Abstract

Title of Dissertation:	THE PCG GENE *POLYHOMEOTIC* CONTROLS CELL PROLIFERATION AUTONOMOUSLY AND NON-AUTONOMOUSLY IN *DROSOPHILA*
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Polycomb group (PcG) proteins are conserved epigenetic regulators that maintain targets at a repressed transcription state. In my dissertation research, I generated ph^{del}, the first real null allele of the *Drosophila* PcG gene *polyhomeotic (ph).* Using this allele, I found that loss of *ph* causes cell over-proliferation in mosaic tissues in a non-autonomous manner, meaning that the mutant cells induce over-proliferation of neighboring wild type cells. I further identified the underlying signaling pathways: Notch signaling is elevated autonomously in ph^{del} cells, which causes the over-production of 3 Upd homologs. These ligands are then secreted and activate the JAK/STAT pathway in neighboring cells, which eventually causes cell over-proliferation. In addition, ph^{del} cells maintain normal cell polarity but undergo invagination to form unique 3 dimensional structures. Such structures are morphologically and functionally similar to epithelia-derived endocrine glands.

Interestingly, *ph*^{505}, another *ph* allele that has long been considered null, causes both autonomous and non-autonomous cell proliferation in mosaic tissues. To explain the discrepancies between *ph*^{del} and *ph*^{505}, I characterized the nature of *ph*^{505}. Data from embryonic lethal stage, rescue by *ph-d*, and exon sequencing all showed that *ph*^{505} is a hypomorph. Functional analysis then proved that the same signaling pathway also underlies non-autonomous proliferation in *ph*^{505} mosaic tissues.

I then showed that *ph*^{505} cells still respond to the Upd ligands they secreted, but *ph*^{del} cell are no longer responsive. This explains why *ph*^{505} cells still over-proliferate but *ph*^{del} cell do not. Next, Real-Time PCR results demonstrated that the JAK/STAT pathway receptor *domeless* has a higher expression level in *ph*^{505} cells than in *ph*^{del} cells, which may explain their different sensitivities to Upd ligands. Finally, genome wide ChIP data in public database suggest that *Notch* may be a direct target of Polycomb Repressive Complex 1, in which Ph is a core component.

My dissertation established that loss of *ph* causes non-autonomous over-proliferation, and elucidated the underlying mechanism. My results also call for a reevaluation of the non-autonomous over-proliferation pathway in *Drosophila*. Finally, the fact that different alleles of the same gene cause tumors in very different ways have certain implications to cancer research and treatment.

The PcG gene *polyhomeotic* controls cell proliferation autonomously and non-autonomously in *Drosophila*

by

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List of Abbreviations

ANT-C:	Antennapedia Complex
bx:	*bithorax*
BX-C:	Bithorax Complex
ChIP:	Chromatin immunoprecipitation
DamID:	DNA adenine methyltransferase identification
dome:	*domeless*
DSP1:	Dorsal switch protein 1
esc:	*extra sex combs*
eyg:	*eyegone*
E(z):	*Enhancer of zeste*
H3K4me3:	Trimethylation at lysine 4 of histone 3
H3K27me3:	Trimethylation at lysine 27 of histone 3
hh:	*hedgehog*
hop:	*hopscotch*
Hox genes:	Homeobox-containing genes
HP2:	Heterochromatin protein 2
JAK:	Janus kinase
ken:	*ken and barbie*
ORF:	Open reading frame
Pc:	*Polycomb*
Pcl:	*Polycomb-like*
ph:	*polyhomeotic*
pho:	*pleiohomeotic*

phol:	*pleiohomeotic like*
PcG genes:	*Polycomb* group genes
PIAS:	Protein inhibitor of activated STAT
PRC1:	Polycomb repressive complex 1
PRC2:	Polycomb repressive complex 2
PRE:	Polycomb response element
Psc:	*Posterior sex combs*
Scm:	*Sex comb on midleg*
Scr:	*Sex comb reduced*
SOCS:	Suppressors of cytokine signaling
STAT:	Signal transducer and activator of transcription
sxc:	*super sex combs*
Su(z):	*Suppressor of zeste*
trxG genes:	*trithorax* group genes
Ubx:	*Ultrabithorax*
upd:	*unpaired*
upd2:	*unpaired 2*
upd3:	*unpaired 3*
vps23:	*vascular protein sorting 23*
vps25:	*vascular protein sorting 25*

Note: Gene names are in Italic. Genes with dominant phenotypes start with a capital letter, whereas genes with recessive phenotypes start with a lower case letter. Corresponding protein names should not be in Italic and always start with a capital letter.

Chapter 1: Introduction

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Hox genes and the PcG genes

Hox genes and animal body plan

One of the most prominent features of many, if not most, animals is that they are segmented. Their body is composed of repetitive segmental units with distinct identities. Such segmentally organized body plans are arguably most obvious in arthropods. Developmental biologists have long been interested in how such body plans are formed and how the identity of each segment is determined.

By the early 1980s, developmental genetic analyses of the fruit fly *Drosophila melanogaster* has accumulated a rich body of knowledge about how the *Drosophila* body plan is determined. It was shown that a group of genes establish the correct number of body segments, but do not seem to specify their identities (Nusslein-Volhard & Wieschaus, 1980). Therefore another class of so-called "selector genes", a term invented by Antonio Garcia-Bellido (Garcia-Bellido, 1975), were proposed to help diversify the segments by specifying their identities (Ingham, 1985). The selector genes are believed to activate different sets of downstream realisator genes in different body segments, which results in diversification of the segments. Based on their homeotic transformation phenotypes, *Hox* genes were believed to be the best candidates for such selector genes.

Homeotic transformations are a special class of mutant phenotypes in which one body part develops into a different structure normally found elsewhere in the body (Gehring, 1998). From a developmental point of view, such phenotypes

suggest the existence of master controllers, which coordinate common downstream genes to form different body structures (Gehring, 1998). The record of such mutants in *Drosophila* could be traced back to the very early days of *Drosophila* genetics. The first *Drosophila* homeotic mutant was discovered by Bridges in 1915 in Thomas Hunt Morgan's famous fly room in Columbia University. In flies homozygous for this mutation, the anterior half of the third thoracic segment was transformed into the anterior half of the second thoracic segment, thus the anterior half of the haltere developed like the anterior wing. This mutation was thus given the name *bithorax (bx)* (Bridges & Morgan, 1923).

In the next several decades, many more homeotic mutants were found in *Drosophila*. A large portion of them affect the identities of posterior body segments, and their corresponding chromosomal loci were mapped to a very small region on the third chromosome, where *bx* resides. This region was named the *Bithorax* Complex (BX-C) (Lewis, 1978). E. B. Lewis performed extensive genetic analyses on this gene complex, and his data indicated that there were 9 genes in this complex, and mutations in each gene caused homeotic transformation in one body segment. Therefore, Lewis proposed that the genes in the *Bithorax* Complex are the determinants of the identities of posterior segments in *Drosophila* (Lewis, 1978). Interestingly, the order of these genes mapped on chromosome corresponds perfectly to the order of the body segments they control (Lewis, 1978). This phenomenon was called colinearity. Lewis also proposed that genes in the BX-C were evolved from a single ancestor by gene duplication, and the duplicated genes were then diversified to control the

identities of different segments (Lewis, 1978). Later, genes in another locus, which is close to the *Bithorax* Complex on the third chromosome, were found to determine the identities of anterior segments. This complex was named the *Antennapedia* Complex (ANT-C) (Kaufman et al, 1980). Genes in these two complexes turned out to have similar properties.

in situ hybridization showed that the genes in the BX-C and the ANT-C are transcribed in specific body segments. In addition, the segments where one gene is transcribed correspond to those that the same gene controls (Akam, 1983; Akam & Martinez-Arias, 1985; Levine et al, 1983). These observations further supported the roles of these genes as the selector genes. Moreover, two independent studies showed that genes in the BX-C and the ANT-C share a conserved region, which was named the homeobox (McGinnis et al, 1984; Scott & Weiner, 1984). These results supported Lewis' duplication followed by diversification hypothesis on BX-C evolution. The homeobox is a 180 base pair DNA sequence that encodes a 60 amino acid protein domain called the homeodomain. Soon after its discovery, several independent studies showed that the homeodomains from different proteins all bind to DNA in a sequence-specific manner (Desplan et al, 1988; Hoey et al, 1988; Muller et al, 1988), suggesting that proteins containing this domain regulate gene expression, consistent with the idea that BX-C and ANT-C genes act as selector genes. Genes in the *Bithorax* Complex and the *Antennapedia* Complex were then named the *homeobox-containing* genes, or the *Hox* genes. *Hox* genes were later found to

be conserved throughout metazoan, and their functions in specifying body segment identity were also proved to be conserved (Lemons & McGinnis, 2006).

PcG genes were originally identified as repressors of *Hox* genes

When *Hox* genes were emerging as the major determinants of segment identity in *Drosophila* and their restricted expression patterns were experimentally determined (Akam, 1983; Akam & Martinez-Arias, 1985; Levine et al, 1983), a remaining question was how such unique expression patterns were established, i. e., how the expression of *Hox* genes themselves was regulated. The first hint of an answer came from the genetic analysis of a locus called *Polycomb (Pc)*, which causes posteriorly directed homeotic transformations of the abdominal segments, similar to those observed in gain-of-function BX-C mutants (Denell, 1978; Puro & NygrÉN, 1975). But *Pc* itself is not linked to the *Bithorax* Complex. Lewis noticed the *Pc* locus, and based on its homeotic transformation pattern, he proposed that the product of *Pc* was a negative regulator of genes in the *Bithorax* Complex (Lewis, 1978).

Pc was not found based on its recessive homeotic transformation phenotype in embryos, but as its name suggested, its dominant extra sex comb phenotype in adults (Lewis, 1947). Sex combs are male specific structures in insects that have a function during mating. They are usually found on the prothoracic (the first thoracic segment) legs and in some species also on the mesothoracic (the second thoracic segment) legs in males, but never on the legs of females. In *Drosophila*, sex combs are normally only found on the prothoracic legs of males.

Flies with extra sex comb phenotypes show sex combs on mesothoracic and even metathoracic (the third thoracic segment) legs. Besides *Pc*, several other mutants were known to cause extra sex combs, and these mutants were collectively called the "extra sex comb mutants" (Hannah-Alava, 1958).

After *Pc* was found to cause homeotic transformations in embryos (Denell, 1978; Lewis, 1978; Puro & NygrÉN, 1975), some other extra sex comb mutants, such as *esc (extra sex combs)* (Struhl, 1981) and *Pcl (Polycomb-like)* (Duncan, 1982), were also shown to cause posteriorly directed homeotic transformations of posterior segments in homozygous embryos, a pattern similar to that seen in *Pc* homozygous embryos. In addition, genetic epistatic analyses suggested that these extra sex comb mutants do not cause homeotic transformations directly, but rather through disrupting the expression of the *Bithorax* Complex (Duncan, 1982; Struhl, 1981). These observations probably led Gerd Jurgens to conduct a large scale forward genetic screen using the dominant extra sex comb phenotype as the readout in trying to isolate more loci that regulate the *Hox* genes (Jurgens, 1985).

In 1985, Jurgens reported 4 extra sex comb loci from his screen (Jurgens, 1985). Of these 4 genes, 3 were completely new, and the 4th one was the same as *Pcl* reported by Duncan (Duncan, 1982). As expected, mutants of these loci also showed similar patterns of embryonic homeotic transformations seen in *Pc*, and such transformations were caused by mis-expression of the BX-C genes (Jurgens, 1985). Thus Jurgens named these extra sex comb loci as the *Pc* group (PcG) genes, because of their shared phenotypes (Jurgens, 1985). For example,

they all cause posteriorly directed homeotic transformation in embryos when homozygous, and they all have dominant extra sex comb phenotype in adults. *esc* is somewhat unique among *Pc* group genes because its extra sex comb phenotype is recessive in adults, but it is otherwise very similar in property to other PcG genes. Interestingly, Jurgens was not the first to notice the similarities among all these genes and to name them as a group. In 1984, one year before Jurgens proposed the name PcG genes, Sato *et. al.* called such genes the Extra Sex Comb loci (Sato et al, 1983). But for some reason, this name was abandoned by the community and the name PcG genes is still in use today. In the following years, many more PcG genes were found in *Drosophila* (Beck et al, 2010).

Although PcG genes were first discovered in *Drosophila*, their homologs were later found in a variety of higher eukaryotic species, from worms to mammals to plants. In these non-*Drosophila* species, PcG homologs also function as transcription repressors to keep their target genes in an "off" state. It is now clear that this group of genes is conserved in multicellular eukaryotes (Köhler & Villar, 2008).

In addition to the Pc Group loci, other loci were found to cause anteriorly directed homeotic transformations of posterior segments, a pattern that is opposite to that of PcG mutants (Forquignon, 1981; Ingham & Whittle, 1980). These genes were named the *trithorax* group (trxG) genes (Kennison, 1993; Shearn, 1989), after its first member, *trithorax* (Ingham & Whittle, 1980). Genetic interaction data also established that the trxG genes regulate the BX-C, and that the PcG genes and

the trxG genes antagonize each other (Ingham, 1983; Shearn, 1989). *trithorax* group genes were also found in a variety of other animal species as well as plants (Köhler & Villar, 2008; Schuettengruber et al, 2007). It should be pointed out that early studies of PcG mutants and trxG mutants generally only looked at their effects on genes in the *Bithorax* Complex, but later it was shown that the same principles also apply to genes in the *Antennapedia* Complex, as well as other PcG/trxG targets. It is now clear that PcG genes repress the transcription of their targets, while trxG genes activate their target transcription (Schuettengruber et al, 2007).

Functions of the PcG genes

PcG targets

Hox genes were the first known PcG targets. However, gradually accumulated evidence indicated that PcG proteins have many more targets throughout the genome. Immunostaining with a polyclonal antibody against Pc showed that Pc binds to about 60 loci throughout the salivary polytene chromosomes (Zink & Paro, 1989). This number exceeds what would be expected if PcG proteins only regulate the *Hox* genes. Besides being found at the BX-C and the ANT-C, which is expected, Pc also binds to many other loci, which represent potential Pc targets. Interestingly, Pc was found at many PcG loci, suggesting that PcG genes might regulate each other. Later, when an antibody against the PcG protein Ph (Polyhomeotic) became available, it was shown that Ph binds to about 80 loci throughout the polytene chromosomes, suggesting that having a large number of binding sites is not Pc-specific, but might be common for all PcG proteins. The authors also performed side-by-side comparisons between Pc and Ph binding patterns. They identified more than 10 extra Pc binding sites, and noticed that Pc and Ph share the majority of binding loci on the polytene chromosomes (DeCamillis et al, 1992). When confocal microscopy became relatively easily available, a study investigated the distribution of 3 PcG proteins, Pc, Ph and Psc (Posterior sex combs), in interphase cells in the embryos (Buchenau et al, 1998). The results largely confirmed the previous observations by showing that each protein is localized to 100 or more loci in each nucleus.

Recently, 3 studies searched PcG binding sites in *Drosophila* using genome-wide profiling methods (Nègre et al, 2006; Schwartz et al, 2006; Tolhuis et al, 2006). 2 to 3 different PcG proteins were chosen in each study, and their binding profiles were individually determined by ChIP or DamID. These studies all concluded that there are about 200 PcG binding sites throughout the *Drosophila* genome, and different PcG proteins bind to similar sets of loci. In addition, one study also showed that many binding loci identified in the new study correlate well with those found on polytene chromosomes (Nègre et al, 2006). The same study also showed that the Pc and Ph targets change during development, suggesting that the PcG genes regulate genes important for development (Nègre et al, 2006). A detailed comparison between results obtained from these three studies, however, found that they only share a limited overlap (Ringrose & Paro, 2007). The reason was believed to be different experimental systems and statistical methods used in each study. This low overlap also suggests that there might be more PcG targets in *Drosophila*.

