ph-p} single heterozygous mothers. Again \textit{barren} did not increase the penetrance of the major categories of mitotic phenotypes.

As discussed in Chapter 2, many of the low-penetrant mitotic defects are indicative of very severe segregation defects that were unable to resolve but were able to override the fallout mechanism. The frequency of these low-penetrant mitotic defects of the \textit{barren-ph-p} double heterozygote was very similar to that of \textit{ph-p} alone. Again, there was no significant spike in the frequency, indicating that \textit{barren} does not increase the expressivity of any of the mitotic phenotypes of \textit{ph-p} mutant embryos.

\textit{vi. PH-P colocalizes with S-phase regulators}

One strategy to conclusively determine at which cell cycle stage PH-P is required for mitotic progression is to look for PH-P co-localization \textit{in vivo} with cell cycle regulators. One obvious candidate to examine is Barren. However, several of the previous experiments suggest that PH-P may be required during S-phase of the cell cycle. PH-P protein is maintained on chromosomes throughout S-phase in early embryos. In addition, the S-phase regulators \textit{PCNA} and Geminin have been shown to interact with \textit{ph}
and Rae28, respectively. Therefore, these cell cycle regulators were examined for co-localization with PH-P protein in early embryos.

Wild-type 0-3 hour embryos were collected, fixed and stained with TOTO-3 as previously described. In addition, they were labeled with an Alexa488-coupled antibody against PH-P and an Alexa568-coupled anti-Barren antibody (gift of H. Bellen). The embryos were examined using confocal microscopy. The signal from the anti-Barren antibody was consistently uninterpretable. There was very little signal from inside the nuclei at any stage, but very high signal in the common cytoplasm (data not shown). Modifications to the staining protocol, antibody titers, antibody-fluorophore coupling protocol and further antibody purification did not improve the signal.

To determine whether the two S-phase regulators PCNA and Geminin co-localize with PH-P in embryos, wild-type 0-3 hour embryos were collected and fixed, and were labeled with anti-PH-P and anti PCNA antibodies as described in Materials and Methods. Anti-PH-P antibodies were directly coupled to Alexa 488 fluorophore, and anti-PCNA to Alexa 568 via Zenon rabbit IgG labeling Kits (Molecular Probes). DNA of embryos was also stained with TOTO-3 nucleic acid dye. Embryos were examined using confocal microscopy. PCNA signal was very abundant on S-phase chromosomes, in agreement with published results (Yamaguchi et al., 1995). Interestingly, there was a high degree of overlap between the PH-P and PCNA signals on S-phase chromatin (Figure 3-11A), indicating colocalization. Most of the punctate PH-P signal overlaps with that of PCNA (arrowheads). In fact, there appear to be very few solitary PH-P signals, indicating a high degree of colocalization. Since the PCNA signal is more abundant, approximately 50% of the PCNA is not co-localized with PH-P. The images were projected from very thin
(0.1 μm) confocal stack (Figure 3-11B), minimizing the chance for colocalization of false positives.

Wild-type embryos were also labeled using Alexa 488-coupled anti-PH-P, and Alexa 568-coupled anti-Geminin (gift of Hui Zhang). Again, embryos at S-phase were examined for co-localization (Figure 3-12). The Geminin signal appears diffuse and there is significant Geminin signal outside the nuclear periphery. This may represent cross-reactivity resulting in background Geminin signal. Though the Geminin signal is abundant, there is modest signal overlap (arrows) between PH-P and Geminin, indicating a low degree of colocalization. Most PH-P signal in the merge panel appears green indicating very little overlap with Geminin, except for a few punctate spots (arrows, bottom panel). These experiments illustrate that the PH-P maintained on chromosomes during S-phase co-localizes with PCNA, a factor involved in elongation during DNA replication.

vii. PH-P and PCNA share similar nuclear compartments.

The images for the previous experiments were generated from 0.1μm confocal sections. This was important to ensure signal overlap represented true co-localization rather than signals from distant confocal sections superimposed from the projection process. However, during data collection, the entire nucleus was scanned from top to bottom to visualize distribution of each signal. Interestingly, a pattern of PH-P signal emerged which suggested that PH-P is compartmentalized in a specific manner during the cell cycle, and is similar to the compartmentalization of PCNA. To illustrate this, confocal sections of the entire nucleus were projected at 90° to visualize a side view of
Figure 3-11. (next page) PH-P partially co-localizes with PCNA. (A) PCNA and PH-P were visualized using polyclonal antibodies (as described in Materials and Methods) in 0-3 hour wild-type embryos. At late S-phase, PCNA signal (blue) is more abundant than PH-P (green), but there is a high degree of colocalization between the two proteins (bottom panel). Most of the punctate PH-P signal overlaps with PCNA signal (arrowheads). (B) Diagram representing very thin (0.1μm) confocal sections used to generate figures in A to avoid overlaps from signal in different planes to appear as colocalization in projected stack. Scale bars represent 10μm in all panels.
Figure 3-11. See legend on previous page.
Figure 3-12. (next page) PH-P partially co-localizes with Geminin. (A) Geminin and PH-P were visualized using polyclonal antibodies (as described in Materials and Methods) in 0-3 hour wild-type embryos. At late S-phase, there is a low degree of colocalization between the two proteins (bottom panel). Only some of the punctate PH-P signal (green) overlaps with Geminin signal (blue, arrowheads). (B) Diagram representing very thin (0.1μm) confocal sections used to generate figures in A to avoid overlaps from signal in different planes to appear as colocalization in projected stack. Scale bar represents 10μm in all panels.
Figure 3-12. See legend on previous page.
the signals from PH-P, DNA and other proteins (Figure 3-13A). Previous images have provided a top view of nuclei, while these 90° projections provide a side view of the nuclei. The right panel of Figure 3-13A illustrates the outline of a hypothetical nucleus in this view.

At telophase and the start of S-phase, DNA is uniformly decondensed, appearing as a homogeneous signal from the top to the bottom of the nucleus when viewed at 90° (Figure 3-13B, top row, left panel. As prometaphase progresses, the replicated DNA condenses and appears more brightly staining. Condensed DNA first appears towards the internal ("bottom") nuclear surface, while the DNA towards the external ("top") surface remains uncondensed. This is likely because this compartment may still be undergoing DNA replication, and does not condense chromatin until replication is complete. As prometaphase progresses and more DNA condenses, the condensing bright signal progresses towards the top surface of the nucleus (top row, middle and right panels).

Interestingly, PH-P distribution is also compartmentalized, residing in the same compartment as uncondensed DNA. At telophase and S-phase, PH-P is most abundant on chromatin, and appears relatively uniformly distributed in the nuclei. As prometaphase progresses, there is less signal towards the internal nuclear surface where DNA is condensing, but consistently bright signal in the compartment where DNA is still uncondensed (middle row, compare to DNA compartments in top row). It is important to note that the relative brightness of DNA and antibody signal differs between panels, but cannot be directly compared to each other due to variations in surface area, nuclear distribution and number of confocal sections used to generate these images. It is only the distribution of the signal within each panel that is important.
Figure 3-13. Figure continued, and Figure legend on next page.
Figure 3-13, continued. PH-P and PCNA share similar nuclear compartments. (A) Schematic illustrating the collection and projection of confocal stacks in two different planes. Collections and projections from the Z axis (left side) are used to generate images with a “top view”, and have been used so far in this thesis. Collections and projections from the Y axis (right side) generate a “side view” of nuclei and reveal nuclear compartments. A hypothetical nucleus is outlined in a black dotted line. (B) Visualizing compartmentalization of DNA, PCNA and PH-P as mitosis progresses from S-phase to prometaphase. Condensing DNA appears brightly stained and is compartmentalized to the bottom half of the nucleus as S-phase progresses. Uncondensed DNA towards the top is in a compartment where both PH-P and PCNA signal is concentrated at late S-phase. Geminin (bottom row) does not appear compartmentalized at late S-phase. A hypothetical nucleus is shown to illustrate the depth of the projected stacks.
The prometaphase compartmentalization of PH-P is mirrored by PCNA. The relatively uniform distribution of PCNA at S-phase is re-distributed to coincide with uncondensed DNA, and high PH-P distribution. Given the major role of PCNA as a processivity factor required for DNA elongation, it’s likely that its distribution at prometaphase to uncondensed DNA reflects ongoing DNA replication in that specific compartment. In addition, PH-P and PCNA co-localization and co-compartmentalization suggests that PH-P requirement for mitotic progression is during DNA replication, and that the cause of mitotic phenotype in ph-p mutant embryos is likely due to defects in S-phase.

viii. PH-P does not co-localize to centromeres.

PH-P was shown to have a specific compartmentalization pattern during mitosis. Centromeres are also highly compartmentalized within the nucleus (Ahmad and Henikoff, 2002). In addition, centromere chromatin structure is epigenetically regulated by a number of chromatin modifiers (Karpen and Allshire, 1997). Though there is no prior evidence that PH-P regulates centromeric chromatin, PH-P and centromeres were labeled in embryos to assay for co-localization. Wild-type 0-3 hour embryos were doubly labeled with Alexa-568 coupled anti-PH-P, and anti-Cid which was detected with Alexa488-coupled secondary antibody. The punctate Cid signals corresponding to the eight centromeres in a 2N nucleus were consistently clustered near each other and towards the outer surface of the nucleus. PH-P signal was never observed to co-localize to centromeric regions (Figure 3-14A). All PH-P signal was independent of Cid (arrows, bottom panel). Since centromeres were perfectly clustered and compartmentalized near the top of the nucleus, they were consistently visible in a single confocal section.
Consequently, these images were generated using a single confocal section, to eliminate the possibility of co-localization false positive results (Figure 3-14B).

**ix. PH-D has a different distribution pattern and behaves differently than PH-P during mitosis.**

As discussed in Chapter 2 mutations in *ph-distal* did not exhibit mitotic defects, suggesting that unlike PH-P, PH-D does not have a role in mitotic progression. However, PHD has such a high degree of sequence similarity to PH-P, they are often classified as the same protein, and are often not distinguished in literature (Hodgson *et al.*, 1997). Any differences in distribution during mitosis may highlight unique roles for each protein.

Wild-type 0-3 hour embryos were labeled with anti-PH-D, an antibody directed against the unique N-terminal region of PH-Distal. DNA was stained with TOTO-3 as described in Materials and Methods. Like PH-P, PH-D is abundant during S-phase (Figure 3-15A, middle right panel) and is removed from nuclei by metaphase. The telophase distribution of PH-D however, differs significantly from PH-P since there is very little co-localization between the two proteins. Though there is some degree of signal overlap (Figure 3-15A, bottom panel, arrows), the majority of PH-P and PH-D signal is independent. A significant amount of PHD signal appears very close to the nuclear periphery (middle right panel, arrowheads), whereas much of the PH-P signal is clustered inside the nucleus, away from the nuclear periphery (middle left panel). Again, the images were generated from thin confocal sections to avoid false co-localization results (Figure 3-15B).
Figure 3-14. (next page) PH-P does not colocalize with centromeres. (A) Centromeres were visualized using anti-Cid antibody and PH-P was visualized as described in Materials and Methods in 0-3 hour wild-type embryos. Centromeres are compartmentalized near the top of each nucleus (See Figure B for illustration). At telophase, there is no colocalization between the two proteins (see arrowheads, bottom panel). (B) Diagram illustrating that single confocal sections used to generate figures in A to avoid overlaps from signal in different planes to appear as colocalization in projected stacks. This figure also illustrates that centromeric signal was consistently found near the top of nuclei, close to the nuclear periphery. Scale bar represents 10μm in all figures.
Figure 3-14. See legend on previous page.
Figure 3-15. (next page) PH-P only partially co-localizes with PH-D (A) PH-P (green) and PH-D (blue) were visualized using polyclonal antibodies (as described in Materials and Methods) in 0-3 hour wild-type embryos. At telophase and S-phase (late telophase shown), PH-D distribution differs markedly from that of PH-P, with a significant amount of PH-D signal very close to the nuclear periphery (arrowheads, right panel middle row). There is only a low degree of colocalization between the two proteins (arrowheads, bottom panel). (B) Diagram representing very thin (0.2 μm) confocal sections used to generate figures in A to avoid overlaps from signal in different planes to appear as colocalization in projected stack. Scale bars represent 10 μm in all panels.
Figure 3-15.
See legend on previous page.
Since the distribution of PH-D at telophase differs from that of PH-P, it was important to determine whether PH-D localized to centromeric regions. Wild-type 0-3 hour embryos were labeled with both Alexa568-coupled anti-PH-D and anti-Cid detected with Alexa488-coupled secondary antibody. Single confocal sections revealed that PH-D does not co-localize to centromeres (Figure 3-16A). Again, much of the PH-D signal localizes toward the nuclear periphery and appears as a ring of PH-D (bottom right panel) while the centromeres are clustered near each other towards the interior of the nucleus (bottom left panel; arrows in all panels mark centromere cluster location). There were no instances of signal overlap (top right panel). A single confocal section was used to generate these images to avoid signal overlap (Figure 3-16B).

By metaphase, PH-D is also removed from chromatin and is redistributed outside the nuclear periphery in the common cytoplasm (Figure 3-17, middle right panel). Interestingly, a brighter punctate PH-D signal is observed at opposite poles of the metaphase nucleus (middle row, arrows), which higher magnification reveals to correspond to the tips of the metaphase spindle (bottom panel, arrows).