PcG binding sites in mammalian cells have also been studied using genome-wide methods by 3 independent groups (Boyer et al, 2006; Bracken et al, 2006; Lee et al, 2006). Of these three studies, two were performed with human cell lines, and one used a mouse cell line. Different PcG proteins were also selected in different studies, and the number of genomic binding sites for each PcG protein varied from about 800 to about 1900. The results showed that different PcG proteins clearly share many binding sites, but each also has their unique ones (Boyer et al, 2006). Again probably because different systems and

statistical methods were used, a low degree of overlap (about 30%) exists among results from these studies (Ringrose, 2007).

Although the number of PcG targets identified in different studies vary dramatically, one thing for sure is that the PcG proteins have a large number of targets, far beyond the *Hox* genes. In addition, it seems each mammalian PcG protein has more than 800 PcG targets, while every *Drosophila* PcG protein binds to about 200 loci (Ringrose, 2007). Recently a study using *Drosophila* S2 cells concluded that in *Drosophila*, PcG complexes also regulate about 1000 target loci (Enderle et al, 2011). Interestingly, this study identified many non-coding transcripts as PcG targets.

Detailed comparison between results from the *Drosophila* studies (Nègre et al, 2006; Schwartz et al, 2006; Tolhuis et al, 2006) and those from the mammalian studies (Boyer et al, 2006; Bracken et al, 2006; Lee et al, 2006) revealed that in each case, 30% to 60 % of identified PcG targets were transcription factors, many of which play important roles in development and differentiation (Ringrose, 2007). In addition, many components of common signaling pathways, such as Notch and Wnt signaling pathways, were also identified as PcG targets (Ringrose, 2007). These findings support the important roles of PcG genes during development.

PcG genes maintain target genes at a repressed transcription state

The study of *Hox* gene expression concluded that *Hox* genes are regulated by maternal factors, gap genes, pair-rule genes, as well as segment polarity genes

(reviewed in Akam, 1987). These genes are only transiently expressed during very early stages of embryonic development, but *Hox* genes are expressed throughout development to adult stages (Maeda & Karch, 2010). Thus other factors must exist to maintain the correct *Hox* gene expression patterns. Such factors were named the maintenance factors, and the segmentation genes that establish the initial *Hox* gene expression patters were called the initiators (Maeda & Karch, 2006).

Results from several studies established that the PcG genes are required for the maintenance of *Hox* gene expression. *in situ* hybridization showed that *Ubx* (*Ultrabithorax*, a member of the BX-C) transcription was initially normal in embryos mutant for *esc*, a PcG gene, but ectopic *Ubx* transcription was later detected in segments where *Ubx* is normally silenced (Struhl & Akam, 1985). Similar observations were also reported for *Pc* embryos (Wedeen et al, 1986). Then another study demonstrated that at the blastoderm stage, *Scr* (*Sex comb reduced*, a *Hox* gene in ANT-C) and *Ubx* are both expressed normally in *ph* embryos, but both are ectopically expressed at a later stage (Dura & Ingham, 1988).

The requirement of PcG protein during the maintenance phase was also demonstrated in a reporter assay, in which the *E. coli* gene *lacZ* was driven by an initiation element from the BX-C. The initiation element itself was able to drive *lacZ* expression in the correct pattern initially, but ectopic *lacZ* expression was seen later. On the other hand, when a PRE (Polycomb Response Element, the DNA element where PcG proteins bind, see below for details) was also included

in the reporter construct, the correct *lacZ* expression pattern can be maintained to later stages (Maeda & Karch, 2010). Further studies demonstrated that this mode of action not only applies to the *Hox* genes, but also applies to other PcG targets (Schuettengruber et al, 2007). Therefore the function of the PcG genes is to keep their target genes in a repressed state.

The biological functions of PcG genes and their roles in diseases

Since the PcG proteins have a large number of targets in species belonging to diverse phylogenetic lineages, it is not surprising that they play a wide range of biological functions. In *Drosophila*, besides their roles in embryonic development, PcG proteins are also required for normal central nervous system development (Smouse et al, 1988; Smouse & Perrimon, 1990). Recently PcG proteins have also been shown to directly regulate Cyclin A expression, therefore linking PcG genes to cell cycle control (Martinez et al, 2006).

In *C. elegans*, genetic screens have been performed to isolate maternal effect genes that are required for germ-line development, so-called the grandchildless loci (Capowski et al, 1991). Several of these loci, for example *mes-2* and *mes-6*, turned out to be PcG homologs (Cao & Zhang, 2004). Studies have shown that the wild type products of these genes are required for maintaining a transcription repressed state of X chromosome in hermaphrodite germ-line (Fong et al, 2002).

In mammals, PcG proteins have been shown to play important roles in the control of cell proliferation (Martinez & Cavalli, 2006), in genomic imprinting

(Delaval & Feil, 2004), in X-chromosome inactivation in females (Edith, 2005), and in the maintenance of embryonic stem cells and adult stem cells (Pietersen & van Lohuizen, 2008). For example, *eed (embryonic ectoderm development)*, the mammalian homolog of *Drosophila esc*, has been shown to be essential for the imprinted repression of several genomic loci in mouse (Mager et al, 2003). Besides, *eed* was also shown to be essential for imprinted X-inactivation in mouse (Wang et al, 2001). Imprinted X-inactivation is a phenomenon that cells in the extra-embryonic lineage always have their paternal X chromosomes repressed, as opposed to random inactivation of either X chromosome in embryonic cells. In *eed-/-* mice, the paternal X chromosomes in extra-embryonic cells become derepressed.

Given the role of PcG genes in regulating so many biological functions, it is not surprising that deregulation of PcG genes causes many diseases. Both gain-of-function and loss-of-function of PcG genes have been linked to various types of cancers (Bracken & Helin, 2009; Sparmann & van Lohuizen, 2006). The first PcG gene linked to cancer was mouse *bmi1*, which is the homolog of *Drosophila Psc (Psterior sex combs)*. Increased *bmi1* expression was shown to correlate lymphomagenesis (Haupt et al, 1991; van Lohuizen et al, 1991). Later, several other PcG genes were also found to be misregulated in cancer. For example, SUZ12 and EZH2 were found to be up-regulated in several types of cancers (Sparmann & van Lohuizen, 2006).

Molecular Mechanisms of PcG repression

PcG proteins form multisubunit DNA binding complexes

In the very early days of PcG research, it was observed that PcG mutants enhance each others' homeotic phenotypes, such that double mutants of any pair of PcG loci showed a stronger expressivity of homeotic transformation than even the strongest allele of a single PcG locus (Jurgens, 1985). This observation led to two different models to explain it, which may not be mutually exclusive. First, PcG proteins may act in a hierarchy similar to a signaling transduction cascade (Jurgens, 1985). Evidence supporting this model includes the finding that Pc binds to other PcG loci on the polytene chromosomes (Zink & Paro, 1989), suggesting that Pc regulate such loci, thereby forming a regulatory hierarchy. Alternatively, PcG proteins may form multisubunit protein complexes, which follows the so-called "mass-action kinetics" (Locke et al, 1988), in order to execute their function in repressing target gene transcription.

Later it was shown that different PcG proteins often colocalize to the same loci on salivary polytene chromosomes (DeCamillis et al, 1992), supporting the hypothesis that PcG proteins form large complexes. The colocalization of PcG proteins on polytene chromosomes also suggests a model of direct transcription repression. Biochemical characterization of PcG proteins showed that many PcG proteins directly interact with each other. For example, Pc and Ph were co-immunoprecipitated in the same protein complex (Franke et al, 1992); Psc was also shown to interact with both Pc and Ph (Strutt & Paro, 1997); and Ph and Scm (<u>S</u>ex <u>c</u>ombs on <u>m</u>idleg) interact with each other with a shared domain

(Peterson et al, 1997). These results further supported the multimeric complex model of PcG repression.

In 1999, a landmark paper in the PcG research field was published, which described the biochemical purification of a multi-subunit PcG protein complex termed the Polycomb Repressive Complex 1 (PRC1), from *Drosophila* embryos (Shao et al, 1999). Further biochemical characterization identified in PRC1 4 major PcG proteins, Pc, Ph, Psc and Scm, as well as several other proteins. PRC1 was shown to be able to block chromatin remodeling by SWI/SNF in vitro, which is homologous to the *Drosophila trithorax* group proteins. Remodeling by SWI/SNF is an important step in transcriptional activation, thus the ability of PRC1 to antagonize SWI/SNF provided mechanistic insight into how this complex directly represses target gene transcription. Follow-up studies established that Pc, Ph, Psc and another protein dRing as core components of PRC1 (Francis et al, 2001; Saurin et al, 2001).

In addition to PRC1, a different PcG complex containing Extra sex combs (Esc) and Enhancer of Zeste, or E(z), was biochemically purified from *Drosophila* (Ng et al, 2000). In 2002, four groups reported simultaneously that this protein complex has a histone methyltransferase activity that methylates histone H3 at lysine 27(Cao et al, 2002; Czermin et al, 2002; Kuzmichev et al, 2002; Müller et al, 2002), thus linking Polycomb repression to the then emerging “histone code” hypothesis, which states that the histone tails could be post-translationally modified by different small molecules, the combination of which forms a "code" that can be read by a set of proteins to affect transcription (Jenuwein & Allis,

2001; Strahl & Allis, 2000). Such a union arguably caused the explosion in PcG studies seen in the past decade. The *Drosophila* system and the mammalian system were both investigated in these four studies, and similar conclusions were obtained, indicating that this complex and its methyltransferase activity are both conserved from flies to mammals. This complex, which also contains Su(z)12 and other accessory proteins, was named the Polycomb Repressive Complex 2 (PRC2) by one of these studies (Kuzmichev et al, 2002).

More PcG complexes were biochemically purified later. This is not surprising, because early evidence suggested the existence of multiple different PcG complexes, mainly based on the observations that although different PcG proteins colocalize at many loci on polytene chromosomes, different loci are often occupied by different PcG proteins (Strutt 1997). Another 3 PcG complex were identified after the report of PRC1 and PRC2, which makes the total number of known PcG complexes being 5 (Beisel 2011).

Recruitment of PcG complexes to target genes

One of the most important questions in the study of Polycomb repression is how PcG complexes are recruited to their target genes. In 1993, a study reported the identification and characterization of several short pieces of DNA from the *Bithorax* Complex, which the authors called the Polycomb Response Elements (PREs) (Simon et al, 1993). They showed that a PRE is bound by PcG proteins in vivo and is sufficient and necessary to repress reporter gene expression (Simon et al, 1993). Later, many such PREs were identified from many PcG

target loci throughout the *Drosophila* genome, based on their ability to repress reporter gene expression (Ringrose & Paro, 2007). No mammalian PREs were identified until recently (Sing et al, 2009; Woo et al, 2010).

Drosophila PREs could be as short as only several hundred base pairs. However, PREs do not have any consensus sequences. In well characterized *Drosophila* PREs, binding sites of Pleiohomeotic (Pho)/Pleiohomeotic-like (Pho-l), GAF and Zeste are frequently found, but not in particular orders (Ringrose & Paro, 2007). A study showed that an artificially designed DNA sequence with all these binding sites alone is not sufficient to make a PRE. However, if a binding site for the protein DSP1 (Dorsal Switch Protein 1) is also included, the artificial sequence behaves similarly to a PRE (Dejardin et al, 2005). The DSP1 binding site was also shown to be present in natural PREs and is required for the activity of at least some of them (Dejardin et al, 2005), but how general this requirement is remains untested. In addition, one group developed an algorithm to predict PREs directly from the genomic sequences (Ringrose et al, 2003). The results had limited accuracy, and showed some overlap with experimental data using ChIP or DamID (Ringrose & Paro, 2007). Therefore it is still not completely clear what makes a PRE.

No core component of PRC1 or PRC2 binds to DNA in a sequence-specific manner (Schuettengruber et al, 2007). Thus, a group of factors called the recruiters were believed to direct these complexes to their target PREs (Schuettengruber & Cavalli, 2009). The model suggests that the recruiters are sequence-specific DNA binding proteins that bind to PREs, and bring the PRC

complexes to their target PREs to repress transcription. Several lines of evidence make Pho and Pho-l attractive candidates for the recruiters: First, Pho and Pho-l are the only PcG proteins that have sequence specific DNA binding activity (Köhler & Villar, 2008; Mohd-Sarip et al, 2002), and Pho is able to direct Pc to DNA (Mohd-Sarip et al, 2002). Second, in PREs there are often Pho/Pho-l binding sites. And third, PRC1 and PRC2 localization on chromatin was impaired in *pho* mutants (Wang et al, 2004b). As mentioned above, PREs also contain binding sites of GAF and Zeste, therefore these factors are also likely to be recruiters.

Biochemical data showed that Pc, a core component of PRC1, selectively binds to trimethylated histone H3 at lysine 27 (H3K27me3) via its “chromodomain” (Fischle et al, 2003; Min et al, 2003). Artificially increasing and decreasing the level of H3K17me3 at a target site in vivo also resulted in enhanced and impaired PRC1 recruitment respectively (Lee et al, 2007; Mujtaba et al, 2008). On the other hand, PRC2 has a histone methyltransferase activity in both *Drosophila* and mammals, with E(z) being the catalytic subunit (Czermin et al, 2002; Kuzmichev et al, 2002). These findings led to a model that explains PcG complex recruitment: First, the recruiters bind to PREs in a sequence specific manner. Next PRC2 is directed to the target sites by the recruiters, and trimethylates nearby nucleosomes at lysine 27 of the H3 subunits. The epigenetic mark H3K27me3 is then being recognized by the chromodomain of Pc, thereby directing PRC1 to the targets. This sequential recruitment model was later experimentally reconstituted in vitro (Wang et al, 2004b).

The model above however, is unlikely to be the only way how PcG complexes are recruited to their targets. For example, H3K27me3 is neither sufficient nor necessary for PRC1 recruitment. On one hand, not all H3K17me3 marked sites are occupied by PRC1 (Schwartz et al, 2006). This is probably because the epigenetic mark H3K27me3 has other functions. On the other hand, there are PRC1 sites that lack PRC2 and H3K27me3 (Schoeftner et al, 2006). Therefore there exists PRC2 independent recruitment of PRC1 to their target sites. The nature of such mechanism remains unknown. Given the large number of PcG target genes, it is not surprising that PRC1 is recruited to different targets by different mechanisms. Therefore H3K27me3 may represent one determining factor in a particular PRC1 recruitment mechanism, which is used by the cells to deploy PRC1 to a subset of PcG targets.

Even for the above model, evidence suggested that there are still some missing parts. For example, the dissociation constant of the Pc chromodomain for H3K27me3 is in the order of micro-molar, an affinity that is still several orders of magnitude lower than a typical regulatory protein-DNA binding (Fischle et al, 2003; Schwartz & Pirrotta, 2007). This suggests that some other factors are involved to enhance the binding between Pc and H3K27me3. Therefore, still much is unknown about the recruitment of PcG complexes to their targets.

Mechanisms of transcription repression of PcG targets

Many PcG proteins have been shown to bind to specific loci on polytene chromosomes, suggesting that these proteins may bind DNA (DeCamillis et al,

1992; Zink & Paro, 1989). As progress in molecular biological techniques grew in the 1980s, several PcG genes were cloned, and their corresponding protein sequences analyzed. Many PcG proteins have been shown to have DNA binding motifs (Bornemann et al, 1996; DeCamillis et al, 1992). These observations suggested that PcG proteins might directly repress their target gene transcription.

There are many ways by which transcription could be repressed by PcG complexes (Simon & Kingston, 2009). For example, PcG complexes may occupy promoter regions to directly prevent RNA polymerase II and/or transcription factors from binding to their corresponding sites. Similarly, PcG complexes may directly block transcription factors from binding to cis-regulatory elements. Or, PcG complexes may compact chromatin to make promoter or cis-regulatory elements physically inaccessible to RNA polymerase II and/or transcription factors. In addition, PcG complexes may induce local looping or long distance interactions between chromatin elements to block transcription elongation. Finally, there is evidence that the space closely below the nuclear envelope is occupied mainly by genes at a repressed state, so this space has been called the repressive compartment (Shevelyov & Nurminsk, 2011). Thus PcG complexes may also direct their targets to such repressive compartments. How PcG proteins repress transcription is another important question in the PcG research field.

PRC2 has a histone methyltransferease that marks nucleosomes with H3K27me3 (Kuzmichev et al, 2002; Müller et al, 2002), and it was recently reported to also recruit histone H3 lysine 4 (H3K4) demethylase to target genes in mammals (Pasini et al, 2008). H3K27me3 and H3K4me3 are associated with

transcriptionally repressed and activated states respectively (Wang et al, 2007), although the molecular mechanisms are not completely clear. Therefore, PRC2 seems to be able to repress transcription by increase trimethylation at H3K27, and at the same time reduce the level of trimethylation at H3K4. However, whether *Drosophila* PRC2 also possesses H3K4 demethylase recruitment activity is not known.

A very interesting observation is that in mammalian embryonic stem cells, many loci are occupied by both H3K27me3 and H3K4me3, thus creating a so-called "bivalent" state (Bernstein et al, 2006), which could be caused by PRC2 bound to those sites. Many of these loci become either activated or repressed as the cells differentiate and commit to a particular lineage (Bernstein et al, 2006). The active loci generally retain the H3K4me3 marks, but the H3K27me3 marks are lost. The opposite holds true for the repressed loci (Bernstein et al, 2006). Surprisingly, these bivalent loci are engaged by paused RNA polymerase II (Stock et al, 2007), meaning that transcription has started, but the RNA polymerase stopped in the middle of the gene during elongation. The PcG protein Ring, which has an E3 ubiquitin ligase activity (de Napoles et al, 2004; Wang et al, 2004a), has been shown to ubiquitylate histone H2A, and this particular modification is necessary for RNA polymerase pausing. Removal of Ring abolished H2A ubiquitylation, and resumed the RNA polymerase to the active transcription state (Stock et al, 2007). Such paused RNA polymerase II was also found in a large number of loci in *Drosophila* (Chopra et al, 2009), but whether or not PcG genes play a role was

not tested in the study. Nevertheless, it is possible that in *Drosophila*, PcG genes also repress their target gene transcription by blocking elongation.

In addition to the above mentioned mechanisms that more or less depend on epigenetic marks, local and long distance chromatin structure might also be regulated by PcG complexes to repress target gene transcription. Early in PcG studies, it was discovered that the PcG protein Pc shares sequence homology with heterochromatin protein-2 (HP2), which is involved in heterochromatin formation (Paro & Hogness, 1991). This led to the hypothesis that PcG complexes heterochromatinize their target genes, therefore repress their expression by physically blocking the DNA. An *in vivo* study showed that the T7 polymerase was able to express a reporter gene inserted into an intron of *Ubx*, a region under PcG regulation, in segments throughout the embryo, no matter if *Ubx* is activated or repressed (McCall & Bender, 1996). This result argued against the heterochromatin model, as it showed that the T7 RNA polymerase has access to the DNA even if the DNA is in a PcG-repressed state. The same study also showed that the yeast Gal4 protein only expressed a reporter inserted into the same region in posterior segments of the embryos, where *Ubx* is normally activated (McCall & Bender, 1996). This observation was interpreted as supporting the heterochromatin hypothesis at the time, but it might also be explained by RNA polymerase pausing. Later, a group used confocal microscope to directly observe the behavior of PcG proteins and discovered that there is no increase in DNA density at loci occupied by PcG complexes, which argues against the formation of long heterochromatin regions at PcG targets (Buchenau

et al, 1998). However, due to the limited sensitivity of confocal microscopy, the possibility of short local heterochromatin could not be ruled out (Buchenau et al, 1998).

Biochemical data also support the idea that PcG complexes do not block DNA accessibility. PRC1 could block chromatin remodeling by the SWI/SNF remodeler (Shao et al, 1999). Therefore PRC1 seems to be able to affect the positioning of polynucleosomes. However, this repositioning was shown not to affect the general accessibility of other factors to DNA, as judged by the sensitivity of DNA to endonuclease (Francis et al, 2001). A recent study however, provided evidence supporting the heterochromatin hypothesis by showing that one mammalian PRC2 variant was able to compact chromatin (Margueron et al, 2008). Mammals have two E(Z) homologs, EZH1 and EZH2. PRC2 containing EZH1 does not have a strong methyltransferase activity, but was able to compact chromatin, and this chromatin compaction activity is independent of the methyltransferase activity. On the other hand, EZH2 containing PRC2 has a strong methyltransferase activity, but is unable to compact chromatin (Margueron et al, 2008). These results suggested that PRC2 might directly repress target gene transcription by chromatin compaction. Since *Drosophila* only has a single E(Z) protein, it remains to be seen if *Drosophila* PRC2 is able to compact chromatin.

To summarize, our understanding on the mechanisms of PcG repression is still far from complete. Current evidence established correlations between PcG repression and histone marks such as H3K27me3, H3K4me3 and histone 2A

ubiquitylation. Although studies are just beginning to reveal how such histone modifications affect transcription, current data have provided evidence showing that H3K27me3 plays a role in PcG recruitment, and H2A ubiquitylation is important for RNA polymerase pausing in mammals. As paused RNA polymerase II is also common in *Drosophila*, it is likely that PcG complexes also block transcription elongation in *Drosophila*. In addition, although it is unlikely that PcG complexes induce the formation of broad heterochromatin domains, evidence suggests that they are able to regulate local nucleosome organization and compact chromatin, and thereby affect transcription.

polyhomeotic is a member of PcG genes

Drosophila polyhomeotic has two redundant functional units

The PcG gene *polyhomeotic (ph)* was first reported in 1985 (Dura et al, 1985). It was discovered in genetic screens for X chromosome mutants that display extra sex combs. Since males are naturally hemizygous, recessive mutants could also be identified among the F1 progeny, which makes the screens much more potent. *ph* was mapped to 2D2-3 on the cytological map. Using their initial *ph* alleles, Dura *et al.* found that *ph* is homozygous viable, and has no embryonic phenotype. Homozygous adult females and hemizygous adult males show diverse homeotic transformations with various penetrance and expressivity. These transformations include wing to haltere tansformation, extra sex combs on second and third legs, and partial transformation of segment A1 to A2, and A4 to A5. In addition, *ph* adults frequently show loss of humerus, a phenotype previously not seen in other *Pc* group genes. On the other hand, antenna to leg transformation, a homeotic phenotype seen in mutants of *Pc* and *Pcl*, two other *Pc* Group genes, is not observed in *ph* mutants.

However, genetic evidence indicated that all *ph* alleles reported in the original study were not amorphic, because many of these *ph* alleles are hemizygous lethal in females (females with the genotype of *ph/Deficiency*), but none is homozygous lethal in females (females with the genotype of *ph/ph*) (Dura et al, 1985). Therefore these *ph* alleles are all hypomorphs (Muller, 1932). In a follow-up study, the authors tried to obtain a *ph* null allele by performing a large scale (likely saturated) mutagenesis (Dura et al, 1987). Their readout in this

mutagenesis was lethality over a small deletion that uncovers the *ph* locus. In two rounds of mutagenesis using EMS and X-ray, they isolated 41 mutants which caused lethality when heterozygous with the small deletion, and the mutants were grouped into 5 complementation groups. 24 mutants turned out to carry *ph* alleles. However, all 24 *ph* alleles were homozygous viable compared to only 2 being homozygous viable out of the other 17 alleles belonging to other nearby genes (Dura et al, 1987).

The high mutability of the *ph* locus (~ 60% of alleles recovered in the above screens are *ph* alleles), as well as the difficulty in obtaining null alleles, led Dura *et. al.* to propose that the *ph* locus is large and complex, and may include two redundant genes (Dura et al, 1987). Based on their hypothesis, a *ph* null allele can only be obtained by two mutagenetic events.

Therefore, The authors mutagenized a chromosome that already bears a viable *ph* allele (ph^{209}), and isolated 6 alleles that are embryonic lethal when over the original ph^{209} allele (Dura et al, 1987). Complementation tests confirmed that the lethality of all 6 alleles was caused by mutations at the *ph* locus. 5 alleles were proved to be amorphic. Southern blots further confirmed that there are indeed two large duplicated DNA sequences within the *ph* locus (Dura et al, 1987).

Based on their genetic analyses, a model of the dosage effect of *ph* was proposed. According to this model, the two *ph* genes are largely functionally redundant. In addition, females normally have 4 copies of the *ph* gene, and two copies are required for viability. Thus if two copies of *ph* are lost, the flies are still

viable, but if three or four copies are mutated, the individuals die during embryogenesis (Dura et al, 1987).

It must be pointed out that although some *ph* alleles Dura *et. al.* isolated had long been used as null alleles, none of them has been characterized molecularly. In fact, recently I have shown that ph^{505}, one allele that has been used extensively as a *ph* null allele, is actually hypomorphic. The first molecularly characterized true *ph* null allele was generated by me during my dissertation research (Feng et al, 2011a) (Also see Chapter 2).

Structure and biochemical function of Ph proteins

The genomic sequence of the *Drosophila ph* locus was determined in 1991 (Deatrick et al, 1991). The sequencing results confirmed that the *ph* locus has two genes that are highly similar. The two genes were named the "*ph proximal unit*" (or *ph-p*) and the "*ph distal unit*" (or *ph-d*). At the time, a partial cDNA clone corresponding to *ph-p* was also available and was sequenced. Comparison between the genomic sequence and the cDNA sequence facilitated the identification of the introns in *ph-p*. Interestingly, the two introns and the corresponding exon-intron junctions are perfectly conserved between *ph-p* and *ph-d*, thus it was assumed that *ph-d* was spliced in the same way. Putative Open Reading Frames (ORFs) were identified from both *ph-p* and *ph-d*. Then Ph-P and Ph-D protein sequences were deduced from the ORFs. (Deatrick et al, 1991).

Protein domain analyses using the putative protein sequences showed that the Ph proteins have a single zinc finger (Deatrick et al, 1991). At the time, it was known that proteins with multiple zinc fingers bind to DNA. Therefore this suggested that if Ph were to bind to DNA, it might do so by forming complexes with other factors, possibly other PcG proteins. However it could not be ruled out that the zinc finger found in Ph had nothing to do with DNA binding, since zinc finger motifs had been found in non-DNA binding proteins (Deatrick et al, 1991). This particular zinc finger domain, which is also found in several other PcG proteins, was later named the FCS zinc finger domain, and has been shown to be required for Ph mediated transcription repression (Wang et al, 2011). Recent structural studies of this domain suggested that it binds to RNA in a non-sequence specific manner (Lechtenberg et al, 2009). Given the accumulating evidence that non-coding RNAs play important roles in PcG mediated repression (Simon & Kingston, 2009), this finding is of particular interest.

In addition to the zinc finger motif, the Ph proteins also have a polyglutamine motif and a serine-threonine rich motif, both of which had been reported to function in transcription activation in other proteins (Deatrick et al, 1991). It is interesting that although PcG proteins function in maintaining a repressed transcription state, the PcG protein Ph has these transcription activation motifs. Ph also has an alpha helix at its C terminus, which was suggested to function in protein-protein interaction (Deatrick et al, 1991). This C terminal domain was later named the SPM domain (Bornemann et al, 1996) or the SAM domain (Ponting, 1995), and its function in protein-protein interaction was experimentally

confirmed. For example, this domain is required to mediate interactions between Ph and Sex Comb on Midleg (Scm), another PcG protein (Kyba & Brock, 1998; Peterson et al, 1997). Moreover, this particular protein-protein interaction and the involvement of the SAM domain are both conserved in mammals (Tomotsune et al, 1999), suggesting that this interaction is functionally important.

The full length cDNA of *ph-p* was reported a year after its genomic sequence was decoded (DeCamillis et al, 1992), and later the full length *ph-d* cDNA was also determined (Hodgson et al, 1997). Their sequences largely confirmed earlier analyses (Deatrick et al, 1991), and also revealed more features of the Ph proteins. For example, Hodgson *et. al.* identified from the Ph sequences a nuclear localization signal, and a putative GTP binding motif. They also showed that Ph-p has a 194 amino acid N-terminal domain that is missing in Ph-d (Hodgson et al, 1997). A schematic of the domain structures of *Drosophila* Ph-p and Ph-d is given in Figure 1-1.

Figure 1-1. Domain structures of *Drosophila* Ph-p and Ph-d
Based on data from DeCamillis *et. al*. (DeCamillis et al, 1992) and Hodgson *et. al.* (Hodgson et al, 1997). Not draw to scale.

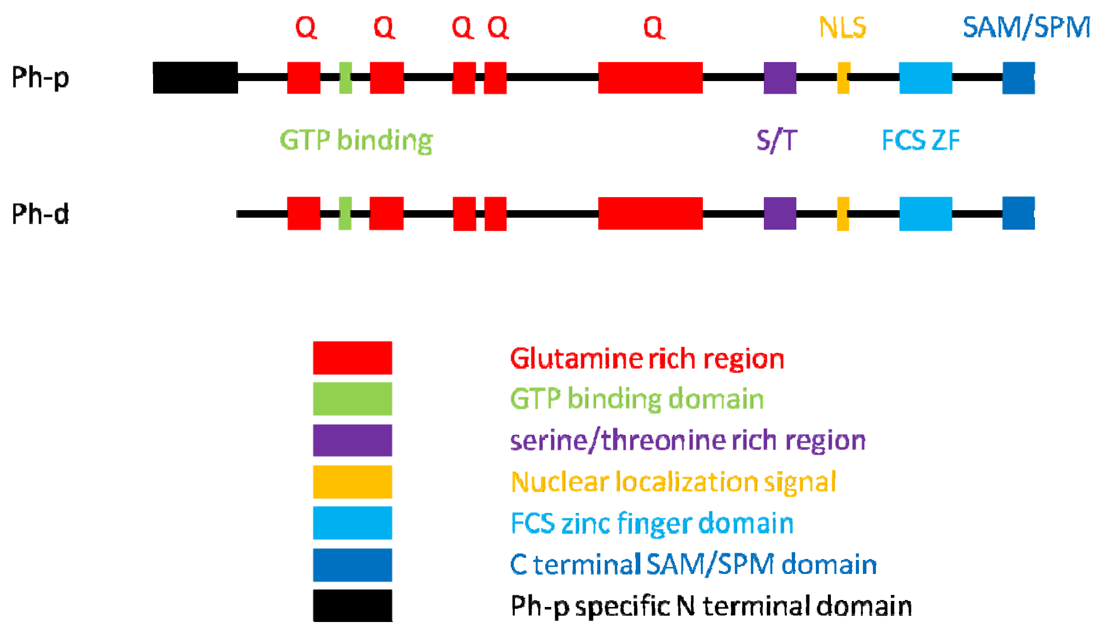

Figure 1-1

In addition to analyzing the *ph-p* cDNA, DeCamillis *et. al.* also raised a polyclonal antibody against a conserved region between Ph-p and Ph-d (DeCamillis et al, 1992). Thus this antibody was expected to recognize both Ph proteins. Using this antibody, the authors showed for the first time, that the Ph proteins localize to the polytene chromosomes. They further showed that Ph binds to a transgene that contains a piece of DNA from the *bxd* locus originally residing in the *Bithorax* Complex (DeCamillis et al, 1992). This demonstrated that the Ph proteins are recruited to specific DNA sequences. Since Ph has no sequence specific DNA binding activity, how it is recruited to specific sequences is an interesting question. The same study also showed that Ph and Pc share many loci on the polytene chromosomes (DeCamillis et al, 1992). Almost at the same time, another study confirmed that Ph and Pc physically interact with each other and form a multimeric protein complex using coimmunoprecipitation (Franke et al, 1992). Later, the first PcG complex, Polycomb Repressive Complex 1 (PRC1), was biochemically purified from *Drosophila* embryo nuclear extracts (Shao et al, 1999). Ph was shown to be one of the core components of PRC1 (Francis et al, 2001; Saurin et al, 2001; Shao et al, 1999).