The major difference in distribution of PH-P and PH-D is observed during relocalization to the chromatin after segregation. PH-P relocalizes after chromosomes have fully segregated at telophase (see Figure 3-5A). In contrast, PH-D relocalization to nuclei appears at very early anaphase (Figure 3-18A). A significant PH-D signal is apparent within the nuclear periphery (outlined in white dotted line). Higher magnification reveals that the PH-D signal is co-localized to early anaphase chromosomes towards the midline of the segregating chromosomes (Figure 3-18B,
Figure 3-16. (next page) PH-D does not colocalize with centromeres. (A) Centromeres were visualized using anti-Cid antibody (green) and PH-D (blue) was visualized as described in Materials and Methods in 0-3 hour wild-type embryos. Centromeres are again clustered near the top of each nucleus (arrowheads, bottom left panel and see Figure B for illustration) while PH-D tends to distribute near the nuclear periphery (arrowheads, bottom right panel). At telophase, there is no colocalization between the two proteins (see arrowheads, top right panel). (B) Diagram illustrating that single confocal sections used to generate figures in A to avoid overlaps from signal in different planes to appear as colocalization in projected stacks. This figure also illustrates that centromeric signal was consistently found near the top of nuclei, close to the nuclear periphery. Scale bar represents 5 μm.
Figure 3-16. See legend on previous page.
Figure 3-17. (next page) At metaphase, the majority of PH-D (blue panel) is removed from the nucleus and is redistributed outside the nuclear periphery, similar to PH-P. A small proportion of PH-D (blue, at arrows) is localized to the tips of the spindle apparatus (green panel, and merge panel at bottom). Arrows are positioned identically in each panel to allow comparison of PH-D positioning. Scale bars represent 10µm in all panels.
Figure 3-17. See legend on previous page.
Figure 3-18. (next page) PH-D relocalization to nuclei occurs earlier than for PH-P. (A) The nucleus at early anaphase is outlined in white dotted line. A proportion of PH-D signal is found within the nucleus (blue, bottom left panel). (B) Increased zoom reveals that the nuclear PH-D signal at anaphase is found near the midline of the segregating chromosomes (arrow, both panels). Scale bars represent 10μm in all panels.
Figure 3-18. See legend on previous page.
This difference in relocalization timing as well as differences in distribution pattern suggest a potential functional difference between PH-P and PH-D.

III. Discussion

There are many possible steps in the cell cycle in which defects lead to a chromatin bridge phenotype at anaphase and telophase. Neither the reported interaction of PH-P with Barren and Topoisomerase II proteins (Lupo et al., 2001) nor the detailed phenotypic analysis of ph-p mitotic defects in Chapter 2 is sufficient to explain the cause of chromatin bridges or the cell cycle stage at which they arise. The experiments in this chapter reveal that the mitotic role of PH-P is likely during S-phase, and is independent of barren’s role in mitotic progression.

i. Segregation is properly initiated in ph-p mutants.

Examination of the mitotic spindle in embryos of ph-p mutants revealed that spindle formation and maintenance are normal. Chapter 2 results demonstrated that metaphase plate chromosomes in all PcG mutants (with the exception of E(z)) are consistently normal, with properly condensed chromosomes correctly aligning at the metaphase plate. This demonstrates that the mitotic events involved in proper chromatid condensation and alignment are unaffected in ph-p mutants. In addition, mapping centromere distribution in bridged nuclei established that since centromeres are most often found at the extreme opposite poles in bridged nuclei, even in cases where the nuclei are joined by very thick chromatin bridges, sister chromatid segregation was normally initiated. Kinetochore structures at centromeres attach the mitotic spindle to
chromosomes and are sites of attachment of sister chromatids to each other at the metaphase plate. Segregation and poleward movement of sister chromatids is initiated at centromeres and is followed by chromatid arm movement to opposite poles. Since segregation appears to be normally initiated, the metaphase to anaphase transition seems to be normal in \textit{ph-p} mutants. These experiments demonstrate that chromatin bridges in embryos from \textit{ph-p} mutant females are composed of unsegregated chromatid arms, and not lagging chromosomes. This eliminates many steps in the cell cycle that may be impaired in \textit{ph-p} mutants.

Attempts to determine whether the unsegregated chromatid arms are due to uncleaved cohesins were unsuccessful due to the lack of a signal from the antibody against the cohesin subunit Scc1 (dRad21). It is still formally possible that chromatid arms within the chromatin bridges are attached by cohesins, but there is no evidence in the literature that cohesins bind chromosomes at sites other than the centromere and pericentric heterochromatin in flies (Valdeolmillos \textit{et al.}, 2004; Warren \textit{et al.}, 2000). Therefore, the unsegregated chromatid arms are likely due to defects in steps other than chromatin condensation, alignment and initiation of segregation.

\textbf{ii. PH-P association with chromatin oscillates during mitosis.}

Examination of PH-P distribution during early embryonic cell cycles revealed that PH-P association with chromatin is mostly restricted to telophase and S-phase of the mitotic cycle. During prometaphase, PH-P loses its association with chromatin and exits the nucleus, and is redistributed to the common syncytial cytoplasm surrounding the nuclei. PH-P remains cytoplasmic until telophase, at which time it re-enters the nucleus.
and re-localizes to chromatin. This oscillation is likely important and must be regulated by a number of factors. The maintenance of PH-P on chromosomes during S-phase makes this stage likely to be the time at which PH-P is required for cell cycle progression.

The oscillation of PH-P in early embryonic cycles agrees with previously published results that examined PH-P distribution in mitotic cells of older (6-14 hour) embryos (Buchena et al., 1998). That study also reported punctate PH-P signal that was abundant in the nucleus at telophase and S-phase, and was mostly cytoplasmic at metaphase. However, the antibody used in that study was generated against a region of ph that is identical between both PH-P and PH-D. Consequently, most isoforms of both proteins were potentially recognized. A later report in which a transgenic fly was generated expressing GFP-PH-P driven by a GAL4 promoter also reported punctate PH-P distribution, but only in embryos past cell cycle 14. There was no examination of GFP-PH-P distribution in mitotic cells. In this study, a PH-P-specific antibody was used, which detects endogenous levels of both PH-P170, the full-length protein and PH-P140, which initiates from an internal methionine situated at position 244 (Hodgson et al., 1997).

The similarity of cycling of both maternal PH-P in 0-3 hour embryos and zygotic PH-P in older embryos suggests that early embryonic mitoses are representative of later cell divisions, and that there is a mitotic role for PH-P later in development. Also, given that zygotic PH-P acts in its capacity as an epigenetic maintenance protein, the fact that there is no change in PH-P oscillation during later embryogenesis suggests that the oscillation, and perhaps a mitotic role, is a necessary component of the epigenetic
mechanism. There is no evidence, however, that maternal PH-P has any maintenance role in early mitoses, as there is no zygotic transcription at that stage. In addition, no parallels may be drawn between the actual chromosomal targets of maternal and zygotic PH-P, especially since zygotic PH-P is presumably targeting chromosomal sites in its capacity as an epigenetic maintenance protein. The possibility also exists that the presence of PH-P in the cytoplasm during prometaphase and metaphase is important to cell cycle progression such that PH-P may act in a cytoplasmic rather than a nuclear capacity.

An oscillation pattern was also observed for PH-D protein, but with notable differences to PH-P. PH-D distribution at telophase and S-phase differs from PH-P such that only a small proportion of colocalization between PH-P and PH-D is detected. This differs from previous reports that demonstrated no difference between PH-P and PH-D binding to polytene chromosomes (Hodgson et al., 1997). However, since it is likely that neither PH protein acts as an epigenetic regulator during the early embryonic nuclear divisions, a distribution difference probably reflects different functions for each protein at this developmental stage. Though both PH-P and PH-D exit the nucleus at prometaphase, a bright punctate PH-D signal is found localized to the tips of the mitotic spindle at metaphase. PH-D also relocalizes to chromosomes at early anaphase in contrast to the telophase relocalization for PH-P. The distribution differences offer further evidence that the two proteins are functionally distinct. Though PH-D mutants do not exhibit any mitotic phenotypes, it cannot be ruled out that the oscillation pattern of PH-D or its localization to spindle tips at metaphase reflect a cell cycle role.
iii. **PH-P acts independently of barren for proper mitotic progression and is likely required during DNA replication.**

PH-P abundance on S-phase chromosomes argues against a functional interaction with barren, a component of the condensin complex that acts at prometaphase to condense replicated chromatin. However, since the two proteins were reported to co-immunoprecipitate from embryonic nuclear extracts, it was still possible that they co-operate to ensure mitotic progression. Attempts to determine whether the two proteins co-localize in vivo were unsuccessful due to the lack of a clear signal from the anti-Barren antibody. An alternate strategy was to examine whether a barren deficiency affected the mitotic role of ph-p in two different assays, namely, by looking for changes in the distribution of PH-P during the cell cycle in a barren mutant background, and by assaying whether barren enhances the mitotic phenotypes of ph-p. Interestingly, the presence of severe chromatin bridges in telophase nuclei inhibited re-entry of PH-P into the nucleus, thereby preventing its re-association with chromatin. This was not a direct effect of the barren deficiency since nuclear re-entry varied between neighbouring nuclei in the same barren mutant embryo, and appeared to depend solely on the severity of chromatin bridges joining nuclei.

If chromatin bridges were to impair PH-P localization to chromatin, a prediction is that PH-P would be unable to localize to the bridged chromatin, but be able to enter the nucleus and localize to properly segregated portions of chromosomes. However, PH-P was prevented from nuclear re-entry in cases where nuclei were joined by very severe chromatin bridges. This suggests that there may be a checkpoint that ensures chromosomes are properly segregated before PH-P and other nuclear factors are re-
imported to the nucleus. Alternatively, a chromatin “signal” may be uncovered on portions of chromosomes that are properly segregated, leading to the import and targeting of PH-P. The results showed that chromosomes were resolved to near completion before PH-P was imported and targeted to chromatin.

The mitotic phenotypes of *ph-p* are not enhanced by a *barren* deficiency. There was no synergistic increase in the penetrance or expressivity of *ph-p* phenotypes. In addition, no new phenotypes were uncovered. The lack of genetic interaction suggests no functional co-operation between *barren* and *ph-p* in mitotic progression. Interestingly, in addition to the lack of synergy, there was also no additive increase in the mitotic phenotypes of double mutants. The penetrance of all mitotic phenotypes in all categories of the double mutants were nearly identical to those of *ph-p* mutation alone. Therefore, the *barren* deficiency was effectively masked. Though the phenotypes of *barren* and *ph-p* are identical as single mutants, identical phenotypes may result from defects in many different unrelated but temporally connected steps of mitosis. Therefore, since the frequency of the mitotic phenotypes of *barren* is masked by those of *ph-p*, the results argue that *ph-p* is epistatic to *barren*.

If *ph-p* is epistatic to *barren*, it likely acts at an earlier stage of the cell cycle than *Barren*, that is, prior to chromosome condensation. This result in conjunction with the fact that most PH-P is removed from nuclei during chromatin condensation argues that PH-P likely acts during S-phase. In fact, PH-P co-localizes with PCNA, a processivity factor required for DNA elongation during replication. Though these results do not show if PH-P and PCNA directly interact, co-localization of PH-P with an elongation factor
defines DNA replication as the cell cycle stage for the mitotic role of PH-P, and predicts that its role in cell cycle progression may be linked to elongation.

**iv. The epigenetic and mitotic roles of PH-P may be related.**

The results in this chapter demonstrate that PH-P has a specific oscillating pattern of association with chromatin during mitosis. Reassociation of PH-P with chromatin after mitosis is impaired when chromatin bridges prevent segregation of chromatid arms. If PH-P localization to chromatin following cell division is imperative to its role as an epigenetic maintenance protein, chromatin bridges that prevent PH-P relocalization to chromatin in later embryonic cell cycles have the potential to impair the inheritance process. The following chapter will address this hypothesis and explore the link between PH-P and other cell cycle regulators.
CHAPTER 4 Exploring the links between cell cycle regulation and epigenetic silencing

I. Introduction

Previous chapters have established that members of the PcG have a role in cell cycle progression. In addition, a variety of cell cycle regulators have been shown to directly interact with PcG proteins, as discussed in Chapter 1. Taken together, these data suggest the hypothesis that cell cycle progression and epigenetic silencing are directly interlinked. Since this study has established that epigenetic silencers such as the PcG are involved in cell cycle progression, the reciprocal question arises, namely are cell cycle regulators involved in epigenetic silencing?

One approach to answer this question is to examine if cell cycle regulators exhibit the criteria used to classify PcG members. A previous report (Lupo et al., 2001) implicated the cell cycle regulator barren as directly involved in epigenetic silencing. Barren protein localizes to PREs and mutations in barren strongly de-repress a mini-white reporter gene controlled by the Fab7 PRE element. These data lead to the hypothesis that barren may itself be a PcG gene since it fulfils some of the criteria used to classify PcG genes, namely recruitment to PREs and de-repression of a PRE-linked reporter gene. To further test the hypothesis that barren is a PcG gene, a test of another PcG criterion was applied. PcG genes not only enhance the homeotic phenotypes of other PcG genes, but either suppress or enhance the homeotic phenotypes of trithorax Group genes, classifying them as either PcG or ETP genes (Gildea et al., 2000; and see Chapter 1).
One strategy to test whether both of these criteria apply to *barren* is to cross *barren* mutants to PcG and trxG mutants and assay for enhancement or suppression of homeotic phenotypes. In PcG adults, anterior to posterior transformation of body segments are phenotypic readouts of the derepression of Hox targets during development. The specific pattern of Hox expression and repression in a body segment leads to that segment’s identity, and de-repression of Hox genes in PcG mutants leads to an expression pattern that is normally found more posteriorly. Abdominal segments that are posteriorly transformed in PcG mutants exhibit bristle patterns or pigment patterns characteristic of more posterior segments. In male flies, the dorsal integument surface (tergite) of the first four abdominal segments (A1 to A4) have a light tan pigment pattern, in contrast to A5 and A6 which are completely dark brown in colour. Posterior abdominal transformations appear as patches of dark pigment in tergites of abdominal segments A1 to A4, reminiscent of A5 and A6. In addition, the bristles on the tergite of segment A1 are very short. In contrast, the posterior tergite borders of segments A2 to A5 exhibit long thick bristles; therefore a posterior transformation of A1 may also exhibit long thick bristles at the posterior margin. Posterior transformation of abdominal segment A6 to more posterior structures appears as completely or partially missing portions of A6 tergite. In wild-type flies, abdominal segment A7 is greatly reduced in size since there are no tergite or sternite (ventral integument surface) structures (reviewed in Kopp and Duncan, 2002).

Male flies also have a pair of structures comprised of a regular row of short bristles called sex combs on the most anterior (prothoracic) pair of legs which function to aid in the grasping of female flies during mating (Coyne, 1985). Sex combs result from the expression of the Hox gene *Sex combs reduced (Scr)* which is normally repressed by
PcG in the more posterior second and third (meso- and metathoracic) pairs of legs. PcG mutants typically exhibit ectopic sex combs on the meso- and metathoracic legs due to de-repression of Scr. Other posterior transformations in PcG mutants may manifest as a full or partial transformation of wing appendages of the second thoracic segment (T2) to the more posterior sensory appendages called halteres normally found on the third thoracic segment (T3). The Hox gene Ultrabithorax (Ubx) is expressed in and regulates haltere development in haltere imaginal discs (and thus T3), but is repressed by PcG proteins in wing imaginal discs (and thus T2) during wing development (Struhl, 1982). De-repression of Ubx in haltere discs by PcG mutations results in partial wing to haltere transformations (Dura et al., 1985; Busturia and Morata, 1988; Smolik-Utlaut, 1990) which appear as patches of thick brown cuticle with or without bristles on the surface of the otherwise thin and translucent wings.

Homeotic phenotypes of trithorax Group mutants are typically posterior to anterior transformations. The most common phenotype is transformation of segment A5 to A4, visible as patches of light tan pigment in contrast to the dark brown pigment of A5. Changes in penetrance and expressivity of anterior homeotic phenotypes were again assayed in double barren-trithorax heterozygotes and compared to sibling trithorax controls. Two available barren alleles were tested, barr\textsuperscript{1305}, a null mutation resulting from a P-element-mediated deletion and barr\textsuperscript{K14014}, a null mutation resulting from P-element insertion (Bhat et al., 1996b). Both of these barren alleles exhibited mitotic phenotypes reported in Chapter 2. The representative PcG members that were chosen were both \textit{ph-proximal} and \textit{ph-distal}, and Polycomb. Two alleles of trxG member \textit{trithorax} were chosen to cross to barren mutants. \textit{trx}\textsuperscript{E13} and \textit{trx}\textsuperscript{Z11} are hypomorphic
alleles that exhibit anterior transformations (Kennison and Tamkun, 1988). \( \text{trx}^{211} \) contains a point mutation in the conserved SET domain and abolishes the histone binding and methyltransferase activity of the SET domain (Breen et al., 1999; Katsani et al., 2001). Genetic crosses between barren and PcG and trxG members will determine if barren further fits the criteria of being a Polycomb Group gene.

Data from Chapter 2 demonstrated that most PcG mutants exhibit mitotic phenotypes. Therefore the common mitotic phenotype of barren and \( ph-p \) mutants previously reported (Lupo et al., 2001) is not unique and can no longer bolster the hypothesis that the mitotic role of \( ph-p \) is via its interaction with barren. In addition, data in Chapter 3 demonstrated that barren does not enhance the mitotic phenotype of \( ph-p \) mutants and is hypostatic to \( ph-p \). These data cannot be explained by the presence of a protein complex containing PH-P140, Barren and Topoisomerase II proteins, therefore, it became imperative to confirm the existence of this complex to allow further purification and biochemical testing. One strategy was to perform co-immunoprecipitations using Kc1 embryonic cell culture nuclear extracts.

Two types of Kc1 cell lines were available for study. One, a stably transfected cell line called F-PH-P-HA expresses Ph-Proximal protein (PH-P) tagged at the N-terminus with FLAG and at the C-terminus with HA epitopes. Both full length 170 kDa PH-P flanked by both epitopes and the shorter 140kDa PH-P tagged with the HA epitope are detected. This cell line over-expresses PH-P and allows for enrichment of PH-P in nuclear extracts and the detection of PHP140 at the exclusion of similarly-sized PH-Distal protein. Since PHP140 was reported to precipitate with Barren in a complex, this cell line is ideal to confirm the existence of the complex. Alternatively, an untransfected
Kc1 cell line expressing endogenous amounts of PH-P isoforms was also used to test for coimmunoprecipitation of Barren, Topo II and endogenous PH-P.

In Chapter 3 it was demonstrated that in embryos, PH-P relocalization to chromatin was delayed, only in nuclei that were joined by thick chromatin bridges in barren mutants (see Figure 3-8). One hypothesis is that the proper cell cycle-dependent distribution of PH-P is critical for its role as an epigenetic regulator. Furthermore, since a segregation defect itself was sufficient to prevent PH-P re-recruitment, another hypothesis predicts that any segregation defect would result in prevention of proper PH-P oscillation on and off chromosomes during the cell cycle and by extension, may affect PH-P-mediated epigenetic silencing. To test whether segregation defects interfere in the role of PH-P as an epigenetic regulator, flies mutant for several genes involved in segregation were tested for genetic interaction with ph-p.

polo kinase is required at several mitotic stages including sister chromatid segregation and cytokinesis, and polo mutants exhibit segregation defects (Herrmann et al., 1998; Donaldson et al., 2001). Pimples and three rows are both required for the degradation of cohesins at anaphase which releases paired sister chromatids. Both pimples and three rows have segregation phenotypes (Stratmann and Lehner, 1996; Leismann et al., 2000; Jager et al., 2001). Each of these segregation mutants was crossed to ph-p mutants and tested for genetic interaction by assaying for enhancement or suppression of the homeotic phenotypes of ph-p. To test whether segregation defects resulting from an S-phase defect also interacts with ph-p, flies mutant for Glutamine synthetase were also crossed to ph-p and tested for genetic interaction. Glutamine synthetase is required for the nucleotide biosynthesis pathway and mutations in Gs result
in incomplete DNA replication and thus segregation defects (Frenz and Glover, 1996). In addition, since the S-phase regulator Geminin was shown to partially colocalize with PH-P protein (See Chapter 3) and murine Geminin was reported to colocalize and interact directly with the murine ph homologue Rae28 (Luo et al., 2004), geminin mutants were also tested for genetic interaction with ph-p.

II. Results

i. barren enhances the homeotic phenotypes of PcG mutants and suppresses the homeotic phenotypes of trx mutants

To assess whether barren fits the criteria of a PcG gene, mutations in barren were tested for genetic interaction with members of PcG and trxG genes. 25 ph-proximal (ph409) virgins were mated with 15 males carrying one of two mutant alleles of barren. Mated flies were turned over into fresh vials daily and resultant adult offspring were examined for penetrance and expressivity of homeotic phenotypes. barren-polyhomeotic double heterozygote male offspring were examined and compared to control siblings mutant for polyhomeotic-proximal alone. All adults which emerged from a given vial were scored, up to approximately 100 double heterozygotes or control adults.

Two barren alleles were available from public stock centers. barr^{L05} is a null P-element induced deletion and barr^K14014 is a null P-element insertion. Both alleles were crossed to ph-p virgins and resultant adults examined for homeotic phenotypes. Both barren alleles enhance the penetrance of ph-p homeotic phenotypes (Table 4-1). The penetrance for all homeotic transformations including ectopic sex combs, abdominal transformations and wing to haltere transformations are all significantly enhanced in the
Table 4-1. *barren* mutations increase the penetrance of *ph-proximal* homeotic phenotypes. Both *barren*<sup>1305</sup> and *barren*<sup>14014</sup> alleles significantly enhance the penetrance of extra sex combs and both A4 to A5 and A6 to A7 abdominal transformations. 25 *ph*<sup>409</sup> virgins were mated with 15 males of each *barren* genotype and turned over daily for 4 days. Progeny were sequestered daily upon emergence and aged for one day in a fresh vial to allow full deposition of body pigment and unfurling of wings. All flies which emerged from a given vial were scored until no more progeny emerged. Enough vials were scored to completion until approximately 100 male flies of the double heterozygous and control *ph*<sup>409</sup> heterozygotes were counted. *barren-ph*<sup>409</sup> double heterozygotes have significantly higher penetrance of homeotic transformations than control siblings heterozygous for *ph*<sup>409</sup> only. Chi-squared analysis (bottom row of each panel) reveals critical Probability values lower than the 0.05 cutoff accepted as statistically significant. The number (n) of progeny scored appears below each genotype.

<table>
<thead>
<tr>
<th>Genotype Scored (n)</th>
<th>Penetrance and Phenotype Scored:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Average # of legs with sex combs</td>
</tr>
<tr>
<td><em>ph</em>&lt;sup&gt;409&lt;/sup&gt;Y; <em>barr</em>&lt;sup&gt;1305&lt;/sup&gt;/+ (99)</td>
<td>5.30</td>
</tr>
<tr>
<td><em>ph</em>&lt;sup&gt;409&lt;/sup&gt;Y; +/CyO (94)</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
</tr>
<tr>
<td><em>ph</em>&lt;sup&gt;409&lt;/sup&gt;Y; <em>barr</em>&lt;sup&gt;14014&lt;/sup&gt;/+ (101)</td>
<td>4.64</td>
</tr>
<tr>
<td><em>ph</em>&lt;sup&gt;409&lt;/sup&gt;Y; +/CyO (94)</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
</tr>
</tbody>
</table>
double heterozygote compared to the *ph-p* control siblings. Sibling *barren* heterozygotes themselves exhibited no homeotic phenotypes (data not shown). Chi-squared analysis reveals that enhancement of all phenotypes reported in Table 4-1 are significant with Probability values less than 0.05. The expressivity of homeotic phenotypes in each individual was also scored (Figure 4-1, see Materials and Methods for scoring criteria). Both alleles of *barren* increase the expressivity of the homeotic phenotypes of polyhomeotic-proximal. For *barren*1305, the increase in expressivity of the abdominal homeotic transformations was modest but the increase in the expressivity of the ectopic sex combs was more pronounced, increasing from an average of 1.62 to 2.88 (Figure 4-1, top panel). The *barr*14014 allele increased the expressivity of abdominal transformations more markedly (Figure 4-1, bottom panel).

To determine whether genetic interaction was limited to *ph-p*, both *barren* alleles were also crossed to two alleles of *Polycomb (Pc)* and adults were examined for homeotic phenotypes, as above (Table 4-2). In *Pc* mutants, the most prevalent homeotic phenotypes were the extra sex combs and wing to haltere transformation. Abdominal transformations were either absent (*Pc*4) or very weakly penetrant (*Pc*4310) and thus not scored. *barren*1305 significantly enhanced the penetrance of ectopic sex combs and wing to haltere phenotype of both *Polycomb* alleles (Table 4-2). *barr*14014 also enhanced the penetrance of the homeotic phenotypes of both *Pc* alleles. In all pairwise combinations, the enhancement of homeotic phenotypes was significant with the exception of the extra sex combs phenotype in the *Pc*4-*barr*14014 double mutant (second panel, in bold and underlined). The penetrance of ectopic sex combs in this case was enhanced, but not significant.
Figure 4-1. Both *barren* alleles enhance the expressivity of each homeotic transformation reported in Table 4-1. The expressivity of homeotic transformations of each fly was scored on a scale of 1 to 4. 1 represents a partial transformation where the transformed area occupies up to 25% of the abdominal segment or 25% of the length of a full sex comb. 2 represents 25-50%, 3 is 50 to 75% and 4 is 75-100% transformation. The average expressivity was calculated and graphed. *barr* modestly enhances the expressivity of A4 to A5 abdominal transformations and significantly increases the expressivity of the extra sex combs phenotype (red bars, double heterozygotes) compared to control siblings (gray bars, *ph* single heterozygotes). Expressivity of all three homeotic transformations is increased by *barr*.

![Graph showing expressivity of various phenotypes](image)
Table 4-2. Both "barren" and "barren" significantly enhance the penetrance of the homeotic phenotypes of two Polycomb alleles. In nearly all cases, Chi-squared analysis reveals the results are statistically significant. In the case of extra sex combs in "barren" and PcA double heterozygotes, the critical P value was calculated to be between 0.05 and 0.1 (shown in bold and underlined) therefore is just above the 0.05 cutoff considered to be statistically significant. However, the wing to haltere transformation was significant.