Recently, it was found that the *Drosophila* Ph proteins are post-translationally modified by another PcG protein Super Sex Combs (Sxc), which is a highly conserved glycosyltransferase (Gambetta et al, 2009). In *sxc* mutants, Ph is not glycosylated. In such a genetic background, while PcG protein complexes are still bound to their target sites, PcG repression of targets is released. These findings indicated that glycosylation of Ph by Sxc is not required for PcG complex

formation or PcG complex recruitment, but it is required for the repression of target gene transcription.

The function of *ph* in normal physiology and in diseases

In *Drosophila*, extensive phenotypic analyses have been performed to characterize the functions of *ph*. Using *ph* hypomorphic alleles, the adult phenotypes have been detailed, which include extra sex combs, wing to haltere transformation, and loss of humerus (Dura et al, 1985). When the first then believed *ph* null allele was isolated, embryonic phenotypes were scored (Dura et al, 1987). *ph* embryonic phenotypes were very similar to those of other PcG genes. For example, the posterior half of the embryos showed posteriorly directed homeotic transformations, consistent with Ph being a negative regulator of *Hox* genes. In addition to homeotic transformations, *ph* mutant embryos also showed massive programmed cell death in their ventral dermis.

Later, two studies reported that *ph* mutant embryos had severe phenotypes in their central nervous system (Smouse et al, 1988; Smouse & Perrimon, 1990), indicating that *ph* is required for normal CNS development in *Drosophila*. More than a decade after, another study revealed additional function of *ph* in *Drosophila* neurodevelopment by showing that *ph* plays a role in mushroom body development (Wang et al, 2006).

In recent years, several studies have linked *ph* to cell cycle control and cell polarity. Martinez *et. al.* used ph^{505}, a *ph* hypomorphic allele then believed to be

amorphic, to show that *loss of ph* in a mosaic tissue causes autonomous cell over-proliferation (Martinez et al, 2009). The authors also observed abnormalities in mutant cell polarity. Later, Gonzalez *et. al.* over-expressed Ph in imaginal discs and observed tissue overgrowth (González et al, 2009). It is interesting that both loss-of-function and gain-of-function of *ph* cause cell over-proliferation. Another study showed that loss-of-function and gain-of-function of *ph* both caused defects in cell polarity and epithelial integrity (Gandille et al, 2010).

ph homologs have been found in other organisms, indicating that it is a conserved gene. The first *ph* homolog in non-*Drosophila* species was identified in mouse, in a cDNA library screen for clones up-regulated by 17-retinoic acid (RA). One clone called number “Rae-28” was chosen for further characterization, and the Rae28 cDNA was shown to be homologous to *Drosophila ph* (Nomura et al, 1994). Later its genomic sequence was also reported (Motaleb et al, 1996). Almost at the same time, another group used yeast two-hybrid to isolate mouse proteins that bind to Bmi1, a vertebrate homolog of the *Drosophila* PcG protein Posterior Sex comb (Psc). They identified a single protein that is homologous to *Drosophila* Ph. Thus they named this protein Mph1 (Alkema et al, 1997). It turned out that Rae28 and Mph1 are the same protein.

Human Ph homologs were isolated in a similar way as Mph1, in a yeast two-hybrid screen for human proteins interacting with Bmi1. However, two proteins were identified that were both homologous to *Drosophila* Ph. So they were named HPH1 and HPH2 respectively (Gunster et al, 1997). HPH1 turned out to be orthologous to MPH1, and a mouse protein orthologous to HPH2 was later

isolated and named MPH2 (Hemenway et al, 1998). Several years later, *mph2* genomic sequence was reported and the gene structure analyzed (Yamaki et al, 2002). A new human Ph homolog called HPH3 was discovered in an attempt to purify the human Polycomb Repressive Complex 1 (PRC1) (Levine et al, 2002). In addition to mammalian homologs of *ph*, a zebrafish gene homologous to *Drosophila ph*, mouse *mph2* and human *hph2* was also reported (Kawamura et al, 2002).

rae28 (mph1) knockout mice have been generated (Takihara et al, 1997). *rae28-/-* mice die perinatally, and show posteriorly directed skeleton transformations, consistent with Rae28 being a repressor of *Hox* genes. Abnormal *Hox* gene expression patterns were also observed in homozygous mice. In addition to skeleton transformations, *rae28* deficient mice also showed abnormalities in neural crest and cardiac morphogenesis, as well as other defects (Takihara et al, 1997). A follow-up study showed that the cardiac phenotypes of *rae28-/-* mice were due to premature turnoff of the cardiac selector gene *nkx2.5* (Shirai et al, 2002). *mph2* knockout mice were also produced, and showed similar skeleton transformations as in *mph1* knockout mice. It was also shown that mutations of *mph1* and *mph2* enhance each other's homeotic transformation phenotypes (Isono et al, 2005).

The first clue to the functions of Ph in humans came from a study that measured *hph1* transcription level during hematopoiesis. It showed that *hph1* RNA level increased as the bone marrow cells mature in vitro, suggesting a possible regulatory role in differentiation (Lessard et al, 1998). Later, a study using *rae28*

knockout mice confirmed that B cell maturation was arrested without Ph (Tokimasa et al, 2001). Studies in mouse also showed that Ph is required for the maintenance of the hematopoietic stem cells (Ohta et al, 2002).

A recent study directly linked Ph to human cancer by showing that Ph has a tumor suppressor activity in osteosarcoma cells (Iwata et al, 2010). The authors used Real-Time PCR to compare the expression level of *hph3* in 10 osteosarcoma cell lines and 42 primary osteosarcoma samples, and correlated *hph3* RNA level with clinical outcomes. They found that lower *hph3* expression level correlated with poorer prognosis. In addition, a missense mutation that impaired the ability of HPH3 to repress target genes was found in 1 of 10 cell lines as well as 4 out of 42 primary samples.

JAK/STAT pathway and non-autonomous cell over-proliferation in *Drosophila*

The JAK-STAT pathway in *Drosophila*

The Janus Kinase/Signal Transducer and Activator of Transcription pathway (JAK/STAT pathway) is a conserved signal transduction pathway in animals. It was originally identified in mammals because of its important functions in the immune system (Fu et al, 1992; Schindler et al, 1992), but later was found to be present in *C. elegans*, *Drosophila melanogaster*, zebrafish and even the slime mold *Dictyostelium* (Hou et al, 2002). The canonical JAK/STAT pathway has four groups of core components: the extracellular ligands, the transmembrane receptors, the receptor associated kinases (the JAK kinases), as well as the downstream transcription factors (the STAT proteins) (Arbouzova & Zeidler, 2006). In mammals, the JAK/STAT pathway plays diverse roles in the immune system, hematopoiesis, cell proliferation and stem cell maintenance. Interestingly, these biological functions are also conserved in *Drosophila* (Hombría et al, 2005).

Extensive studies have established this pathway's major mode of activation. The pathway receptors themselves do not have a kinase domain, but they are associated with the JAK kinases. Ligand binding activates the JAK tyrosine kinases, which phosphorylate themselves, as well as the receptors to form docking sites for the STAT proteins. The STAT proteins are then recruited to these docking sites and are phosphorylated by the JAK kinases. Unphosphorylated STAT proteins exist as monomers and reside in the cytoplasm. Upon phosphorylation, STAT proteins dimerize and are transported into the

nucleus, where they acts as transcription activators to induce the expression of downstream targets (Arbouzova & Zeidler, 2006).

The mammalian genomes encode 4 JAKs, 7 STATs, several receptors and a variety of ligands (Kisseleva et al, 2002). Therefore there is a considerable degree of complexity and redundancy (Carbia-Nagashima & Arzt, 2004; Kisseleva et al, 2002). On the contrary, the *Drosophila* genome encodes a single transmembrane receptor Domeless (Dome) (Brown et al, 2001; Chen et al, 2002), a single JAK kinase Hopscotch (Hop) (Binari & Perrimon, 1994), a single STAT protein State92E (Hou et al, 1996; Yan et al, 1996), and three extracellular ligands Unpaired (Upd) (Harrison et al, 1998), Unpaired 2 (Upd2) (Gilbert et al, 2005; Hombría et al, 2005) and Unpaired 3 (Upd3) (Agaisse et al, 2003). This simplicity makes functional characterization of this pathway much easier. Nevertheless, because this pathway is conserved from flies to mammals, knowledge gained from studying this pathway in flies is likely to be applicable to mammals.

In addition to the above mentioned core components, many JAK/STAT pathway regulators, both positive and negative, have been identified in *Drosophila*. BRWD3 is an example of positive regulator, whereas the SOCS (Suppressors Of Cytokine Signaling) proteins, PIAS (Protein Inhibitor of Activated STAT) and Ken (Ken and Barbie) are well characterized negative regulators (Arbouzova & Zeidler, 2006). Some of these regulators were identified based on sequence homology to their mammalian counterparts, such as the *SOCS* genes (Rawlings

et al, 2004) and *pias* (Betz et al, 2001; Mohr & Boswell, 1999), while others were identified in genome wide screens.

In *Drosophila*, the JAK/STAT pathway has been shown to be sensitive to dosage effects, such that heterozygosity of a pathway component often greatly suppresses a pathway gain-of-function phenotype, such as cell over-proliferation. This property has been used to perform a large scale genetic screen to identify dominant modulators of the JAK/STAT pathway (Bach et al, 2003). Dozens of modulators were identified, and a follow-up study functionally characterized one hit, the gene encoding Ken and Barbie (Ken), and established it as a negative regulator of the JAK/STAT pathway (Arbouzova et al, 2006).

Recently, two cell culture based genome wide RNAi screens were performed to systematically search for JAK/STAT pathway regulators (Baeg et al, 2005; Muller et al, 2005). These two studies had led to the discovery of, among other modulators, Ptp61F, a phosphatase that down-regulates the JAK/STAT pathway activity and a long sought missing part. Surprisingly however, although these two screens were both valid, as they both identified known regulators, their results showed a very limited overlap. Among 121 and 90 hits identified from the two screens, only 6 were found in both. The possible reasons for such a low overlap level were discussed in detail elsewhere (Müller et al, 2008). Briefly, it was believed that because different reporter systems were used in the two screens, one screen tends to identify positive regulators, whereas the other tends to identify negative ones.

Non-autonomous cell over-proliferation in *Drosophila*

The unparalleled genetics toolkit of *Drosophila* makes phenotype-based forward genetic screens an invaluable strategy to study the molecular mechanisms of biological phenomena. One classic example of dissecting molecular mechanisms using forward genetic screens in *Drosophila* is the identification of tumor suppressors. Using Flp-FRT based mosaic techniques that are only available in *Drosophila*, geneticists have conducted many large scale F1 screens to search for mutants that disrupt potential tumor suppressors. Such screens generally use the size of the fly eye as the readout, and identify those individuals with enlarged eyes. Since these screens are F1 screens, millions of individuals can be scored, which makes it very easy to reach saturation. This strategy has led to the identification of dozens of *Drosophila* tumor suppressors, most of which are grouped into several signaling pathways, such as the Insulin Receptor (InR) pathway, TSC pathway, mTOR pathway, and the recently identified Hippo pathway. It also turned out that many tumor suppressors first identified in *Drosophila* are also conserved in humans. Thus such studies have provided important insights into cancer research. It must be pointed out that the opposite strategy, meaning that using shrunken eye as the desired phenotype to screen for oncogenes, is generally considered infeasible. This is because mutations in many genes, for example housekeeping genes, can cause the eyes to become smaller, so such screens would generate intolerably high false positives. On the other hand, if a mutation causes the eye to overgrow, generally it disrupts a tumor suppressor (Hariharan & Bilder, 2006).

Most currently identified *Drosophila* tumor suppressors have cell autonomous effects, meaning that the mutant cells in a mosaic tissue become super proliferative. However, there are a few cases where a mutation or mutations have been shown to have non-autonomous effects on cell proliferation. This means that the mutant cells in a mosaic tissue do not over-proliferate, instead they induce surrounding wild type cells to become highly proliferative. Before my dissertation research was published, there were 8 studies reporting 5 different cases of non-autonomous over-proliferation in *Drosophila*.

The study conducted by Lee *et. al.* (Lee et al, 2002) was probably the first to document non-autonomous over-proliferation in *Drosophila*, which was caused by mutations in *hyperplastic discs (hyd)*, which encodes ubiquitin ligase. The authors further showed that Hedgehog (Hh) expression was elevated in the mutant clones, which was at least partially responsible for the non-autonomous over-proliferation phenotype. But they did not identify the complete non-autonomous proliferation signaling pathway. Later, a group described that *Notch* gain-of-function in clones of cells causes non-autonomous cell over-proliferation (Reynolds-Kenneally & Mlodzik, 2005). Then 3 groups independently found that mutations that inactivate the gene *vps25* (vps stands for vascular protein sorting), whose product plays important roles in endocytosis, cause non-autonomous over proliferation (Herz et al, 2006; Thompson et al, 2005; Vaccari & Bilder, 2005). Moreover, a group also reported that mutants of *vps23*, which functions in the same pathway as *vps25* in endocytosis, also induce non-autonomous over-proliferation (Moberg et al, 2005). Besides, a study showed that mutations in

uba1, which encodes the E1 ubiquitin-activating enzyme, could cause non-autonomous proliferation under certain conditions (Lee et al, 2008). In addition, a study showed that non-autonomous proliferation is a synthetic phenotype caused by elevated activities in both the JNK and the Ras/Raf pathways (Uhlirova et al, 2005).

Among the 8 studies mentioned above, except for the first and the last ones, the same pathway was shown to cause non-autonomous cell proliferation in the remaining 6 cases: Notch activity is increased autonomously in the mutant cells, which causes the mutant cells to over-express the JAK/STAT pathway ligand Upd. The ectopically increased Notch activity has been suggested to cause Upd over-expression either directly or indirectly via the transcription factor Eyegone (Eyg). Upd is a secreted ligand that activates the JAK/STAT pathway in neighboring wild type cells, which causes their over-proliferation. It is interesting that mutations in components of the endocytosis pathway cause non-autonomous over-proliferation. It has been suggested that impaired Notch protein recycling might be the cause of increased Notch activity, which eventually causes non-autonomous over-proliferation (Herz et al, 2006). This hypothesis is plausible because ubiquitylation plays an important role in protein degradation (Hershko & Ciechanover, 1998), and impaired ubiquitylation pathway was also found to cause accumulation of Notch, which eventually induces non-autonomous cell proliferation (Lee et al, 2008).

Chapter 2: Loss of *ph* causes non-autonomous over-proliferation by activating the Notch-Upd homologs-JAK/STAT pathway

Abstract

The first true molecularly characterized *ph* null allele, ph^{del}, was generated, and its phenotypes were characterized. In mosaic tissues, loss of *ph* causes tissue over-proliferation. However, such over-proliferation shows a non-autonomous pattern, meaning that the mutant cells themselves do not over-proliferate, but they could stimulate over-proliferation of surrounding wild type cells. Cellular abnormalities in *ph* mosaic tissues were then characterized. Wild-type cells show elevated cell proliferation level, while mutant cells show impaired differentiation, decreased cell proliferation and increases apoptosis. Another surprising finding was that the *ph* mutant cells do not lose their normal cell polarity, but undergo invagination and form a unique 3 dimensional structure, which morphologically and functionally resemble secretory glands. Moreover, the signaling pathways underlying *ph* induced non-autonomous over-proliferation were determined. Briefly, Notch activity is autonomously increased by loss of *ph*, causing over-expression of all three *upd* homologs, which encode JAK/STAT pathway ligands. These ligands then activate the JAK/STAT pathway in surrounding wild-type cells and cause their over-proliferation.