<table>
<thead>
<tr>
<th>Genotype Scored (n)</th>
<th>Phenotype Scored:</th>
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<tbody>
<tr>
<td></td>
<td>Average # of legs with sex combs</td>
</tr>
<tr>
<td>+/Y; barrL305/+; PcA/+ (93)</td>
<td>3.20</td>
</tr>
<tr>
<td>+/Y; +/CyO; PcA/+ (76)</td>
<td>2.15</td>
</tr>
<tr>
<td>X² P value</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>+/Y; barrK14014/+; PcA/+ (98)</td>
<td>2.52</td>
</tr>
<tr>
<td>+/Y; +/CyO; PcA/+ (101)</td>
<td>2.05</td>
</tr>
<tr>
<td>X² P value</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>+/Y; barrL305/+; PcXT109/+ (103)</td>
<td>3.96</td>
</tr>
<tr>
<td>+/Y; +/CyO; PcXT104/+ (89)</td>
<td>2.45</td>
</tr>
<tr>
<td>X² P value</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>+/Y; barrK14014/+; PcXT109/+ (112)</td>
<td>3.25</td>
</tr>
<tr>
<td>+/Y; +/CyO; PcXT104/+ (121)</td>
<td>2.55</td>
</tr>
<tr>
<td>X² P value</td>
<td>&lt; 0.05</td>
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</table>
The expressivity of the sex combs phenotype was also scored in all crosses. Expressivity of the wing to haltere transformation was not scored because in all cases, the phenotype was very mild, exhibiting several small cuticular patches on the wings with nearly no variability. In addition, wing defects in the control embryos were occasionally difficult to discern as the curled wings resulting from the Curly of Oster (CyO) marker occasionally have a crumpled appearance and minute dark patches which appear to be necrotic tissue. As a result, only easily discernible patches of shiny light brown cuticle occasionally exhibiting sensory bristles were scored for penetrance of the wing to haltere transformation. Nevertheless, the expressivity of extra sex combs was enhanced markedly (Figure 4-2) in each double heterozygote.

Female offspring of the barren-Polycomb crosses were also scored for the wing to haltere phenotype. Females are typically not scored in PcG crosses but in this case, the wing to haltere phenotype was visible. Both alleles of barren significantly enhanced the penetrance of the wing to haltere phenotype of both alleles of Polycomb used in the assay (Table 4-3).

In previous chapters it was shown that ph-distal mutant embryos do not themselves exhibit mitotic defects and do not enhance the mitotic phenotypes of barren mutants. To determine whether there is genetic interaction between barren and ph-distal, both barren alleles were crossed to ph-distal (ph^{401}) virgins and assayed for enhancement of homeotic phenotypes as before. Interestingly, neither barren allele enhances the penetrance of the homeotic phenotypes of ph-distal (Table 4-4). The expressivity of each of the phenotypes scored was also very similar, indicating no genetic interaction (Figure 4-3).
Figure 4-2. Both barren alleles enhance the expressivity of the extra sex combs phenotype in both alleles of \( Pc \). The expressivity of the wing to haltere phenotype appeared to be consistently mild in both the double heterozygotes and control siblings of all crosses and were therefore not scored. Again, red bars represent the double heterozygotes while grey bars represent the control \( Pc \) single heterozygotes.
Table 4-3. Both *barren* alleles significantly enhance the penetrance of the wing to haltere homeotic phenotype of both *Polycomb* alleles in female progeny. Female progeny were tallied and the penetrance of the wing to haltere phenotype was scored. In all pairwise combinations of both *Polycomb* and both *barren* alleles, the enhancement of wing to haltere transformations was significant as judged by Chi-squared analysis.

<table>
<thead>
<tr>
<th>Genotype Scored</th>
<th>Phenotype Scored: Partial wing to haltere</th>
</tr>
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<tbody>
<tr>
<td>yw/+; <em>barr</em>&lt;sup&gt;L305&lt;/sup&gt;/+; *Pc&lt;sup&gt;d&lt;/sup&gt;/+ (105)</td>
<td>31%</td>
</tr>
<tr>
<td>yw/+; *+/CyO; Pc&lt;sup&gt;d&lt;/sup&gt;/+ (80)</td>
<td>3.8%</td>
</tr>
<tr>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>yw/+; <em>barr</em>&lt;sup&gt;L305&lt;/sup&gt;/+; *Pc&lt;sup&gt;XT104&lt;/sup&gt;/+ (116)</td>
<td>28%</td>
</tr>
<tr>
<td>yw/+; *+/CyO; Pc&lt;sup&gt;XT104&lt;/sup&gt;/+ (94)</td>
<td>7.6%</td>
</tr>
<tr>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>yw/+; <em>barr</em>&lt;sup&gt;K4014&lt;/sup&gt;/+; *Pc&lt;sup&gt;d&lt;/sup&gt;/+ (99)</td>
<td>73%</td>
</tr>
<tr>
<td>yw/+; *+/CyO; Pc&lt;sup&gt;d&lt;/sup&gt;/+ (106)</td>
<td>17%</td>
</tr>
<tr>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>yw/+; <em>barr</em>&lt;sup&gt;K4014&lt;/sup&gt;/+; *Pc&lt;sup&gt;XT104&lt;/sup&gt;/+ (96)</td>
<td>52%</td>
</tr>
<tr>
<td>yw/+; *+/CyO; Pc&lt;sup&gt;XT104&lt;/sup&gt;/+ (82)</td>
<td>23%</td>
</tr>
<tr>
<td>X&lt;sup&gt;2&lt;/sup&gt; P value</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
Table 4-4. Neither barren\textsuperscript{L305} nor barren\textsuperscript{K14014} allele significantly enhances the penetrance or expressivity of the homeotic phenotypes of \textit{ph-distal}. Extra sex combs and A1 to A2 and A4 to A5 homeotic transformations were scored. Calculation of P-values indicates that there is close to 50% to nearly 100% probability that the results may have arisen by chance, therefore are not significant.

<table>
<thead>
<tr>
<th>Genotype Scored (n)</th>
<th>Phenotype Scored:</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Average # of legs with sex combs</td>
<td>A1 to A2 transformation</td>
<td>A4 to A5 transformation</td>
</tr>
<tr>
<td>\textit{ph}\textsuperscript{401}w/Y; \textit{barr}\textsuperscript{L305}/+ (115)</td>
<td>2.03</td>
<td>32%</td>
<td>75%</td>
</tr>
<tr>
<td>\textit{ph}\textsuperscript{401}w/Y; +/CyO (60)</td>
<td>2.0</td>
<td>38%</td>
<td>72%</td>
</tr>
<tr>
<td>\textit{X}\textsuperscript{2} P value</td>
<td>&gt;0.9</td>
<td>0.1&lt;P&lt;0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>\textit{ph}\textsuperscript{401}w/Y; \textit{barr}\textsuperscript{K14014}/+ (93)</td>
<td>2.00</td>
<td>45%</td>
<td>75%</td>
</tr>
<tr>
<td>\textit{ph}\textsuperscript{401}w/Y; +/CyO (94)</td>
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</tr>
<tr>
<td>\textit{X}\textsuperscript{2} P value</td>
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<td>&gt; 0.9</td>
<td>0.1&lt;P&lt;0.5</td>
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</table>
Figure 4-3. Neither barren$^{L305}$ nor barren$^{K14014}$ allele enhances the expressivity of the homeotic phenotypes of ph-distal. In all cases, the expressivity of each phenotype is very similar in the barren - ph$^{401}$ double heterozygotes and the control ph$^{401}$ siblings.
Enhancement of PcG phenotypes and the previous findings that Barren is localized to PREs and is required for silencing (Lupo et al., 2001) suggests that barren may itself be a PcG or ETP group gene. To test whether barren interacts genetically with trxG genes, both alleles of barren were crossed to two alleles of trithorax, $trx^{E13}$ and $trx^{211}$. $trx$ typically has posterior homeotic transformations most often visible as patches of light pigment reminiscent of anterior abdominal segments within segment A5 which is normally darkly pigmented. Interestingly, $barr^{L305}$ suppressed the penetrance and expressivity of the homeotic phenotype of $trx^{E13}$ (Table 4-5, top panel). There was significant reduction in the penetrance of the A5 to A4 transformation from 50.5% to 22.3%. In addition, expressivity of the phenotype was lower in the double heterozygote than in $trx$ control siblings (Figure 4-4 A). This genetic interaction suggests that barren does fit into the classical definition of a Polycomb Group gene by enhancing other PcG homeotic transformations and suppressing those of trxG. Interestingly in two separate experiments, when barren$^{K14014}$ allele was crossed to $trx^{E13}$, many eggs were laid in all vials, but no progeny survived (Table 4-5, second panel).

Both barren alleles were also crossed to a second $trx$ allele, $trx^{211}$. Interestingly, both alleles of barren crossed to $trx^{211}$ resulted in very low numbers of offspring (table 4-5, bottom panels). A typical cross is turned over 8 times resulting in approximately 150 emerged adults per vial for a total of 800-1000 adults emerged per cross. The $barr^{L305}$. $trx^{211}$ cross resulted in many unhatched eggs and a total of only 105 emerged adults. No pupal cases with unemerged pharate adults were observed in any of the vials. Only 14 male $trx$-barren double heterozygotes and 8 male $trx$ control siblings emerged in total. Though these flies were scored for penetrance and barren again appears to suppress the
Table 4-5. barren$^{L305}$ significantly suppresses the penetrance of the homeotic phenotypes of $trx^{E13}$ (top panel). Only this combination of barren$^{L305}$ and $trx^{E13}$ resulted in enough male progeny (n) to allow statistical analysis. There were no hatched larvae nor emergent adults from two attempts at crossing barren$^{K140I4}$ and $trx^{E13}$ (second panel). Many eggs were laid in all 10 vials the parents were turned over into, but no larvae were observed. Similarly, crossing both barren alleles to $trx^{Z11}$ resulted in very few hatched progeny. Although it appears as if barren suppresses the penetrance of the A5 to A4 abdominal transformations (bottom panels), the low numbers of progeny (N) prevented statistical analysis.

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<th>Genotype Scored (n)</th>
<th>Phenotype Scored: A5 to A4 transformation</th>
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<td>50.5%</td>
<td>$+/Y; +/CyO; trx^{Z11}+/+$ (8)</td>
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<td>37% *</td>
</tr>
<tr>
<td>$+/Y; +/CyO;trx^{Z11}+/+$ (8)</td>
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</tr>
<tr>
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<td>39.1%</td>
<td>X$^2$ P value</td>
<td>NA</td>
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</table>

* The cross of 20 $trx^{Z11}/TM3.Sb$ virgins to 15 yw/Y; barr$^{K140I4}/CyO$ males yielded many eggs laid and very few hatched progeny. Only 105 adults emerged from 10 vials.

NA
penetrance of A5 to A4 abdominal transformations (Table 4-5, bottom panels), the low numbers of progeny prevent concluding that this interaction is significant. Examination of the distribution of resultant genotypes from this cross reveals that there is no great fluctuation of resultant progeny from expected Mendelian ratios (Figure 4-4 B, top panel) which was confirmed by Chi-squared analysis (data not shown). Similar to barr$^{L305}$, the barr$^{K14014}$ cross yielded 254 emerged adults from 10 vials. Only 23 male barren-trithorax double heterozygotes and 23 male trithorax control siblings emerged. Though it appears that barren suppresses the abdominal transformation phenotype of trx$^{Z11}$, low progeny numbers again prevent conclusions about the significance of the data. Distribution of sibling genotypes again is very close to expected Mendelian ratios (Figure 4-4B, bottom panel).

**ii. barren does not co-immunoprecipitate with any Ph isoform, or Topoisomerase II.**

The data in previous chapters demonstrated that of the Polycomb Group, a mitotic phenotype is not restricted to polyhomeotic and is also not dependent on barren. The evidence argues that a functional Polyhomeotic-Barren complex is not sufficient to explain the presence of mitotic defects in other PcG mutants, nor the evidence that the mitotic defects in ph-proximal likely arise at S-phase. To confirm the results of Lupo et al. (2001) that Barren protein and the PH-P 140kDa isoform co-immunoprecipitate in a complex with Topoisomerase II, immunoprecipitations using several types of nuclear extracts were performed. At the time these experiments were performed, the antibodies distinguishing PH-P from PH-D that were described in Chapter 3 were not available. Since PH-P140 and PH-Distal are a similar size, an alternative strategy to enrich for and
Figure 4-4. (A) *barren*\textsuperscript{L305} significantly suppresses the expressivity of the A5 to A4 homeotic phenotype of *trx*\textsuperscript{E13}. (B) The crosses between both *barren* alleles and *trx*\textsuperscript{Z11} reported in Table 4-5 yielded very few surviving progeny. Examining the distribution of the genotypes of the emerged adults (*barren*\textsuperscript{L305} in top pane and *barren*\textsuperscript{K14014} in bottom panel) reveals a distribution relatively close to the expected Mendelian ratio as judged by Chi-squared analysis (data not shown).
detect PH-P isoforms was used. Nuclear extract from a stably transfected Kc1 embryonic cell line expressing a FLAG- and Hemagglutinin-tagged PH-P under the control of a metallothionein-inducible promoter (called F-PH-P-HA) was used (Wang and Brock, 2003). In this cell line, the ph-proximal cDNA is flanked by the FLAG epitope at the 5’ end and an HA epitope at the 3’ end. In this cell line, two isoforms of PH-P are expressed, a 170kDa full-length PH-P (PHP170) flanked by both epitopes and a 140kDa PH-P (PHP140) fused to a C-terminal HA epitope, which results from the internal initiation of Methionine at position 244 (Figure 4-5A, Hodgson et al., 1997). Since Lupo et al. (2001) observed the 140kDa isoform co-immunoprecipitated with Barren and Topo II, the a-HA antibody was used to immunoprecipitate the nuclear extract. 50μg of nuclear extract from the F-PH-P-HA Kc1 cell line was immunoprecipitated with a-HA antibody. Western blots using α-HA, α-Topo II and α-Barren (gifts of H. Bellen) antibodies revealed that neither barren nor Topo II immunoprecipitate with PH-P140 (Fig 4-5B).