Loss of *ph* causes non-autonomous overgrowth in mosaic eyes

In a previous study, an EMS-induced recessive lethal mutant, *l(X)MB342,* was isolated and was shown to alter cell fates in the *Drosophila* brain (Wang et al, 2006). Subsequently, my advisor, Dr. Jian Wang, found that this mutant also caused cell over-proliferation in mosaic eyes (Figure 2-1B), and my PhD dissertation project was to study the underlying mechanisms. *l(X)MB342* is a deficiency line that uncovers a ~40 kb genomic region and fails to complement with 3 complementation groups (*ph*, *Pgd* and *wapl*; Figure 2-1A). To determine which gene(s) were responsible for the enlarged eyes, I examined the mosaic eye phenotypes of various mutant lines within this genomic region. No eye enlargement was observed to result from any *Pgd* or *wapl* alleles (Figure 2-2A). However, I observed various different, even opposite, phenotypes in eyes mosaic for different *ph* alleles that were all reported to be *ph* null alleles (Dura et al, 1987) (Figure 2-2B).

The *ph* locus consists of two tandemly duplicated genes, *ph-p* and *ph-d*, which are functionally redundant (Deatrick et al, 1991). All currently known *ph* alleles were generated by multiple rounds of mutagenesis to inactivate both genes, and their nature of mutations has not been clearly characterized at the molecular level (Dura et al, 1987). These features may explain why different *ph* alleles have different phenotypes, and they also highlight the need for a *ph* null allele with a clean genetic background. Therefore, I generated a *ph* deficiency line, ph^{del}, from two viable *P*-element insertion lines using an FRT-based genetic technique (Parks et al, 2004). In ph^{del}, the *ph* locus was completely deleted without disrupting the surrounding genes (Figure 2-1A and 3-2). As expected, ph^{del} failed

to complement with several known *ph* alleles but complemented with genes adjacent to the *ph* locus on both sides. The phenotypes of ph^{del} mosaic eyes were indistinguishable from those of *l(X)MB342* (Figure 2-1B and 2-1C). Moreover, a transgene, *p*{*ph-d*$^{+}$}, that carries a 10 kb *ph-d* genomic fragment, could rescue the lethality and over-proliferation phenotype of ph^{del} (Figure 2-1B and 2-3). Together, these data demonstrate that loss of Ph causes overgrowth in mosaic eyes.

Next MARCM analysis (Lee & Luo, 1999) was performed using ph^{del} in eye discs. Although ph^{del} mosaic discs were remarkably enlarged at the third instar larval stage, homozygous mutant cells (positively labeled by GFP) occupied an even smaller proportion of *ph* mosaic eye discs than did control clones in wild type mosaic discs (Figure 2-1C), suggesting that *ph* causes over-proliferation in a cell non-autonomous manner (i.e., only in wild-type cells). To test this idea, I labeled the *ph* chromosome with w^{-} and the wild type chromosome with w^{+}. In the resulting mosaic eyes, most, if not all, ommatidia were red. This is in contrast to wild type mosaic eyes, which exhibited patches of white and red ommatidia (Figure 2-1D). Conversely, when the *ph* chromosome was labeled with w^{+} and the wild type chromosome was labeled with w^{-} in ph^{del} mosaic eyes, I observed mainly white eyes with a few red cells, which might represent heterozygous cells that did not undergo recombination (Figure 2-1D). These results provide further evidence that loss of Ph induces non-autonomous overgrowth and suggest that *ph* cells do not differentiate into ommatidia.

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Figure 2-1. *ph*^{del} clones in mosaic eyes induce non-autonomous overgrowth.

(**A**) Diagram of the genomic region surrounding the *ph* locus showing DNA fragments that are deleted in the two deficiency lines, *l(X)MB342* and *ph*^{del}. (**B**) Scanning electron microscopy images showing mosaic eyes with different genotypes. Mosaic eyes of *l(X)MB342* and *ph*^{del} induced by *ey-flp* are enlarged and misshapen. The overgrowth phenotype of *ph*^{del} mosaic eyes can be suppressed by one copy of the *p*{*ph-d*$^{+}$} transgene, but the rescued eye has a rough surface. Two copies of *p*{*ph-d*$^{+}$} transgene are needed for a full rescue (Figure 2-3). (**C**) Mosaic eye imaginal discs were dissected at the wandering larval stage and stained with DAPI (blue). Clones homozygous for wt or *ph*^{del} were marked by GFP (green). Compared to wt, the *ph*^{del} mosaic eye discs are larger overall but *ph*^{del} clones occupy a smaller portion of the disc. One copy of the *p*{*ph-d*$^{+}$} transgene suppresses the overgrowth phenotype of the *ph*^{del} mosaic eye disc and restores normal morphology of *ph*^{del} clones. (**D**) Adult eyes of *ey-flp*-induced mosaics of wt and *ph*^{del} show that *ph*^{del} mosaic eyes are overgrown and lack mutant clones. When *ph*^{del} is associated with a w^{-} chromosome, the mosaic eye is largely red. Conversely, when *ph*^{del} is associated with a w^{+} chromosome, the mosaic eye is largely white.

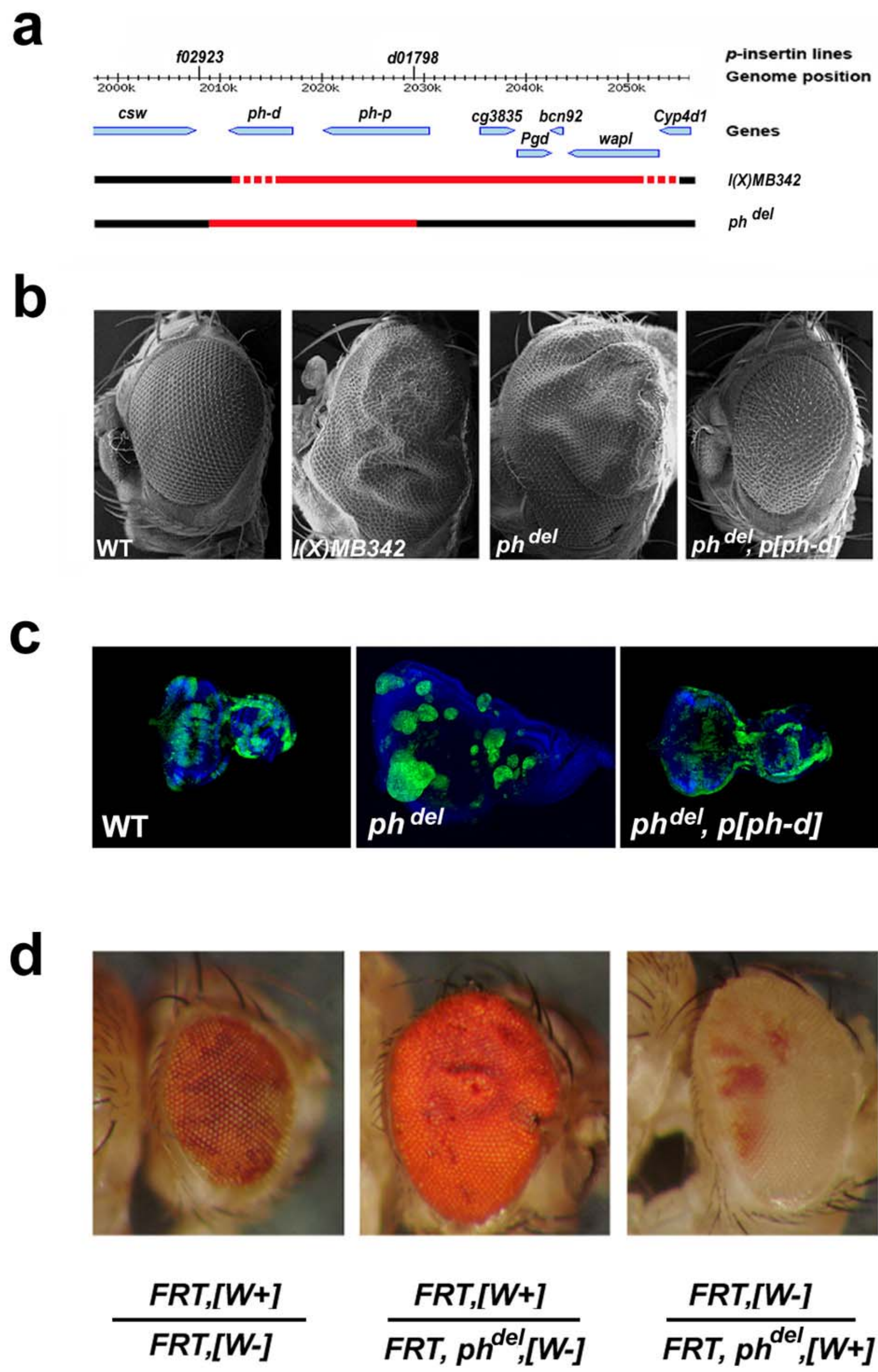


Figure 2-1

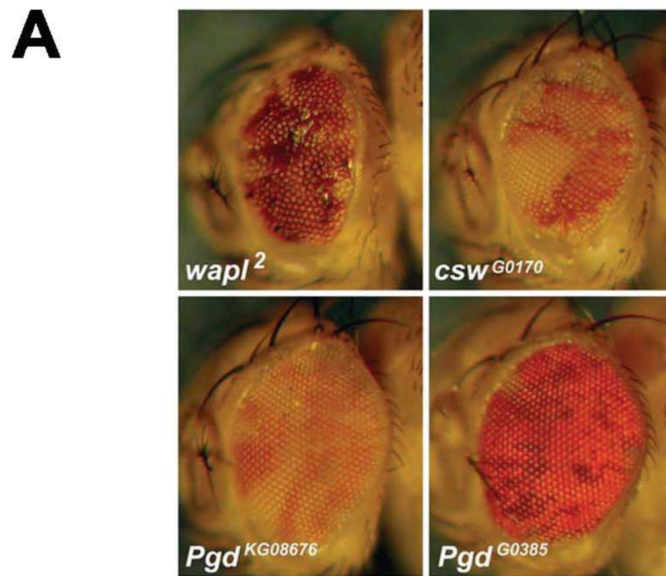
Figure 2-2. Phenotypes of eyes mosaic for genes around *ph* and various *ph* alleles

(A) Mosaic analyses using null alleles of genes other than *ph* within the *l(X)MB342* deletion region. $wapl^{2}$ mosaic eyes are smaller than wild type and have a rough surface. csw^{G0170}, $Pgd^{KG08676}$, and Pgd^{G0385} mosaic eyes are all phenotypically wild type.

(B). Allele variations of *ph* phenotypes. The known *ph* null alleles were all generated by multiple rounds of mutagenesis to inactivate both the *ph-p* and the *ph-d* genes. ph^{503} and ph^{505} were generated by EMS-treatment of $ph\text{-}d^{209}$, an EMS-induced *ph* allele (Dura et al, 1987). ph^{600} and ph^{602} were generated by X-ray treatment of $ph\text{-}d^{401}$, an X-ray induced mutant (Boivin et al, 1999). The molecular nature of ph^{503}, ph^{600}, and ph^{602} is not characterized and that of ph^{505} is shown in Figure 2-8.

Mosaic eyes of ph^{503} and ph^{505} are enlarged and abnormal, like those of *l(X)MB342* and ph^{del} (Figure 2-1B). In contrast, mosaic eyes of ph^{600} and ph^{602} are smaller than wild type.

ph^{503} and ph^{505} have similar genetic backgrounds and similar phenotypes. ph^{600} and ph^{602} also have similar genetic background and similar phenotypes. These facts suggest that the variable phenotypes of the different *ph* alleles (ph^{503} and ph^{505} *versus* ph^{600} and ph^{602}) are caused by differences in the nature of the mutations, different genetic backgrounds, or the combination of these two factors.



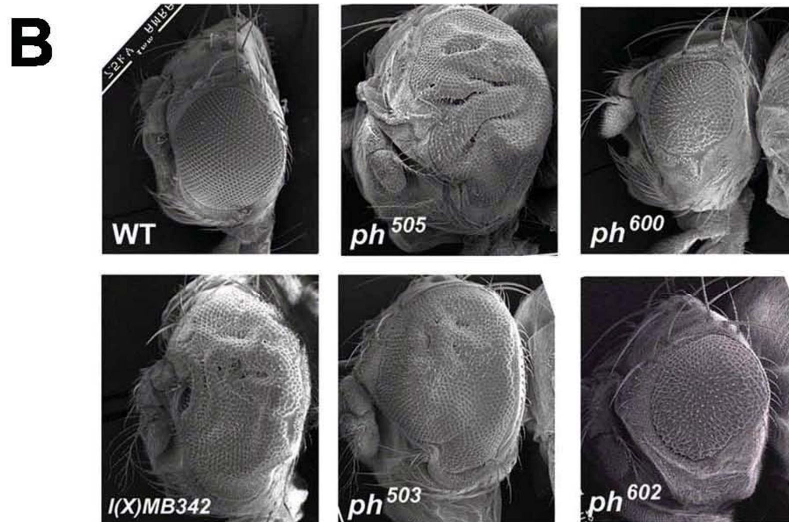


Figure 2-2

Figure 2-3. Phenotypes of *ph*^{del} and *ph*^{505}, but not *ph*^{600} mosaic eyes are rescued by a *ph-d* transgene

wild type, *ph*^{del}, *ph*^{505}, and *ph*^{600} mosaic eyes were induced by *ey-flp*. A transgene, *p*{*ph-d*$^{+}$}, which carries a 10 kb genomic DNA fragment that includes the whole *ph-d* gene, was made either heterozygous (1 copy) or homozygous (2 copies) on the 2nd chromosome of mosaic flies. In the wild type background, neither one copy nor two copies of the *p*{*ph-d*$^{+}$} transgene caused any gain-of-function phenotype in the eyes. One copy of *p*{*ph-d*$^{+}$} transgene could suppress the over-proliferation phenotype of *ph*^{del} mosaic eyes. However, the rescued *ph*^{del} mosaic eyes have a rough surface. Two copies of *p*{*ph-d*$^{+}$} transgene are necessary to fully restore normal morphology of *ph*^{del} mosaic eyes. In contrast, one copy of *p*{*ph-d*$^{+}$} transgene is sufficient to rescue all phenotypes of *ph*^{505} mosaic eyes. Surprisingly, the transgene *p*{*ph-d*$^{+}$} did not rescue the small-eye phenotype of *ph*^{600} mosaic eyes. These data suggest that the over-proliferation phenotype of *ph*^{del} and *ph*^{505} mosaic eyes is caused by loss of *ph*, but the small-eye phenotype of *ph*^{600} mosaic eyes may be caused by an additional mutation associated with this allele.

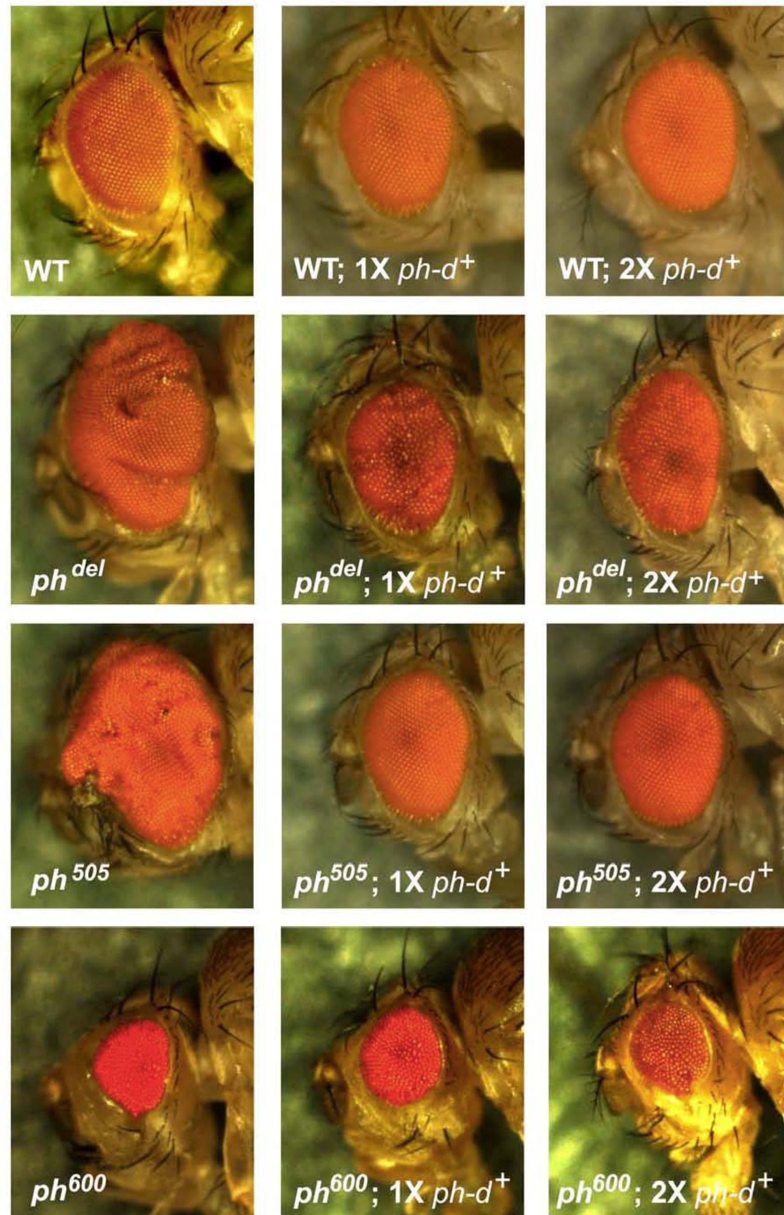


Figure 2-3

Aberrant cell proliferation, apoptosis, differentiation and morphology in *ph*^{del} mosaic discs

Cell proliferation, apoptosis, and differentiation in *ph* mosaic discs were then characterized using different molecular markers by Dr. Jianhua Huang, a former postdoc of the Wang lab. BrdU labeling and PH3 staining, which mark the S and M phases of mitotic cells, respectively, revealed that over-proliferation occurred only in the wild type cells (GFP negative) of the *ph*^{del} mosaic eye discs (Figure 2-4 and 2-5A). On the other hand, TUNEL staining showed that apoptosis was increased in *ph* mutant clones (Figure 2-4). Consistent with the fact that *ph* cells are missing from the adult ommatidia (Figure 2-1D), *ph*^{del} clones posterior to the morphogenetic furrow were found to be negative for Elav (Figure 2-4 and 2-5B), indicating that they do not differentiate normally. My advisor, Dr. Jian Wang, then tracked the fate of *ph* cells through the late developmental stages, and the results showed that some were retained in the adult brains as clusters of undifferentiated cells attached to the surface of optic lobes (Figure 2-5C).

Remarkably, *ph*^{del} clones in the mosaic eye discs formed unique single-cell layer cavities. As indicated by the apical and subapical complex marker aPKC (Wodarz et al, 2000), the apical side of *ph* cells faced the inner surface of the cavities (Figure 2-6A). *ph*^{del} clones in wing and leg discs also formed similar cavity-like structures (Figure 2-6B). aPKC antibody staining indicates that although *ph* clones form such unique three dimensional structures, individual *ph* cells still maintain their normal apical-basal cell polarity.

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Figure 2-4. ***ph^{del}*** **clones stimulate over-proliferation of surrounding wild type cells but cause autonomous apoptosis and defective differentiation.** Confocal images showing eye imaginal discs of wandering larvae containing clones of wt and *ph^{del}* (green) labeled for BrdU (red), PH3 (red), TUNEL (red), and Elav (red). DAPI was used to mark nuclei (blue). Mosaic discs of *ph^{del}* have increased BrdU and PH3 labeling throughout the discs and elevated TUNEL labeling in *ph^{del}* clones. Elav staining is absent from *ph^{del}* clones posterior to the morphogenetic furrow.

Note: Experiments related to this figure were performed by Dr. Jianhua Huang

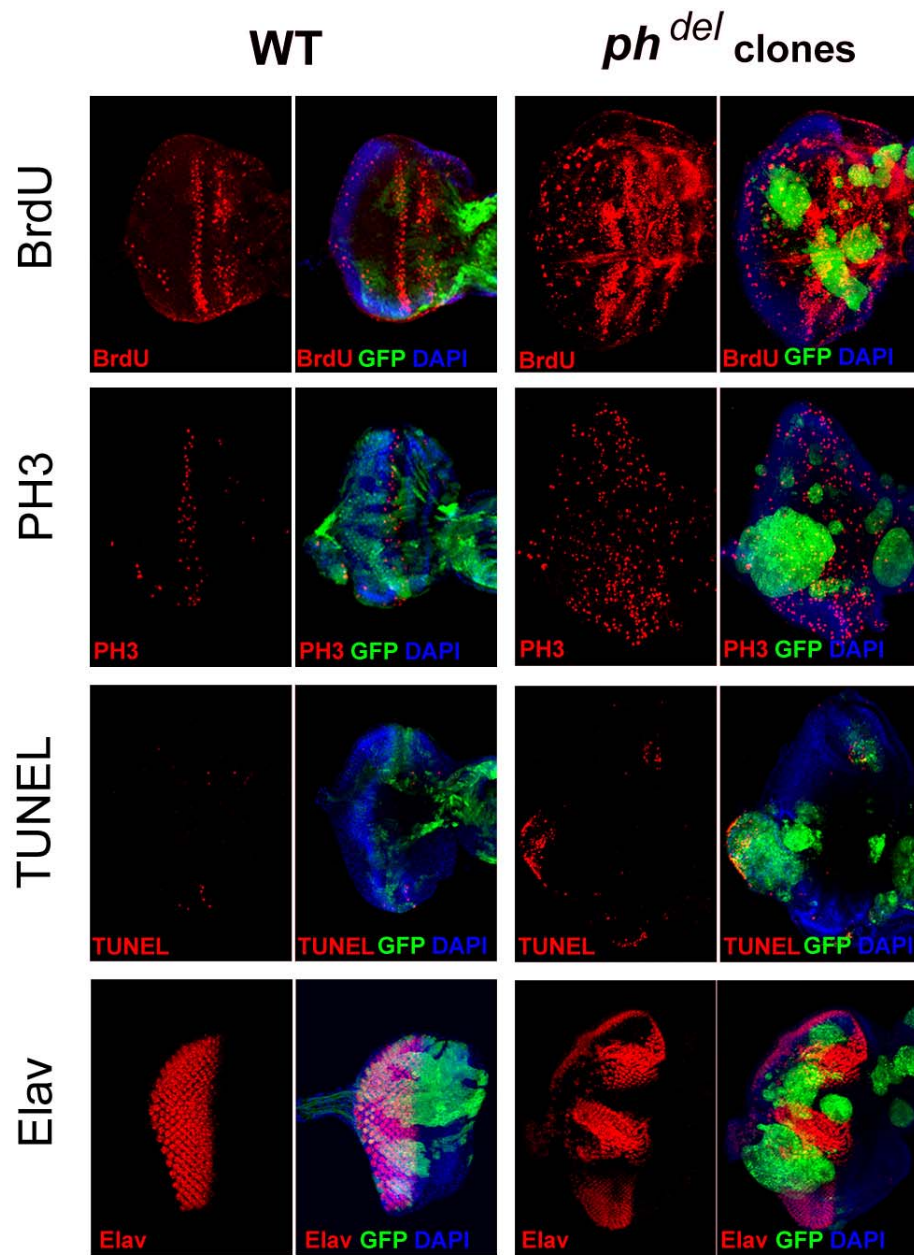


Figure 2-4

Figure 2-5. ***ph*^{del} clones induce over-proliferation of surrounding wild-type cells but *ph* cells fail to differentiate into photoreceptor neurons**
(A). A single focal plane of the confocal image showing a section through *ph*^{del} clones in mosaic eye disc. PH3 antibody staining (red) indicates that the majority of cell proliferation occurs in wild-type cells (GFP-) rather than in *ph*^{del} cells (GFP+). Notably, wild-type cells that are adjacent to *ph*^{del} clones do not show higher proliferation rate.

(B). A single focal plane of the confocal image showing a section through *ph*^{del} clones in mosaic eye disc. Elav antibody staining (red), which labels the differentiated photoreceptor neurons, indicates that *ph*^{del} cells (GFP+) fail to differentiate into photoreceptor neurons.

(C). Cell fate tracking showing that *ph*^{del} cells remain undifferentiated. Wild-type and *ph*^{del} clones were induced by *ey-flp* and were labeled with GFP. Brain-optic lobe complexes were dissected at the adult stage. In wild type mosaic eyes, some GFP+ eye-antenna cells differentiate into photoreceptor neurons, olfactory neurons, or gustatory neurons. The cell bodies of these peripheral neurons were removed during dissection. Their axons that innervate the optic lobe, antennal lobe, or subesophageal ganglion were observed. However, GFP+ axons in the optic lobe, antennal lobe, and subesophageal ganglion of in *ph*^{del} mosaic eyes were not detected. Instead, clusters of GFP+ cells attached to the surface of optic lobe and regions between the brain and optic lobe were found.

Note: (A) and (B) were performed by Dr. Jianhua Huang. (C) was performed by Dr. Jian Wang.

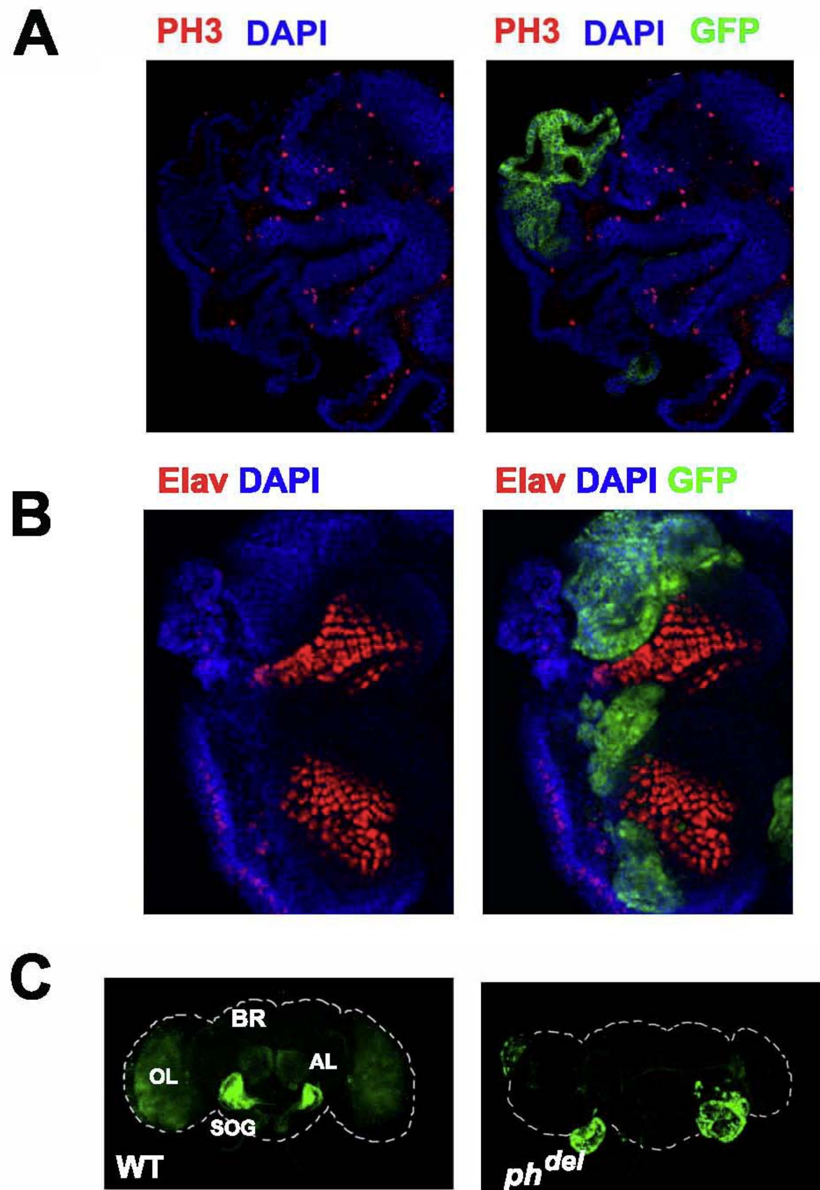


Figure 2-5

Figure 2-6. *ph*^{del} clones form cavity-like structures in mosaic imaginal discs
(A) *ph*^{del} clones form unique 3 dimensional structures in mosaic eye discs. Clones were induced by *ey-flp.* Mosaic eye discs were dissected at the wandering larval stage and stained with aPKC antibody (red) and DAPI (blue). Confocal image of a single section of *ph*^{del} mosaic eye dics shows that *ph*^{del} clones form cavity-like structures with aPKC on the inner surface.

(B) *ph*^{del} clones form similar unique 3 dimensional structures in wing and leg discs, indicating this morphology is not eye disc specific. Clones were induced by *hs-flp* at the early 2nd instar larval stage. Wing (upper panel) and leg (bottom panel) imaginal discs were dissected at the wandering larval stage and stained with aPKC antibody (red) and DAPI (blue). Confocal images show the whole discs (left) and higher magnification of the *ph*^{del} clones (right). Note the cavity-like structures with aPKC staining on the inner surface.

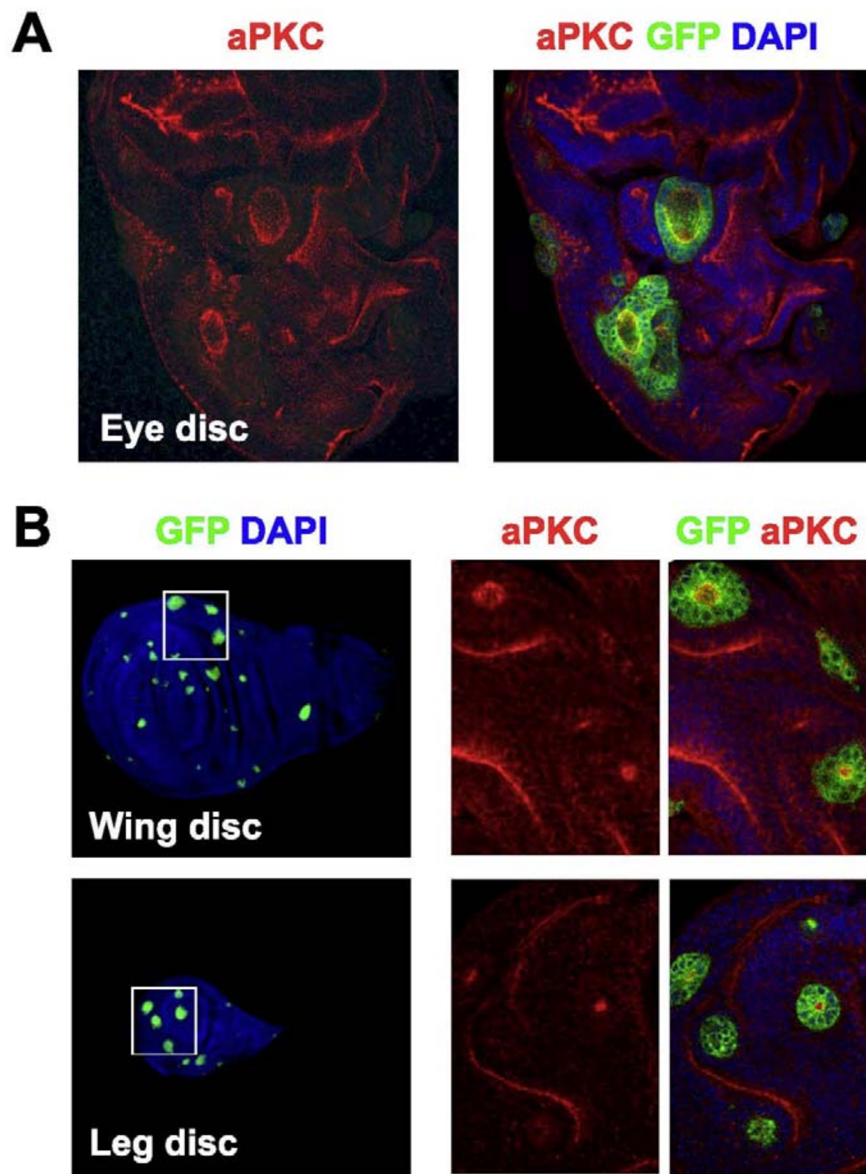


Figure 2-6

Notch is required for *ph*-induced overgrowth

In *Drosophila*, mutations in several tumor suppressor genes, such as *ept*, *vps25*, and *Uba1*, have been reported to cause non-autonomous overgrowth (Herz et al, 2006; Lee et al, 2008; Moberg et al, 2005; Thompson et al, 2005; Vaccari & Bilder, 2005). In all such cases, the Notch-Eyg-Upd-JAK/STAT pathway was reported to be involved. Notch activity is increased in the mutant clones, which induces over-expression of the ligand Upd through the transcription factor Eyg. Upd is then secreted from the mutant cells and activates the JAK/STAT pathway in neighboring cells, inducing over-proliferation. Therefore, I first investigated whether *ph* cells induced non-autonomous overgrowth through the same signaling pathway.