Though the F-PH-P-HA cell line overexpresses both the 170 and 140kDa isoforms of PH-P, the expression of PH-P170 is greatly favoured over PH-P140 (Figure 4-5B, input lanes). The immunoprecipitation was repeated using nuclear extracts from a non-transfected Kc1 cell line expressing endogenous levels of all PH isoforms. The α-Barren antibody was used to immunoprecipitate and Western blots using α-Barren, α-Topo II and α-E5 (which recognizes most isoforms of PH-P and PH-D, including PH-P140) were used to detect the presence of all three proteins. Again, no PH isoform immunoprecipitated with Barren (Figure 4-5C), in agreement with the results from the F-PH-P-HA nuclear extracts. Interestingly, Barren also did not immunoprecipitate
Topoisomerase II in any of the co-IP experiments. Because all co-IP results were negative, a control experiment was performed to rule out the possibility that generation of nuclear extracts failed to inactivate proteases or otherwise resulted in substandard extract and also to confirm that the co-immunoprecipitation procedure would detect *in vitro* interactions. PH-P and Polycomb proteins were previously shown to physically interact and are members of the PRC1 PcG complex (Kyba and Brock, 1998; Shao, *et al.*, 1999). From the Kc1 nuclear extracts, a-Polycomb antibody successfully immunoprecipitated correctly-sized isoforms of polyhomeotic (Figure 4-5D) indicating that the nuclear extract was of acceptable quality and the co-IP procedure does detect possible *in vitro* interactions.

To address the possibility that a PH-P-Barren complex is not abundant, Kc1 nuclear extracts were fractionated to enrich for the Barren, PH-P and TopoII components, and the immunoprecipitations were performed again. Figure 4-6 A shows the fractionation profile of 50mg (total protein) nuclear extract applied to Biorex70 ion exchange column (BioRad Laboratories). 20 fractions at each of 0.1M, 0.18M, 0.3M 0.6M and 0.85M KCl concentrations were collected and protein concentration were determined by BioRad Protein Assay. Peak fractions and fractions with protein concentrations of at least 50% of the peak fraction were pooled. Western blots of all the salt elutions revealed that the 0.18M salt fraction eluted the highest concentration of PcG proteins PH-P and Polycomb, as well as Barren. In addition, all detectable Topo II was eluted in the 0.18 fraction (Figure 4-6B). An immunoprecipitation using α-Barren antibody from the 0.18M fraction again did not precipitate any Polyhomeotic isoform nor any detectable Topoisomerase II (Figure 4-6C).
Figure 4-5. Figure continued and Figure legend on following page.
Figure 4-5. TopoisomeraseII and PH-P do not coimmunoprecipitate with Barren from Kc1 nuclear extracts. (A) A stably transfected Kc1 embryonic cell line called F-PHP-HA (Wang and Brock, 2003) expresses two isoforms of PH-P. The FLAG and HA epitopes flanking the PH-P cDNA result in the 170 kDa PHP170 fused to both epitopes and the 140 kDa PHP140 which results from internal initiation from Methionine 244 (Hodgson et al., 1997) and is fused to the C-terminal HA epitope only. (B) barren and TopoisomeraseII do not co-immunoprecipitate with PH-P from F-PHP-HA nuclear extracts. Immnoprecipitation with the α-HA antibody did not precipitate Barren nor TopoisomeraseII. Figure shows results of immunoprecipitation with α-HA at 1:1000. The input (left) lanes demonstrate that though both isoforms of PH are detected, the full-length 170 kDa PHP170 (marked by asterisk) is greatly favoured over the 140 kDa PHP140 isoform (marked by dagger). Precipitates were run on a 7.5% SDS-PAGE gel and a Western blot was performed with α-HA, α-Barren and α-TopoII each at 1:10000. Goat α-rabbit secondary antibody coupled to horseradish peroxidase was used at 1:5000. Subsequent to the chemiluminescence reaction, neither a long (right panel) and short (left panel) exposure of the blot to film resulted in any detectable barren or TopoII. (C) Co-immunoprecipitation attempt using nuclear extracts from a non-transfected Kc1 cell line which expresses endogenous levels of all PH isoforms. α-barren antibody at 1:500 did not precipitate any detectable levels of TopoisomeraseII or any detectable PH isoform from 75μg (middle lane) or 750μg (right lane) nuclear extract. The E5 antibody was generated against an epitope common to both ph-proximal and distal and therefore recognizes most isoforms of ph-proximal and distal. The input lane of the E5 Western (left lane, top panel) contains the endogenous PHP170 band (marked by 1) and a doublet which contains both PHP 140 and PH-distal (marked by 2). Ph-Distal (PH-D) is approximately 150 kDa. No isoform of either PH-P or PH-D was precipitated by α-barren. The two bands visible in the right lane of the E5 Western blot (marked by asterisks) representing immunoprecipitation from 750μg nuclear extract are non-specific since they also appear in the TopoisomeraseII blot. (D) Control immunoprecipitation using α-Polycomb antibody successfully immunoprecipitates PH, a known interactor, demonstrating that the nuclear extract and immunoprecipitation procedures do not result in degraded proteins or false negative results.
Figure 4-6. (next page) anti-Barren does not immunoprecipitate PH-P or TopoisomeraselI from a fraction enriched for PH, Barren and TopoisomeraselI by a Biorex70 ion exchange column. (A) 50mg (total protein) of nuclear extract from KCl cells was applied to a Biorex70 ion exchange column and fractions were eluted at 0.1M, 0.18M, 0.3M, 0.6M and 0.85M KCl. 20 fractions at each salt cut (X axis) were collected and assayed for protein concentration (OD A595, Y axis). Peak fractions and shoulder fractions with protein concentrations greater than or equal to 50% of peak for each salt fraction were pooled (filled circles). (B) 20μg of protein from each fraction was run on a 7.5% SDS-PAGE gel and Western blots revealed the greatest concentration of PH and Barren were in the 0.18M fraction. In addition, all detectable TopoisomeraselI eluted in the 0.18M fraction. (C) Immunoprecipitation attempt from 500μg (total protein) of 0.18M fraction using anti-Barren antibody. Again, Barren antibody at 1:500 did not precipitate any detectable PH-P or TopoisomeraselI as judged by Western blot. A non-specific band from the E5 blot appears in the IP (right) lane corresponds to the non-specific band appearing in previous IP attempts (See Figure 4-5C).
Figure 4-6. Figure legend on previous page.
iii. cell cycle mutants which exhibit segregation defects enhance the homeotic phenotypes of ph-proximal

Barren was originally discovered as a cell cycle regulator. Data in this chapter suggests that barren may also be considered a PcG gene because it fulfils many of the criteria of PcG genes including enhancing the homeotic phenotypes of other PcG genes and suppressing those of trithorax. Since a reported interaction between barren and PH-P could not be confirmed, it became likely that Barren and Topoisomerase II are not keystone components of the interplay between the PcG and cell cycle progression. To investigate the hypothesis that mutations in any cell cycle gene that disrupts segregation will enhance homeotic phenotypes of ph-proximal, a variety of cell cycle regulators was chosen. Three genes directly involved in sister chromatid segregation: polo kinase, pimples and three rows were chosen. In addition, Glutamine synthetase was chosen because its mutants indirectly affect the segregation process. Gs is required for the nucleotide biosynthesis pathway and its mutants cause incomplete DNA replication and subsequent segregation defects (Frenz and Glover, 1996). Interestingly, all four cell cycle regulators enhanced the penetrance of the homeotic phenotypes of polyhomeotic-proximal (Table 4-6). In nearly all cases the enhancement was significant as judged by Chi-squared analysis. The only exception was the A4 to A5 abdominal transformation in thr/ph409 double mutants (in bold and underlined). Though the penetrance of the transformation is modestly enhanced, it is not significant. It is interesting that Gs mutants, whose segregation phenotypes are indirect due to incomplete DNA replication, also enhance ph-p homeotic phenotypes. Interestingly, mutations in the gene encoding PCNA (which inhibits complete DNA replication) also enhances the penetrance of the
Table 4-6. (next page) A variety of cell cycle regulators which themselves exhibit mitotic but not homeotic phenotypes significantly enhance the penetrance of the homeotic phenotypes of ph-proximal mutants. Segregation mutants polo and pimples (top two panels) both enhance the penetrance of the homeotic phenotypes of ph-proximal. In addition, both alleles of the segregation mutant three rows (third and fourth panel) enhance the penetrance of the homeotic phenotypes of ph-proximal. Finally, the Glutamine synthetase mutation (which affects the nucleotide biosynthesis pathway and was previously shown to have segregation defects) also significantly enhances the penetrance of extra sex combs and both the A4 to A5 and A6 to A7 abdominal transformation of ph-proximal. In nearly all cases, the genetic interactions were calculated to be statistically significant. The only exception was the A4 to A5 transformation in thr-ph-proximal double mutants (shown in bold and underlined). However, both the extra sex combs and A6 to A7 phenotypes as well as all three phenotypes in the thr-ph-proximal double mutants were significant.
Table 4-6. Genetic interactions between *ph-p* and cell cycle regulators

Table legend on previous page.

<table>
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<tr>
<th>Genotype Scored (n)</th>
<th>Phenotype Scored:</th>
<th>Average # of legs with sex combs</th>
<th>A4 to A5 transformation</th>
<th>A6 to A7 transformation</th>
</tr>
</thead>
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</tr>
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</tr>
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homeotic phenotypes of polyhomeotic-proximal (Hodgson and Brock, unpublished observations). Expressivity of each phenotype reported in Table 4-6 was also scored for all crosses (Figure 4-7), and increased in each double heterozygote compared to control siblings. These data indicate that proper segregation of dividing cells during development is crucial to the inheritance of Hox patterning.

*ph-proximal* was also crossed to two available alleles of *geminin*. Geminin is an S-phase regulator which ensures replication origins only fire once per round of DNA replication. In flies, the mitotic phenotype of a *geminin* deficiency is overreplication as well as metaphase arrest and mild anaphase segregation defects (Quinn et al., 2001). As discussed in Chapter 3, the murine homologue of Geminin was shown to co-localize and directly interact with a murine PH homologue, Rae28 (Luo et al., 2004). This study has also shown PH-P protein to partially co-localize with Geminin during S-phase in early embryos. To determine if *geminin* interacts genetically with *ph-proximal*, both *geminin* alleles were crossed to *ph*^409^ and resultant offspring were assayed for homeotic phenotypes. Interestingly, both *geminin* alleles suppressed the penetrance of the homeotic phenotypes of *ph-proximal* (Table 4-7). The *geminin*^K9107^ allele significantly suppressed the ectopic sex combs and abdominal transformation phenotypes of *ph*^409^ as judged by Chi-squared analysis (Top panel). Similarly, the *geminin*^K1404^ allele also significantly suppressed the extra sex combs and abdominal transformation phenotypes of *ph*^409^ (bottom panel). Both *geminin* alleles suppressed the expressivity of these phenotypes (Figure 4-8A), though the effects in *geminin*^K9107^ were more modest.

Chromatin bridges have previously been reported for *Drosophila geminin* mutants (Quinn et al., 2001) but examination of the mitotic phenotypes of *geminin* using the
Figure 4-7. The cell cycle regulators which enhance the penetrance of the homeotic phenotypes of \textit{ph}-proximal (reported in Table 4-6) all enhance the expressivity of those homeotic phenotypes. In some cases, the enhancement was modest (for example see A4 to A5 in the \textit{polo}^{0167}-\textit{ph}^{409} cross, top panel), while in others the enhancement was more significant (see extra sex combs phenotype, same panel). The overall trend in all cases was an increase in the expressivity of the homeotic phenotypes of \textit{ph}-proximal.
In contrast to the cell cycle regulators reported in Table 4-6, geminin significantly suppresses the homeotic phenotypes of ph-proximal mutants. The mitotic role of Geminin ensures a single round of replication during S-phase and mutants of geminin exhibit overreplicated DNA. Both the gem$^{K9107}$ allele (top panel) and the gem$^{K14019}$ allele suppresses the penetrance of the extra sex combs and both the A4 to A5 and A6 to A7 abdominal transformations of ph$^{409}$. Again, in nearly all cases, the genetic interactions were calculated to be statistically significant. The only exception was the A6 to A7 transformation in gem$^{K14019}$-ph$^{409}$ double mutants (shown in bold and underlined).

<table>
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<th>Phenotype Scored:</th>
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<th>A4 to A5 transformation</th>
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criteria outlined for PcG mutants in Chapter 2 revealed a very low penetrance and expressivity of segregation defects (Figure 4-8B, top panel), similar to what was seen in control wild-type embryos. While low-expressivity segregation defects were observed where several nuclei pairs were joined by chromatin bridges (Fig 4-8C, top panel) severe chromatin bridges as were reported for PcG mutants were never observed in either geminin mutant. The penetrance and expressivity of nuclear fallout was significantly above those observed in wild-type embryos (Figure 4-8B, bottom panel) consistent with a cell cycle mutant. The predominant mitotic phenotype observed were puffs of uncondensed chromatin (Figure 4-8C, bottom panel) consistent with DNA overreplication and metaphase arrest as was previously reported (Quinn et al., 2001). These genetic interactions demonstrate that a variety of cell cycle regulators which affect segregation also affect epigenetic regulation by ph-p.