Genetic interaction assay and Real-Time PCR (performed by Dr. Jianhua Huang) results both suggested that the Notch-Upd-JAK/STAT signaling pathway might also play a key role in *ph*-induced non-autonomous overgrowth (Figure 2-7A). To further verify the role of Notch and Upd signaling in *ph*-induced overgrowth *in vivo,* I generated *ph-N* and *ph-upd* double mutant lines (See Methods section in Chapter 6 for details). As shown in Figure 2-8A, the size of *ph-N* mosaic eyes was comparable to that of wild type, indicating that the overgrowth phenotype of *ph* mosaic eyes is significantly, if not completely, suppressed by the loss of *Notch* from *ph* mutant clones. At the same time, I noticed that *ph-N* mosaic eyes were still largely red. Since the wild type chromosome was w^+ and the *ph-N* chromosome was w^-, this result indicates that *ph-N* cells were also missing from the mosaic eyes (Figure 2-8A). To rule out the possibility that *ph-Notch* double mutant cells died prior to the induction of over-proliferation, *ph-N* clones in

mosaic eye discs at the wandering larval stage were examined by Dr. Jianhua Huang. *ph-N* clones were viable and morphologically identical to *ph* mutant clones, but the overall size of *ph-N* mosaic discs was significantly smaller than that of *ph* mosaic discs (Figure 2-8A). Moreover, PH3 staining showed that cell proliferation of *ph-N* mosaic discs was reduced to the wild type level (compare Figure 2-8A to Figure 4), while TUNEL staining showed that the *ph-N* cells still underwent higher rates of apoptosis (Figure 2-8A). These results demonstrate that Notch activity is required for *ph*-induced non-autonomous over-proliferation, but not for the *ph*-induced autonomous increase in apoptosis and defective differentiation. Moreover, the cavity-like morphology was retained in *ph-N* clones.

Figure 2-7. Genetic interaction experiments suggest that Notch-Upd pathways may be involved in *ph*-induced non-autonomous over-proliferation

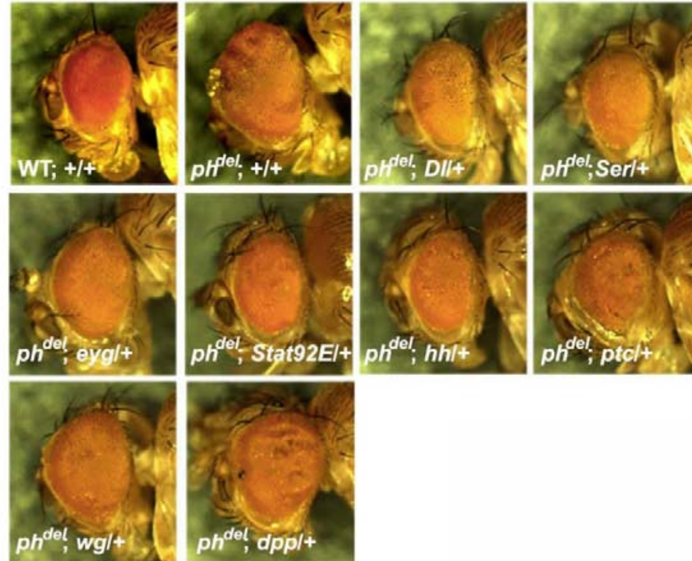
(A) The possible roles of candidate genes on ph^{del} induced over-proliferation phenotype were tested by genetic interaction. The rationale is that heterozygosity of a component of the involved pathway would suppress the over-proliferation phenotype. I discovered that the over-proliferation phenotype of ph^{del} mosaic eyes was suppressed by removing one copy of *Dl*, *Ser*, *eyg*, *Stat92E*, *hh*, or *ptc*, but it was not suppressed by removing one copy of *wg* or *dpp*. These results suggest that the Notch-Eyg-Upd homologs-JAK/STAT and HH-Ptc pathways may be involved in *ph*-induced non-autonomous over-proliferation.

(B) To verify the involvement of Notch-Eyg-Upd homologs-JAK/STAT and Hh-Ptc pathways in *ph*-induced non-autonomous over-proliferation, Real-Time PCR was conducted to measure the expression of genes related to these two signaling pathways in ph^{del} mosaic eye discs. There were no significant changes in the expression levels of *hh* and *ptc*, but increased expression of genes related to Notch-Upd-JAK/STAT signaling pathway, including *eyg*, *Stat92E*, *N*, *upd*, *upd2*, and *upd3,* was observed.

To study the discrepancy between genetic interaction and real-time PCR results, I used MARCM to drive the expression of *hh*-RNAi or *ptc*-RNAi in ph^{del} mutant clones. The resulting eyes were indistinguishable from ph^{del} mosaic eyes (data not shown). Therefore, I only consider that the Notch-Upd-JAK/STAT signaling pathway, but not the hedgehog pathway.

Note: Real-Time PCR was performed by Dr. Jianhua Huang

A



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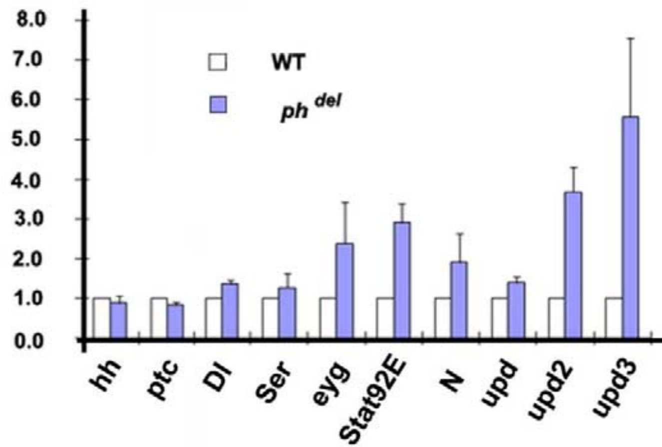

Figure 2-7

Figure 2-8. Notch and Upd homologs are involved in *ph*-induced non-autonomous cell over-proliferation

(**A**) In mosaic eyes, the removal of *Notch* from *ph* mutant clones (ph^{del}-*N* double mutant) totally suppressed the eye overgrowth phenotype. Concomitantly, increased PH3 labeling in the *ph* mosaic eye discs is also eliminated. However, the autonomous apoptosis phenotype and cavity-like structure of *ph* clones are retained. (**B**) Removal of *upd* from *ph* mutant clones (ph^{del}-*upd* double mutant) alone does not suppress either the eye overgrowth phenotype or increased PH3 labeling in *ph* mosaic eye discs. However, removal of all three *upd* homologs from *ph* mutant clones (ph^{del}-$upd^{d1\text{-}3}$ double mutant) has the same effect as ph^{del}-*N* double mutant. (**C**) Elevated levels of Notch protein are detected in the inner surface of ph^{del} clones and massive amounts of Upd protein are detected within the lumen of ph^{del} clones.

Note: PH3 staining and TUNEL staining were performed by Dr. Jianhua Huang.

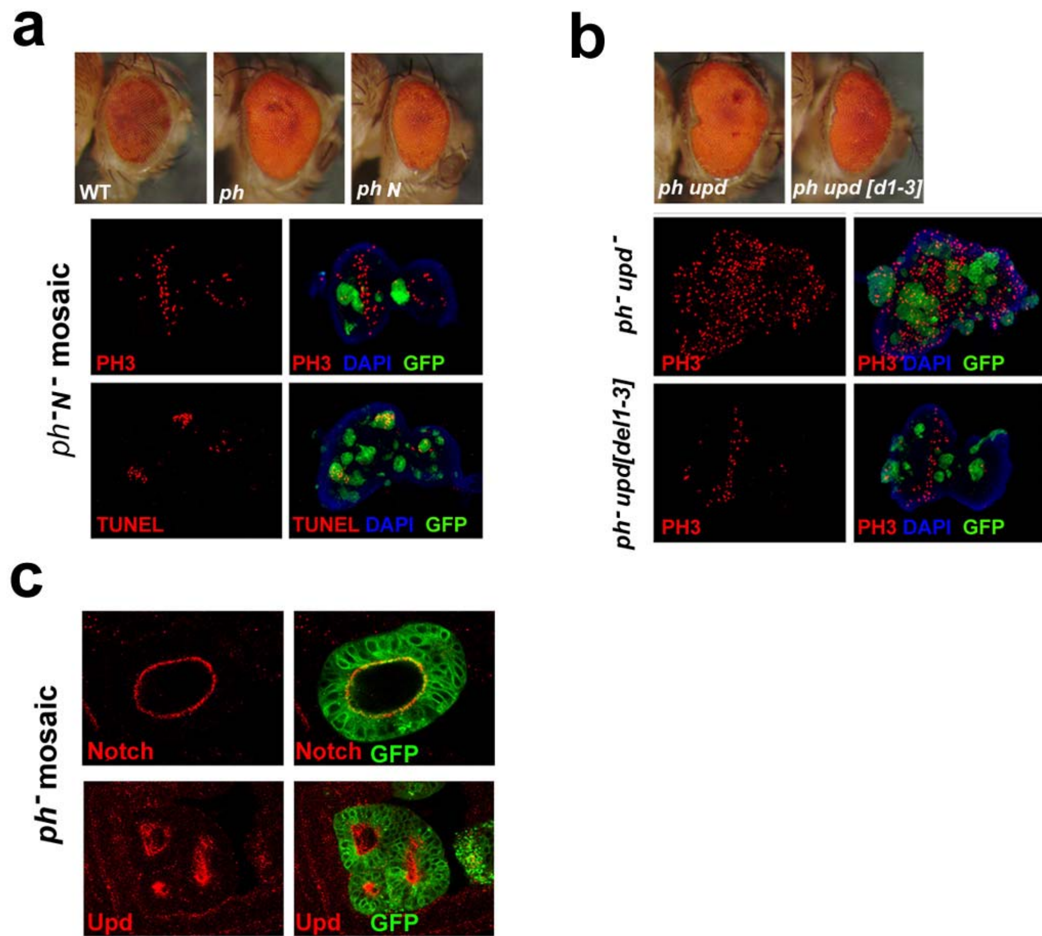

Figure 2-8

Three Upd homologs are functionally redundant in mediating *ph*-induced overgrowth

Next, I examined the mosaic eye phenotypes of *ph-upd* double mutant. Unexpectedly, *ph-upd* mosaic eyes were phenotypically indistinguishable from *ph* mosaic eyes (Figure 2-8B). Thus, removal of *upd* from *ph* clones is not sufficient to block *ph*-induced non-autonomous overgrowth. These results at first appear to be inconsistent with our genetic interaction data that suggest involvement of the JAK/STAT pathway (Figure 2-7A). In the *Drosophila* genome, however, there are three genes that encode potential JAK/STAT pathway ligands, *upd*, *upd2* and *upd3*, which form a cluster (Agaisse et al, 2003; Gilbert et al, 2005; Harrison et al, 1998; Hombría et al, 2005). I therefore postulated that these three *upd* homologs might contribute redundantly to *ph*-induced overgrowth. To test this hypothesis, I created a deficiency line, $upd^{d1\text{-}3}$, which deletes the genomic region from X:18133021 to X:18206733 that includes genes of all three JAK/STAT ligands. I then generated a ph^{del}-$upd^{d1\text{-}3}$ double mutant line. ph^{del}-$upd^{d1\text{-}3}$ mosaic eyes were significantly smaller than *ph* mosaic eyes (Figure 2-8B), and the PH3 signal (stained by Dr. Jianhua Huang) in *ph*-$upd^{d1\text{-}3}$ mosaic discs was similar to that of wild type discs (Figure 2-8B). These observations indicate that the three Upd homologs indeed act redundantly in *ph*-induced overgrowth.

Finally, I asked whether the Notch and Upd proteins were increased in ph^{del} mosaic eye-imaginal discs. As seen in Figure 2-8C, I observed that Notch protein accumulated strongly on the inner surface of *ph*-induced cavity-like structures (i.e., the apical domain of *ph* cells). Meanwhile, the Upd protein was secreted into the lumen of the cavities.

Chapter 3: Different *ph* alleles cause over-proliferation in different patterns

Abstract

ph^{505} had long been believed to be a *ph* null allele, based on classic genetic analyses. However, sequencing results and functional data demonstrated that it is actually a hypomorphic allele. Interestingly, although *ph*^{del}, the true *ph* null allele, and *ph*^{505}, a hypomorphic *ph* allele, both cause mosaic tissue over-proliferation, the patterns of such over-proliferation are different. While *ph*^{del} only causes non-autonomous over-proliferation, *ph*^{505} causes both autonomous and non-autonomous proliferation. Further characterization showed that the same signaling pathway is involved in non-autonomous over-proliferation induced by both alleles.

ph^{505} induces both autonomous and non-autonomous cell over-proliferation

Based on their studies using *ph^{505}*, a long-accepted *ph* null allele, Martinez and colleagues (Martinez et al, 2009) recently reported that loss of *ph* induces cell-autonomous overgrowth in mosaic eye discs, which obviously conflicts with my results. Therefore, I conducted a series of comparative studies on the phenotypes of *ph^{del}* and *ph^{505}* mosaic eyes. On one hand, I found that *ph^{del}* is phenotypically different from *ph^{505}*. For example, *ph^{505}* clones in mosaic eye discs did over-proliferate cell-autonomously, but *ph^{del}* clones did not (Figure 3-1A compared to Figure 2-1C). Moreover, *ph^{del}* clones formed single cell layer cavities (Figure 2-6), but *ph^{505}* clones did not. On the other hand, I also found that *ph^{del}* and *ph^{505}* mosaic eyes were common in other aspects. The overgrowth phenotypes of both *ph^{del}* and *ph^{505}* were fully suppressed by the *p{ph-d^+}* transgene (Figure 3-1A and 2-3), suggesting that they are both caused by loss of Ph. In addition, like *ph^{del}* mosaic eyes, the enlarged *ph^{505}* mosaic adult eyes were mainly composed of wild type cells. As shown in Figure 3-1B, when *ph^{505}* was associated with a *w^-* chromosome, the mosaic eye was largely red. Conversely, when *ph^{505}* was associated with a *w^+* chromosome, the mosaic eye was largely white. Elav antibody staining (by Dr. Jianhua Huang) indicated that *ph^{505}* cells did not differentiate into photoreceptor neurons (Figure 3-1C). Therefore, I infer that to form the enlarged adult eyes, wild-type cells within *ph^{505}* mosaic eyes must also over-proliferate. In short, *ph^{del}* and *ph^{505}* mosaic eyes both overgrow, but *ph^{del}* induces non-autonomous cell over-proliferation, whereas *ph^{505}* induces both

non-autonomous and autonomous cell over-proliferation. Nevertheless, for both alleles, the adult ommatidia are composed of wild type cells only.