III. Discussion

The data in previous chapters argued that the PcG of epigenetic silencers may have a role in cell cycle progression and raised the possibility that cell cycle progression and epigenetic silencing are directly linked. The data presented in this chapter also argues for the reciprocal scenario, namely that cell cycle regulators may have a role in epigenetic silencing. The report by Lupo et al. (2001) in conjunction with data in this chapter argue that barren may technically be considered a PcG gene because it fulfills many of the criteria of the PcG discussed in Chapter 1. Lupo et al., (2001) have shown that Barren is localized to PREs and a barren deficiency derepresses a mini-white reporter gene linked to the Fab7 PRE demonstrating that Barren is required for epigenetic
Figure 4-8. (next page) (A) Both alleles of *geminin* suppress the expressivity of the homeotic phenotypes reported in Table 4-7. Again the suppression was modest in some phenotypes, but the overall effect is suppression in all cases. (B) Examination of the mitotic defects of early embryos of *geminin* mutant females by staining DNA with TOTO-3 nucleic acid dye (as described in Chapter 2) revealed no highly penetrant or expressive chromatin bridge phenotypes at anaphase or telophase, compared to wild-type control embryos. This result is consistent with the hypothesis that severe segregation defects enhance the homeotic phenotypes of *ph*<sup>409</sup>. (C) Examples of mitotic phenotypes encountered in *geminin* mutants. Low-expressivity chromatin bridges (arrows) similar to those encountered in wild-type embryos (top panel). More commonly, metaphase chromosomes with puffs of uncondensed chromatin (arrowheads) consistent with an overreplication phenotype were encountered (bottom panel).
Figure 4-8. Figure legend on previous page.
silencing. This study further supports the hypothesis that *barren* is a PcG gene since two *barren* alleles were shown to enhance the adult homeotic phenotypes of both *ph-proximal* and *Polycomb*, and suppress the homeotic phenotypes of *trithorax*. These two categories of genetic interactions have been hallmarks for categorizing genes as members of the Polycomb Group. Over 40 PcG are thought to exist though only 20 have been identified and characterized. 40 PcG genes is likely an overestimation (Brock and Fisher, 2005) but it is possible that some will be identified in future studies as cell cycle regulators. In addition, a subset of cell cycle regulators will likely fit the criteria of PcG members. Though *barren* adult flies do not themselves exhibit homeotic phenotypes, the strong enhancement of the penetrance and expressivity of *ph-p* and *Pc* argues for a role in epigenetic silencing. The PcG genes *Suppressor 2 of zeste (Su(z)2)* and *Enhancer of Polycomb (E(Pc))* also do not exhibit homeotic phenotypes but enhance those of other PcG genes (Campbell *et al.*, 1995; Cheng *et al.*, 1994; Sato *et al.*, 1984; Soto *et al.*, 1995; Stankunas *et al.*, 1998). The PcG criterion that was not tested is whether Barren has the ability to recruit other PcG proteins to PREs. Though Lupo *et al.* (2001) reported that Barren co-immunoprecipitates with PHP140kDa isoform, those findings were not substantiated in this study, though many repeated attempts were made. The lack of interaction between PH-P and Barren does not however exclude the possibility that Barren interacts with other PcG proteins nor that Barren may recruit PcG proteins to PREs. Those possibilities were not tested in this study.

Interestingly, immunoprecipitations in this study using the α-Barren antibody did not precipitate any detectable Topoisomerase II. This is contrary to the findings of Lupo *et al.*, (2001). Barren and Topoisomerase II were also reported to interact directly in
pulldown and yeast 2-hybrid assays, and purified Barren enhances the decatenation activity of purified Topo II in vitro, suggesting that Barren is required for TopoII activation (Bhat et al., 1996b). However, an in vivo interaction has yet to be demonstrated. Other studies have also reported a lack of interaction between Barren and Topo II homologues. In Xenopus a direct interaction between the homologues of Barren and Topoisomerase II was not detected (Hirano and Mitchison, 1994; Hirano et al., 1997) and localization of Topo II to replicated chromosomes was not dependent on the presence of the Xenopus Barren homologue (Cuvier and Hirano, 2003). Rather, condensin appears to be required to condense chromatids after TopoII has been localized to axial chromosomal structures. In addition, in Saccharomyces cerevisiae, Barren and Topoisomerase II are recruited to the axial structures of chromosomes at different times and Barren mutants do not exhibit chromosomal breakage, suggesting that Barren is not required for the decatenation activity of Topo II (Lavoie et al., 2000). Irrespective of whether the Barren-TopoII interaction is species-specific, in this study, Barren did not co-immunoprecipitate with PH-P in repeated attempts. There is a possibility that the Barren-PH-P interaction was not detected because it may be cell-cycle dependent and thus not abundant in nuclear extracts from unsynchronized cells. In summary, data from this and previous chapters argue against a physical PH-P-barren interaction and argue that the genetic interaction is possibly indirect.

The data presented in Chapter 3 Figure 3-8 demonstrated that the presence of thick chromatin bridges between telophase nuclei of early embryos was sufficient to prevent the timely re-localization of PH-P protein to chromatin at telophase. This delay in relocalization coupled with evidence that the barren-ph genetic interaction is likely
indirect predicts that other cell cycle regulators may also interact genetically with the PcG. A variety of cell cycle regulators were crossed to ph-proximal to test this prediction. Interestingly, all cell cycle regulators tested interacted genetically with ph-proximal. A summary diagram appears in Figure 4-9.

The cell cycle mutants which have been reported to exhibit chromatin bridges all enhance the homeotic phenotypes of ph-p including the segregation mutants polo, pimples and three rows, the condensin barren and glutamine synthetase which is required for nucleotide synthesis and thus to properly replicate DNA. PCNA also enhances ph-p homeotic phenotypes but mitotic phenotypes of PCNA mutants have not been tested. PCNA was previously reported to enhance the homeotic phenotypes of another PcG member, cramped (Yamamoto et al., 1997). The correlation between exhibiting segregation phenotypes at mitosis and enhancement of PcG homeotic phenotypes argues that the segregation defect plays a key role in the epigenetic silencing. This role is likely indirect rather than a direct physical interaction between PH-P protein and any one of this diverse group of cell cycle regulators.

Data in previous chapters argued for an S-phase mitotic role for PH-P and proposed that the segregation phenotype of ph-p mutants may be due to incomplete replication of DNA. Interestingly, both geminin alleles suppress the homeotic phenotype of ph-p. The observation that geminin mutants do not exhibit highly penetrant and expressive chromatin bridges supports the previous hypothesis that it is the presence of segregation defects that enhance homeotic phenotypes of PcG. This may explain why geminin does not enhance PcG homeotic phenotypes while other cell cycle regulators which do have segregation phenotypes significantly enhanced homeosis. The mitotic
Figure 4-9. Summary of cell cycle regulators which interact genetically with ph-proximal mutants. Most cell cycle regulators tested enhance the homeotic phenotypes of ph$^{409}$ and correlate with having highly penetrant segregation defects in early embryos (shown in green). All genetic crosses were performed in this study with the exception of PCNA which are unpublished observations by Jacob W. Hodgson in our laboratory (grey box). Interestingly, only geminin suppressed the homeotic phenotypes (shown in red), which correlates with the fact that geminin does not exhibit segregation defects significantly above those from wild-type embryos. Furthermore, a hypothesis is that geminin's mitotic phenotype of overreplication may partially rescue the proposed underreplication of ph-proximal, leading to suppression of homeotic phenotypes.
function of *geminin* is to ensure replication origins do not reactivate once they have fired, thus ensuring a single round of replication per cell cycle. *geminin* mutants overreplicate portions of their genome (Quinn *et al.*, 2001). A hypothesis is that the overreplication caused by the *geminin* deficiency partially suppresses the homeotic phenotypes in *ph-p* mutants because the overreplication partially relieves the proposed underreplication in *ph-p* mutants. This hypothesis therefore predicts that the epigenetic role of *ph-p* is directly related to its role in mitosis and that homeotic phenotypes of *ph-p* mutants result from mitotic defects. The data that most PcG mutants exhibit severe mitotic defects in early embryos coupled with the data that a wide variety of cell cycle regulators enhance homeotic phenotypes are consistent with this hypothesis. In fact, since the biochemical function of many PcG proteins is unknown, future studies may further blur the categories of what PcG genes are and what cell cycle regulators are. However, the S-phase role of *ph-p* and the hypothesis that *ph-p* mutants have underreplicated DNA remains to be tested.
CHAPTER 5 General Discussion

I. Cell cycle regulation is likely a component of epigenetic silencing

As discussed in Chapter 1, the inheritance of transcriptional states involves three distinct stages: interpreting transcriptional status to establish epigenetic marks, transmitting the epigenetic marks across a cell division, and interpreting the epigenetic marks in the daughter cells to re-establish correct transcriptional status. These stages of inheritance will be considered distinct from the role the PcG have in maintaining transcriptional repression of their targets within a nucleus between mitoses, and will be the focus of this Discussion. Since the aforementioned stages of inheritance coincide with the many regulatory events governing mitosis, it is very likely that the two processes are linked, and that epigenetic inheritance depends on cooperation with cell cycle regulation. Prior to this study, there has been little evidence that cell cycle progression and epigenetic inheritance are linked. The data in this thesis demonstrate that the PcG have a role in cell cycle progression, and proposes that cell cycle regulation is a necessary component of epigenetic inheritance.

II. A model to link cell cycle regulation and epigenetic silencing

Most PcG mutants tested exhibited severe chromatin bridges and a variety of other mitotic phenotypes. Though not every PcG mutant was tested, those that were represented a diverse subset that encompassed EPCs, ETPs, PRC1, and PRC2 members (See Chapter 2 Introduction). The ubiquity and similarity of the phenotypes within this subset makes it likely that many other PcG mutants will also exhibit mitotic defects. As Discussed in Chapter 2, these phenotypes are shared by a number of cell cycle regulators
which act at many stages of the cell cycle. This study has not formally ruled out the possibility that cell cycle regulators are PcG targets and that the mitotic phenotypes of PcG mutants are indirect. However, evidence from the subsequent chapters argues for an intimate link between the PcG and cell cycle regulation that argues for a more direct role for the PcG in cell cycle progression. The temporal oscillation of PH-P protein on and off chromosomes during the cell cycle argues that the timing of the co-localization of PH-P with chromatin is tightly regulated, and suggests that the time frame during which PH-P colocalizes with chromatin is functionally important. In these early cell cycles, PH-P co-localization with chromatin is from late telophase to late S-phase and argues that PH-P has a functional role at these stages (see Chapter 3 discussion). Furthermore, the timing of the re-localization of PH-P to chromatin following mitosis is also tightly regulated and is likely crucial for PH-P function. Reassociation of PH-P with chromatin after mitosis was prevented when thick chromatin bridges linked sister nuclei. This demonstrates that proper cell cycle progression is crucial for PH-P localization, and therefore likely crucial for PH-P function within the next cell cycle.

If the timing of PH-P localization to chromatin is imperative to its role as an epigenetic maintenance protein, chromatin bridges which prevent PH-P relocalization to chromatin in later embryonic cell cycles have the potential to impair the inheritance process. This argues that maintenance of silencing is dependent on proper cell cycle progression. In other words, proper cell cycle progression maintains the proper oscillation of PcG proteins off and onto chromatin after cell division and is thus critical for epigenetic inheritance (Figure 5-1, top and middle panels). This model argues that any disruption in cell cycle progression that results in the prevention or delay in the
Figure 5-1. (next page) Proposed models for interplay between cell cycle regulation and epigenetic maintenance mediated by PH-P. Top panel. Proposed mechanism for inheritance of epigenetic states in a wild-type embryo. Chromosomes are depicted as black lines and PcG proteins as red ovals. Proper segregation of sister chromatids at anaphase and telophase (1) facilitates the proper reassocation of PcG proteins with their targets at telophase (2). Proper reassocation allows targets in next cell cycle to remain silent (3) leading to proper inheritance of epigenetic pattern (4). Middle panel. Proposed mechanism for failure to maintain epigenetic states due to segregation defects. A likely S-phase event in \( ph-p \) mutants (1a) or a defect at any cell cycle stage (1b) which leads to severe segregation defects (2) prevents the proper segregation of chromosomes and prevents or delays the proper reassocation of PcG proteins with targets at telophase (3). Prevention or delay of PcG reassocation allows the de-repression or enhances the derepression of some PcG targets (4) and results in the loss of inheritance of the epigenetic pattern (5). This model proposes that the mitotic phenotype observed for PcG mutants leads to derepression and homeotic phenotypes. In addition, it proposes that the loss of proper reassocation of PcG proteins at telophase results in the de-repression of PcG targets. Both these predictions remain to be tested. Bottom panel. Proposed mechanism explaining how S-phase regulation by PH-P affects its role as an epigenetic regulator. A proposed role of PH-P during S-phase (1) affects DNA replication, perhaps by affecting replication timing of its targets (2a, 2c) as suggested by the report that most PcG targets are late-replicating (Zhimulev et al., 2003) or via a role during elongation (2b) as suggested by the co-localization with PCNA. These S-phase roles may directly influence maintenance of silencing in the next cell cycle (3) since many silenced targets are correlated with late replication and late replication is correlated with maintenance of silencing. Another possibility is that regulation of replication timing or elongation directly affects temporal regulation of PcG oscillation on and off chromosomes during mitosis (4) which in turn ensures proper inheritance of epigenetic states (5) as discussed in the previous models. In addition, PH-P may affect other cell cycle stages in addition to DNA replication including condensation, cohesion and segregation which may all ensure proper temporal regulation of PH-P distribution or targeting (6) during the subsequent cell cycle.
Figure 5-1. See legend on previous page.
reassociation of PH-P or other PcG with chromatin at the proper time may result in derepression of PcG targets in the subsequent cell cycle and therefore loss of the epigenetic inheritance pattern. The enhancement of PH-P homeotic phenotypes by a variety of cell cycle regulators which themselves exhibit chromatin bridges (See Chapter 4) supports this hypothesis. None of this subset of cell cycle mutants (which act during DNA synthesis, chromosome condensation and sister-chromatid segregation) exhibits homeotic phenotypes of their own. Therefore, the enhancement of homeotic phenotypes of PcG mutants is indirect and proposed to be solely due to the segregation defects of these cell cycle regulators, as predicted by the model. In addition, geminin mutants (which have a very low penetrance and expressivity of chromatin bridges, similar to wild-type) did not enhance adult homeotic phenotypes of ph-p, consistent with the model. In summary, only cell cycle regulators which exhibit segregation phenotypes enhance the adult homeotic phenotypes of PcG mutants, therefore, proper segregation at mitosis is a necessary component of epigenetic inheritance.

III. A proposed cell cycle role for Polyhomeotic

The model presented above argues that proper cell cycle progression is a crucial component of the epigenetic inheritance process. However, the data in this thesis also argues that PH-P (and by extension, the PcG) itself has a direct role in cell cycle progression. ph-p (and many other PcG mutants) exhibits severe mitotic defects and co-localizes with S-phase regulators. Data in Chapter 3 showed partial co-localization between Geminin and PH-P protein, in agreement with reports that the murine Geminin homologue co-localizes with and physically interact with a murine ph homologue, Rae28
(Luo et al., 2004). In addition, a partial colocalization with PCNA protein was also demonstrated. These results argue in favour of an S-phase role for PH-P.

If the cycling of PH-P protein on and off chromosomes is crucial for PH function as an epigenetic regulator, one hypothesis is that PH-P itself directly regulates cell cycle progression solely to ensure its own proper oscillation on and off chromosomes during cell division, thereby ensuring proper relocalization to targets during the next cell cycle. In other words, PH has a direct role in mitosis to ensure mitotic stages progress smoothly, akin to checkpoint proteins. This proposed function is likely via interactions with cell cycle regulators, as was demonstrated by the colocalization of PH-P protein with the S-phase regulators Geminin and PCNA. Interaction with the replication-licensing factor Geminin suggests a role in replication timing and interaction with PCNA suggests a role in elongation during replication (see next section). This hypothesis predicts that perhaps the cell cycle function of PH is to facilitate complete DNA replication, thereby ensuring the mitotic cycle progresses smoothly and the relocalization of PH to chromosomes is secured in the next cell cycle. Therefore, the mitotic defects of ph mutants may be due to incomplete DNA replication and appear as a chromatin bridge phenotype. Interestingly, geminin mutants partially suppressed the homeotic phenotypes of polyhomeotic mutants. Given that geminin mutants overreplicate DNA during S-phase, it is possible that the suppression of homeotic phenotypes is due to overreplication by geminin partially relieving the proposed underreplication of ph-p mutants. If PH-P has a role in DNA replication during S-phase, it is likely that this role may be on a small scale at PH-P targets, rather than globally across the genome. Figure 5-1, bottom panel illustrates the possible S-phase roles of PH-P and how these may affect epigenetic inheritance.
This hypothesis therefore predicts that the epigenetic role of PH-P is directly related to its role in mitosis and that homeotic phenotypes of ph-p mutants result from mitotic defects, specifically, incomplete replication. Therefore, the mitotic role of PH-P is necessary for inheritance of the transcriptional status of its targets. In other words, the molecular functions of PH-P that ensure mitotic progression directly result in maintenance of silencing in the next cell cycle. A hypothesis linking replication and epigenetic inheritance will be further discussed below.

IV. Epigenetic silencing and replication timing

DNA replication is the only time during the cell cycle when the entire genome and epigenome of each cell is surveyed. Therefore, if transcriptional status for given genes is to be interpreted and marked for epigenetic inheritance, DNA replication is the ideal stage to do so. It is likely that Maintenance Proteins interact with the replication machinery at the replication fork to facilitate inheritance of transcriptional states of their specific targets.

It has been generally accepted that in metazoa, transcribed euchromatin replicates during early S-phase and silenced heterochromatin replicates in late S-phase (Gilbert, 2002). In yeast, there is no correlation between expression and replication timing (Raghuraman et al., 2001), and replication is initiated from a well-defined consensus sequence. In contrast, there is no metazoan consensus sequence for replication origins, but there is a correlation between replication timing and gene expression, suggesting that origin choice and replication timing of certain genes has an epigenetic component (Gilbert, 2002). In Drosophila, origin choice may change during development, further
evidence that origin activity is epigenetically controlled (Aggarwal and Calvi, 2004). In Drosophila, examination of 40% of the genome found a strong correlation between early replication and gene expression, and late replication and repressed genes (Schubeler et al., 2002). It is unclear however if the chromatin structure of silent loci physically prohibits early replication (Gilbert, 2002) or if maintenance proteins are responsible for timing and dictating origin choice and the assembly of replication foci at their targets. Whichever model is correct, it is equally unclear whether replication timing in turn, dictates the inheritance of specific chromatin states. The favoured model implies that silent chromatin is self-perpetuating because it is only able to replicate late due to its inherent inaccessibility, and in turn, late-replicating loci only assemble silent chromatin due to the presence and availability of specific chromatin assembly factors and modifiers during late S-phase (Gilbert, 2002). In fact, different subsets of HATs, HDACs and other chromatin modifiers are localized to replication forks during early and late S-phase (reviewed in McNairn and Gilbert, 2003).

This model implies that since chromatin dictates timing and timing dictates the structure of chromatin after replication, the epigenetic component in origin choice and timing is a built-in feature of chromatin structure and suggests that the cell cycle effect of epigenetic factors such as the PcG is strictly due to establishment and maintenance of chromatin structure. Indeed early replicating and active chromatin are marked by the same histone modifications, and late replicating and inactive chromatin also share the same set of histone modifications (Arney and Fisher, 2004). In yeast, histone acetylation state is a direct determinant of replication timing. A mutation in the Rpd3 histone deacetylase (HDAC) or tethering of histone acetyltransferase Gcn5 causes both histone
hyperacetylation and early firing of normally late-firing replication origins (Vogelauer et al., 2002). These data make it difficult to dissect the functional differences between chromatin modification for the purpose of altering gene expression from the purpose of origin choice and timing and thus support the above model in which replication timing and chromatin structure are essentially dual features of a single set of regulatory events.

However, replication timing is only generally early or late, with origins firing continuously throughout the duration of S-phase (Raghuraman et al., 2001) making the above model difficult to reconcile for all genes. In addition, if replication timing and origin choice does dictate inheritance and assembly of specific chromatin structures in the next cell cycle, the data in this thesis suggest that it is likely that maintenance proteins have a direct role in this process, perhaps via chromatin modification. It was recently shown that Polycomb protein tethered to a reporter construct containing a Drosophila origin sequence locally inhibited origin activity (Aggarwal and Calvi, 2004). These results demonstrate that an epigenetic maintenance protein actively modulates the activity of nearby origins. It was also previously reported that most PcG targets (including those of PH and PC) are late-replicating (Zhimulev et al., 2003). However, it remains to be demonstrated whether a silent PcG target replicates late, and whether replication timing is correlated with PcG binding and target expression.

The fact that PcG targets are late replicating presents an intriguing hypothesis that PcG proteins not only modulate origin activity, but also specify origin localization. Since PcG proteins bind to PRE modules in Maintenance Elements, perhaps maintenance elements serve as origin sequences, dictated epigenetically by PcG proteins. There is no consensus sequence for Drosophila origins, rather, DNA topology in the form of
negatively supercoiled DNA facilitates the targeting of ORC (Origin Recognition Complex) proteins to origins (Remus et al., 2004). ORC subunit binding within PREs and Maintenance Elements may be demonstrated by Chromatin Immunoprecipitation (ChIP) analysis. The above hypothesis further predicts that deletions in PRE modules may alter ORC binding and origin firing at PcG targets. However, if silencing and late replication timing are co-dependent, it may not be possible to dissect sequences which affect replication timing, but not silencing of the target gene, and vice versa. It was previously shown that deletion of PRE sequences results in immediate loss of memory as silencing of a reporter gene was lost in the next cell cycle in a Drosophila cell line (Sengupta et al., 2004). It would be interesting to determine if this immediate loss of memory due to deletion of PRE sequences correlates with an immediate change in replication timing. A similar experiment would be to compare replication timing of PcG targets before and after knockdown of PcG proteins using RNAi in synchronized cells.

Specifying origin choice and replication timing of targets does not exclude additional mitotic roles for PH-P. Replication timing appears to be necessary but not sufficient to specify transcriptional status since there are exceptions to the correlation, and since replication during S-phase is continuous, events other than timing likely specify transcriptional status (Gilbert, 2002). In addition, specifying timing and origin choice may not be sufficient to direct complete DNA synthesis at replication foci. Studying chorion gene amplification in Drosophila revealed that replication initiation and elongation are temporally and functionally distinct processes (Claycomb et al., 2002). This raises the possibility that maintenance proteins may have roles during both initiation and elongation. Perhaps Maintenance Proteins facilitate elongation and the passing of
replication forks through their targets via an interaction with PCNA. As demonstrated in this thesis, the co-localization of PH-P and PCNA suggests a role for PH-P in elongation during replication. Incomplete DNA replication proposed for ph-p mutants is consistent with this hypothesis. It was previously shown that PCNA interacts genetically with PH-P (Hodgson and Brock, unpublished observations) and colocalizes with the PcG protein cramped (Yamamoto et al., 1997). It is therefore possible that PCNA interacts physically or colocalizes with other Maintenance Proteins to facilitate elongation at their specific targets.

V. The role of other PcG proteins

Most PcG mutations tested exhibited mitotic phenotypes identical to those of ph-p. However, chromatin bridges may arise from defects in any cell cycle stage. Nevertheless, given the strong correlation between replication timing and silencing and that most PcG targets are late-replicating (Zhimulev, 2003), it is possible that other PcG proteins will have a similar mitotic role as was proposed for PH-P. A report recently demonstrated that mutations in the PcG genes Sex combs extra and Sex combs on midleg (as well as ph) reduced proliferation of ovarian follicle cells in Drosophila (Narbonne et al., 2004) suggesting other PcG members are also required for cell cycle progression. In mammals, PcG mutants cause a number of cell proliferation defects (reviewed in Jacobs and van Lohuizen, 2002). Also, as previously discussed, Polycomb protein was shown to modulate origin activity in vivo (Aggarwal and Calvi, 2004). Taken together, these data suggest a global role for the PcG in S-phase regulation and proliferation.
In addition, if other PcG proteins are shown to oscillate on and off chromosomes as was shown in this thesis for PH-P, and previously for PH-P, PSC and PC (Buchenau et al., 1997) and for cramped (Yamamoto et al., 1997), it is possible that they may also be involved in the progression of cell cycle stages other than replication and may interact with a variety of cell cycle regulators to ensure that the temporal regulation of association with chromatin is maintained.

VI. The role of trxG and other epigenetic regulators in mitosis

Mutations in the trxG gene Trithoraxlike (which encodes GAGA factor) exhibit mitotic defects in embryos similar to those observed in PcG mutants (Bhat et al., 1996a) including severe segregation defects and giant nuclei in addition to defective chromosome condensation. Though other trxG mutants have not been formally surveyed for mitotic defects in early and later embryogenesis, it is likely that mutations of trxG members other than Trithoraxlike will also exhibit mitotic defects. Some trxG members such as lid and ash1 and ash2 exhibit small imaginal discs indicative of proliferation defects (Gildea et al., 1999; Tripoulas et al., 1994). Perhaps future studies will uncover further links between the trxG and cell cycle progression.

VII. Cell cycle proteins regulating Hox expression.

This discussion has focused on links between epigenetic inheritance and cell cycle progression and has proposed models for a possible DNA replication role for PH-P which facilitates inheritance of transcriptional states of its target genes. These models proposed that PcG proteins interact with cell cycle regulators during the inheritance process.
However, cell cycle regulators may also function outside of mitosis to regulate expression of MP targets. In mammals Geminin behaves as a PcG protein. LOF and GOF studies specifies the anterior boundary of *Hoxb9* transcription in the chick neural tube, and in mice, Geminin interacts with Rae28 (a PH homologue) and with Hox proteins themselves (Luo *et al*., 2004). In *Drosophila*, the condensin subunit Barren was localized to PREs (Lupo *et al*., 2001), raising the possibility that it may function to locally compact DNA at maintenance elements, though there is no evidence to support this hypothesis. Proteins regulating sister chromatid cohesion may also play a role in PcG target regulation. ORD, which is required in *Drosophila* to maintain sister chromatid cohesion interacts and colocalizes with the PcG protein RING1 (Balicky *et al*., 2004). In addition, Pds5, a cohesin cofactor also required for chromatid cohesion co-purified in a complex with PH-P (Wang and Brock, unpublished observations). Though there is no evidence to suggest these interactions are functional in repression and maintenance of PcG targets, cohesins may function locally to facilitate interactions between Maintenance Elements on homologous chromosomes or between promoters and MEs to regulate Hox or other target gene expression. Interaction between ME modules and promoters is a favoured model for regulating Hox expression (See Introduction). In addition, interaction and pairing between PREs in *trans* has long been thought to be an important component of repression and silencing of PcG targets (Kassis, 2002). The proteins which bind PRE modules required for pairing include PcG proteins but have not all been identified. It is possible that cohesins and cohesion cofactors play a role in pairing PRE modules in *trans*. Future studies may uncover much more overlap between the events that define PcG-mediated silencing and mitosis. In addition, since the
biochemical activities of many PcG proteins are still unknown, further blurring of what defines PcG proteins and cell cycle proteins is predicted.
CHAPTER 6 Materials and Methods

I. Genetic Crosses

i. Virgin collection

Parental strains were reared at 20°C in bottles and vials containing cornmeal-agar medium supplemented with live yeast paste. Parental strains were maintained by frequent flipping to prevent overcrowding. Virgin females were collected within 8 hours of eclosure to prevent mating with sibling males. Virgins are larger in size than mature females and appear white because epidermal pigments have not yet been deposited. In addition, a greenish plug (food waste) is visible in the abdomen prior to mating maturity. The virgins were sequestered and aged for four days to ensure virginity and prevent contamination of pending genetic crosses. Males were collected within 24 hours of eclosure.

ii. Crosses

All crosses were performed at 25°C in vials containing standard cornmeal-agar medium supplemented with live yeast paste. 25 virgins and 15 males of appropriate genotype were added and vials were turned over daily. Once offspring had reached third instar wandering stage, a small piece of Kimwipe tissue was placed in bottom of vial to soak up excess moisture and ensure newly eclosed progeny did not drown and skew results. Progeny were aged for 24 hours before scoring to allow sufficient time for pigment deposition/darkening. This step was necessary as newly eclosed flies are nearly white, and assaying for homeotic transformations include scoring patches of pigmented cuticle in ectopic positions. Flies were collected and scored daily. Each vial was scored
to completion (all progeny had eclosed) until progeny numbers were at least \( n=80 \) for either the double heterozygote or control.

**iii. Scoring of homeotic phenotypes and statistical analysis.**

Double heterozygous and control male progeny of crosses were scored for presence of homeotic transformations. The homeotic phenotypes are described in detail in Chapter 4 introduction. The penetrance and expressivity of each homeotic phenotype was recorded in a data table. Penetrance was noted as the presence or absence of homeotic mutations. Expressivity was scored on a scale from 1 to 4. For extra sex combs phenotype, the value 1 was assigned to ectopic sex combs judged to have a length of 25% or less than wild-type, 2 was 25-50%, 3 was 50-75% and 4 was a full sex comb, or 100% expressivity. The values were then averaged for the double heterozygote and the control males. The same scale was used for abdominal transformations. The area of transformation for the abdominal segment was judged as a percentage of the total dorsal abdominal surface area from 25% and less (Value = 1), 25 to 50% ( = 2), 50 to 75% (= 3) and transformation of the full segment (Value = 4). The average expressivity was calculated for each abdominal transformation. Genetic interaction between tested mutations was assessed for significance using a standard Chi-square test reporting a \( P \) value of 0.05.

**II. Embryo Staining**

**i. Embryo collection.**

Procedures were modified from Ashburner (1989). 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 “laying plate”, a 50mm Petri dish containing a semi-solid surface of 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 embryos were collected for fixation, with frequent changing of laying plates. 0-3 hour embryos were collected by allowing females to lay for 3 hours, after which time the laying plate was removed and embryos were immediately processed for fixation. Embryos collected from genetic crosses followed an identical procedure, except 200 aged virgins (see virgin collections above) and 50 males of the appropriate genotype were added to the chambers.

ii. Embryo fixation.

Embryos on laying plates were immediately washed into a nylon sieve to remove traces of yeast. The sieve was then immersed into 50% bleach for 2 minutes to dissolve the outer chorion layer. Embryos were then washed for one minute with cold Embryo Wash Buffer (120mM NaCl; 0.02% Triton X-100) followed by a one minute wash with water. Embryos were immediately transferred into 5mL of 3.7% formaldehyde fixative in 1X Phosphate Buffered Saline (Sambrook et al., 1989). Since embryos are buoyant, the fixative was overlayed with 5mL heptane to ensure contact with fixative. Heptane also 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 30 seconds and allowed to
settle. Floating embryos (not devitellinized) were removed and the remaining fixed
devitellinized embryos were washed in 5 mL methanol twice to remove vitelline
envelopes and other debris from solution. Embryos were either immediately processed
for staining or stored in methanol at –20°C.

iii. *Embryo rehydration and preparation for staining.*

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.

iv. *Nucleic acid staining of fixed embryos.*

Embryos were collected, fixed and rehydrated as described above. PBT solution
was removed with a Pasteur pipette and embryos were overlayed with 40μL of 10mg/ml
RNaseA (Sigma) and placed in 37°C water bath for two hours. Embryos were rinsed
twice with 5ml PBT and then stained with the nucleic acid dye TOTO-3 (Molecular
Probes) at 2μM (1:500 dilution of 1 mM stock solution) in PBT for 20 minutes at room
temperature. Tubes were covered with aluminum foil to prevent bleaching of
fluorophore. Embryos were washed twice for 10 minutes each in 1 ml PBT, shielded
with foil. PBT was removed and embryos were suspended in Glycerol mounting medium
(1x PBS in 90% glycerol) containing 2% Dabco solution as an antifade agent (Sigma) or
in Vectashield mounting medium (Vector Laboratories). Embryos were mounted onto
cleaned glass slides and coverslips were sealed with nail polish. Slides were kept at 4°C
in the dark until viewing with a confocal microscope.
v. Immunostaining of fixed embryos.

Fixed embryos were rehydrated, RNase A-treated and rinsed as above. Primary antibody titre was determined by serial dilution and visualization by confocal microscopy to determine optimal staining. In cases where antibodies were obtained from a published source, the published titre was used as the initial dilution, and then optimized according to the signal. Primary antibodies were either directly labelled or visualized by fluorophore-coupled secondary antibodies. Direct labeling was via the Zenon mouse IgG or rabbit IgG labeling kits (Molecular Probes, according to manufacturer’s instructions) and in all subsequent steps, tubes containing embryos were wrapped in foil to prevent photobleaching of labeled antibodies. The antibody titres used to generate figures in this thesis were as follows: rabbit α-PCNA 1:200; rabbit α-geminin 1:100 (gift of Hui Zhang); chicken α-CID 1:50 (gift of G. Karpen); rabbit α-Polyhomeotic-Proximal N-terminal unique region 1:500 to 1:2000; rat α-Polyhomeotic-Distal C-terminal unique region 1:500 to 1:2000; mouse α-tubulin (Sigma) 1:2000, rabbit α-Geminin at 1:500 and rabbit α-PCNA at 1:1000. Embryos were incubated with primary antibody for 90 minutes at room temperature on a rotator. Embryos were then washed three times in 5ml of freshly prepared PBT for 20 minutes each wash. If labeled antibodies were used, a subsequent 5 minute wash step in 5ml PBS was added, followed by re-fixing of embryos in 4% formaldehyde solution in PBS for 15 minutes at room temperature to prevent loss of fluorophore-antibody conjugation (as per manufacturer’s directions). Unlabeled antibodies were visualized using fluorophore coupled secondary antibody. Rabbit derived antibodies were visualized using goat anti-rabbit antibodies coupled to fluorophores Alexa 488 or 568 (Molecular Probes), each at 1:2000 to 1:5000. Rat
derived antibodies were visualized using goat anti-rat Alexa 488 or 568 at 1:5000, and \( \alpha \)-CID was visualized using goat \( \alpha \)-chicken Alexa 488 antibody at 1:2000. Embryos were incubated with secondary antibody diluted in PBT at a final volume of 500\( \mu \)l for 2 hours at room temperature on a rotator, followed by a minimum of 4 washes in 5ml PBT for 30 minutes each. Overnight washes were occasionally performed at 4°C, but did not affect quality of the signal. Immunostained embryos were then treated with TOTO-3 nucleic acid dye and mounted on slides as outlined above.

**vi. Preparation and staining of larval brains.**

Protocols were modified from Henderson, (2004). Brains (including larval brain hemispheres and ventral nerve cord) from third-instar wandering-stage larvae were dissected in 0.7% NaCl. All attached tissue including imaginal discs was removed before proceeding. Brains were placed in a 50mm petri dish containing 0.7% NaCl and a drop of 1mM colchicine for 90 minutes at room temperature, then transferred to a drop of hypotonic solution (0.5% tri-sodium citrate dihydrate) for 10 minutes at room temperature, followed by immersion in fixative solution (11:11:12 methanol/acetic acid/distilled water) 45% acetic acid for 20 seconds. Brains were transferred to a drop of 45% acetic acid on a clean siliconized coverslip for 2 minutes. Brains not treated with hypotonic shock and colchicine were immediately fixed. Brains were squashed with a glass slide and the coverslip removed. Squashed brains adhered to the glass slide, which was then frozen in liquid nitrogen for 30 seconds, and immediately placed in 100% ethanol to dehydrate brains. Slides were air-dried and stored at 4°C for further processing. Slides were rehydrated in 2x SSC (300mM NaCl, 30mM sodium citrate, pH 7.0) for 5 minutes and immersed into a freshly made 2X SSC containing 0.2\( \mu \)g/mL
DAPI for 5 minutes. Slides were briefly rinsed in 2x SSC and allowed to air-dry in darkness to prevent photobleaching. Dry brains were adhered onto a coverslip containing 15μl of Vectashield mounting medium and the coverslip was sealed with nail polish. Slides were stored at 4°C in darkness until visualization with confocal microscopy.

**vii. Confocal microscopy and image rendering.**

Embryos stained with nucleic acid stain TOTO-3 only were visualized using a Bio-Rad MRC-600 Laser Scanning Confocal Microscope or a Bio-Rad Radiance Plus Laser Scanning Confocal Microscope. Antibody-stained embryos and DAPI-stained embryos were visualized using a Bio-Rad Radiance 2000 Multiphoton Confocal Microscope. Laser power, Gain, Iris and Offset levels were determined for each antibody titre and saved for subsequent staining experiments. All embryos from a given collection were scanned. Cell cycle stages and mitotic phenotypes were recorded in a data table. Confocal slices were collected and projected using NIH Image software. Figures were generated using Adobe Photoshop software.

**III. Preparation of nuclear extract, column chromatography and immunoprecipitations.**

**i. Cell culture, nuclear extracts and column chromatography.**

The *Drosophila* embryo-derived cell culture line KC1 and the transfected KC1 line F-PHP-HA (see Chapter 4) was used for preparation of nuclear extract. Cells were grown in spinner cultures as per Cherbas and Cherbas (1998) in Sf-900 serum-free medium (Gibco). Preparation of nuclear extracts, Biorex70 column chromatography, collection and visualization of proteins was performed entirely according to Hodgson et
Protein quantification of nuclear extracts and Biorex fractions were performed using BioRad Protein Assay and comparison to a standard curve.

**ii. Immunoprecipitations and Western analysis.**

Appropriate amount of nuclear extract was diluted in HEMG0.1 buffer (25mM HEPES-K\(^+\) pH 7.6, 0.1 mM EDTA, 0.1mM EGTA, 12.5 mM MgCl\(_2\), 15% glycerol, 100mM KCl, 0.1% NP40) and full complement of protease inhibitors utilized in preparation of nuclear extracts (Hodgson *et al.*, 1997). Diluted extract was incubated with 20 \(\mu\)l HEMG0.1-equilibrated Protein A Sepharose beads (Pharmacia) for 1 hour at 4°C to preclear extract. Supernatant was collected and appropriate precipitating antibody was incubated with the cleared extract for 60-90 minutes at 4°C on a rocking platform. 10\(\mu\)l fresh Protein-A Sepharose was added and incubated as above for 60 minutes. Beads were sedimented at 2000g for 2 minutes and washed 4x with HEBG0.15 wash buffer (25mM HEPES-K\(^+\) pH 7.6, 0.1mM EDTA, 15% glycerol, 0.1% NP40, 150mM KCl, and full complement of protease inhibitors). Washed beads were resuspended in 10\(\mu\)l HEBG0.15 and 10\(\mu\)l SDS sample buffer and heated at 65°C for 10 minutes to release beads. Sample was centrifuged briefly and supernatant was applied to SDS polyacrylamide gels for separation of proteins in preparation for Western blotting. SDS gels were blotted to Hybond-C Extra membranes (Amersham). Membranes were washed with Wash Buffer (10 mM Tris-HCl pH 7.9, 150mM NaCl, 0.05% Tween-20) for 15 minutes and blocked with Blocking Buffer (10mM Tris-HCl pH 7.9, 150mM NaCl, 5% nonfat milk) for 60 minutes at room temperature, followed by 5 minute wash with Wash Buffer. Primary antibody at appropriate concentration was diluted in Blocking Buffer supplemented with 0.05% Tween and incubated with filter for 90 minutes to 3 hours at
room temperature. Filters were washed 3X with wash buffer for 20 minutes each, then incubated with appropriate secondary antibody coupled to horseradish peroxidase, diluted in Block Buffer plus 0.05% Tween, for 90 minutes at room temperature. Filters were washed 6X in large volumes of Wash Buffer for 10 minutes each, followed by wash with 10mM Tris-HCl/150mM NaCl for 10 minutes, and developed with Amersham ECL kit. Precipitating antibody concentrations used were as follows: α-HA at 1:1000, α-Barren at 1:500, α-Polycomb at 1:250, α-E5 at 1:2500. Primary antibody concentrations for Western analysis were used as follows: α-HA, α-Barren and α-TopoII all at 1:10000, α-Pc at 1:5000 and α-E5 at 1:20000. Secondary antibodies were used at 1:5000 to 1:10000.
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