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Figure 3-1. ***ph^{505}* clones in mosaic eyes induce non-autonomous cell over-proliferation**

(**A**) Phenotypes of *ph^{del}* and *ph^{505}* mosaic discs. Wt, *ph^{505}*, and *ph^{del}* mosaic eye discs dissected from wandering larvae were stained with DAPI (blue). Homozygous mosaic clones are labeled with GFP (green). Both *ph^{505}* and *ph^{del}* mosaic eye discs are larger than that of wt, but *ph^{505}* cells have over-proliferated and occupy a major portion of the eye disc, while *ph^{del}* cells occupy a small portion of the disc. However, the overgrowth phenotype of both *ph^{505}* and *ph^{del}* mosaic eye discs can be suppressed by the *p*{*ph-d*$^{+}$} transgene. (**B**) Like *ph^{del}* mosaic eyes (Figure 2-1D), the adult *ph^{505}* mosaic eyes are mainly composed of wt cells. When *ph^{505}* is associated with a w^{-} chromosome, the mosaic eye is largely red. Conversely, when *ph^{505}* is associated with a w^{+} chromosome, the mosaic eye is largely white. (**C**) *ph^{505}* cells do not differentiate normally. *ph^{505}* mosaic eye discs of wandering larval stage were stained with Elav antibody (red). *ph^{505}* cells (GFP-positive) are Elav negative, indicating they do not differentiate into photoreceptor neurons.

Note: Elav staining was performed by Dr. Jianhua Huang

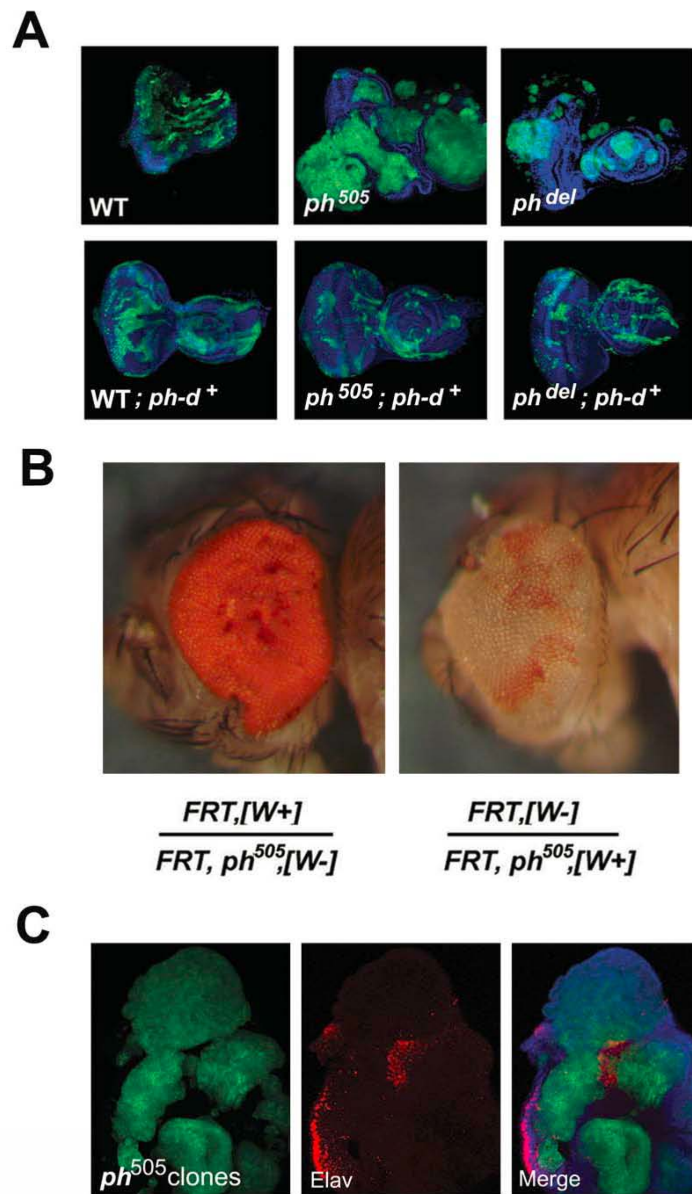

Figure 3-1

ph^{505} is not a completely null allele

In an attempt to clarify why the two *ph* alleles caused different phenotypes, I first compared the nature of the mutations in *ph*^{del} and *ph*^{505} at the molecular level. *ph*^{505} is a mutation created by two-round of EMS treatment (Dura et al, 1987). Therefore the genomic region of *ph*^{505} that contains *ph-d* and *ph-p* genes was sequenced (together with Dr. Jian Wang). The results showed that this *ph* allele carried two non-sense mutations in Q^{398} of *ph-d* and Q^{749} of *ph-p* (Figure 3-2A). On the other hand, DNA sequencing also verified that *ph*^{del} was a deficiency line that uncovered all exons of *ph-d* and *ph-p* except for the first exon of *ph-p*, which only encodes 12 amino acids (Figure 3-2A).

Clearly, *ph*^{del} is a null allele. However, the truncated Ph proteins encoded by *ph*^{505} may retain partial Ph function. To test this possibility, I examined the lethal phase of *ph*^{505} and *ph*^{del}. Both *ph*^{505} and *ph*^{del} mutants died in the embryonic stage, but *ph*^{505} mutants died later than *ph*^{del} mutants (Figure 3-2B). Moreover, I also noticed that two copies of *p*{*ph-d*$^{+}$} transgene were required to fully rescue the adult viability and mosaic eye phenotypes of *ph*^{del}, but one copy of the same transgene was sufficient to rescue these phenotypes of *ph*^{505} (Figure 2-5). Taken together, I conclude that *ph*^{del} is a *ph* null allele, but *ph*^{505} is not. This difference at the molecular level may contribute to the different cellular phenotypes of these two *ph* alleles.

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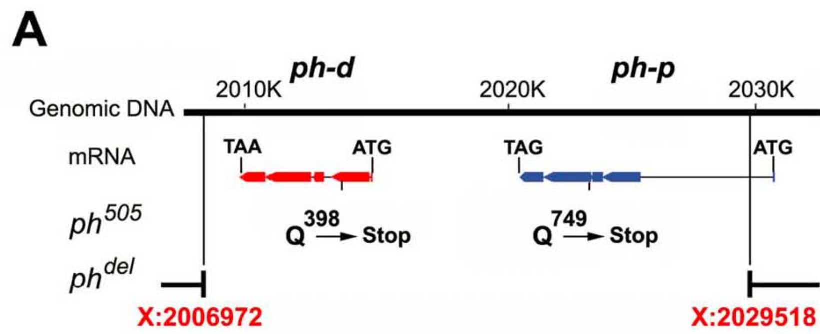
Figure 3-2. *ph*^{505} is not a completely null allele

(A) Nature of mutation of *ph*^{505} and *ph*^{del}. DNA sequencing data revealed that *ph*^{505} carried non-sense mutations in Q^{398} of *ph-d* and Q^{749} of *ph-p*. The breakpoints of the *ph*^{del} deficiency line, X:2006972 and X:2029518, were also verified by DNA sequencing. All exons of *ph-d* and *ph-p* except for the first exon of *ph-p*, which only encodes 12 amino acids, were deleted in *ph*^{del}. **(B)** Phenotypic analyses of *ph*^{del} and *ph*^{505}embryos. Both *ph*^{del}/Y and *ph*^{505}/Y hemizygous males are embryonic lethal. All *ph*^{del}/Y embryos die at a similar stage soon after the germ band retraction initiation. These embryos also show defects in dorsal closure. *ph*^{505}/Y embryos die after germ band retraction has finished, with many of them able to develop clear grooves between segments.

Note: *ph*^{505} sequencing was performed together with Dr. Jian Wang.



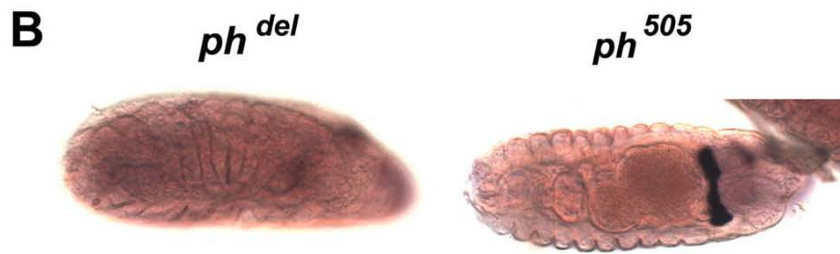


Figure 3-2

The same pathway accounts for *ph*^{505} induced non-autonomous over-proliferation, and is also required for *ph*^{505} induced autonomous over-proliferation

I next addressed why a *ph* null allele and a *ph* hypomorphic allele both caused tumors but in such different ways. For this purpose, I tested whether the same signaling pathway underlay non-autonomous over-proliferation induced by both *ph*^{del} and *ph*^{505}. The functions of Notch and Upd homologs in the *ph*^{505} mosaic eyes were examined with the same strategy used for *ph*^{del}. A *ph*^{505}-*Notch* double mutant line was generated by meiotic recombination. The mosaic eyes of this double mutant line were essentially of the same size as wild type eyes (Figure 3-3, D *vs.* F). The mosaic eye discs had normal size and normal cell proliferation level, as shown by PH3 staining, which marks mitotic cells (Figure 3-3, H *vs.* J). Moreover, the size of *ph*^{505}-*Notch* clones was significantly reduced when compared to *ph*^{505} clones (Figure 3-3, I *vs.* J). These results showed that when *Notch* was removed from *ph*^{505} clones, not only non-autonomous over-proliferation but also autonomous over-proliferation were suppressed.

I next recombined *ph*^{505} with *upd*$^{d1\text{-}3}$, a deficiency line that lacks all three *upd* homologs in the *Drosophila* genome (Feng et al, 2011a). Mosaic analyses were then performed using this double mutant line. *ph*^{505}-*upd*$^{d1\text{-}3}$ mosaic eyes were significantly smaller than *ph*^{505} mosaic eyes and were comparable to wild type eyes (Figure 3-3, D, E and G), indicating that tissue overgrowth was largely suppressed. PH3 staining of the double mutant mosaic eye discs showed that these discs had relatively normal size and cell proliferation level (Figure 3-3, H *vs.* K). Most importantly, *ph*^{505}-*upd*$^{d1\text{-}3}$ clones were also drastically reduced in size

(Figure 3-3, I *vs.* K). These results demonstrated that Upd homologs are required for both non-autonomous and autonomous cell over-proliferations induced by *ph*^{505}.

Figure 3-3. *Notch* and *upd* homologs are required for both autonomous and non-autonomous over-proliferation induced by ph^{505}.

(A-C) ph^{del}, a *ph* null allele, only induced non-autonomous over-proliferation, while ph^{505}, a *ph* hypomorphic allele, induced both autonomous and non-autonomous over-proliferation. Mosaic eye discs of wild type allele (A), ph^{del} (B) and ph^{505} (C) were analyzed. *ey-flp* was used to induce mosaics, and mutant cells were positively labeled by GFP (green) using MARCM (Lee & Luo, 1999). DNA was stained by DAPI (blue). (D-G) The removal of *Notch* or all three *upd* homologs from ph^{505} cells suppressed the enlarged eye phenotype induced by ph^{505}. Adult eyes mosaic for wild type allele (D), ph^{505} (E), ph^{505}-*Notch* (F) and ph^{505}-$upd^{d1\text{-}3}$ (G) were analyzed. *ey-flp* was used to induce mosaics. $upd^{d1\text{-}3}$ is a deletion that lacks all three *upd* homologs (Feng et al, 2011a). To remove *Notch* or all three *upd* homologs specifically from ph^{505} cells in mosaic eyes, ph^{505}-*Notch* and ph^{505}-$upd^{d1\text{-}3}$ double mutant lines were generated and were used to perform mosaic analyses (F and G). (H to K) *Notch* and *upd* homologs are required for not only non-autonomous but also autonomous over-proliferation induced by ph^{505}. Eye discs mosaic for wild type allele (H), ph^{505} (I), ph^{505}-*Notch* (J) and ph^{505}-$upd^{d1\text{-}3}$ (K) were stained with PH3 (red), a mitotic marker. *ey-flp* was used to induce mosaics, andmutant cells were positively labeled by GFP (green). DNA was stained with DAPI (blue). Note that when *Notch* or all three *upd* homologs were removed from ph^{505} cells, both non-autonomous and autonomous over-proliferation was suppressed.

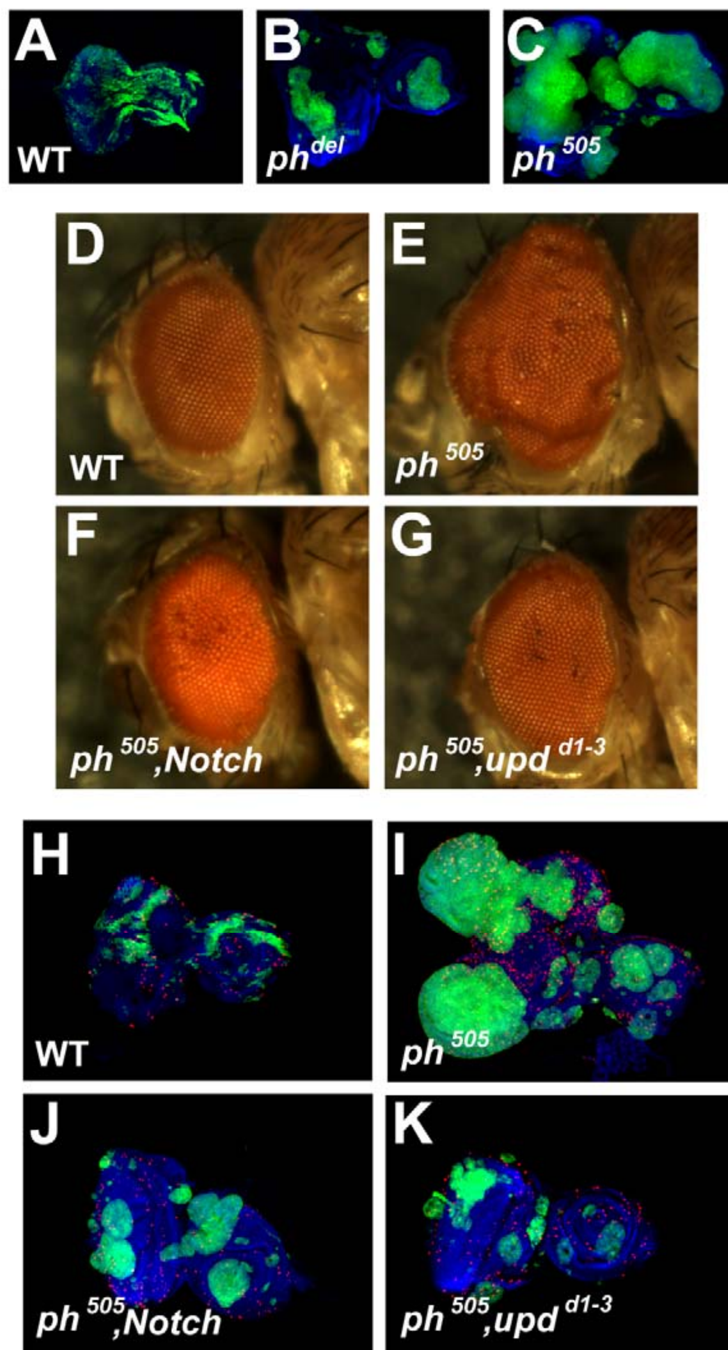


Figure 3-3

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Chapter 4: Cells mutant for different *ph* alleles differentially express *dome* and respond differently to Upd ligands

Abstract

In *ph*^{505} mosaic tissues, when the three upd homologs were specifically removed from the mutant cells, not only non-autonomous over-proliferation, but also autonomous over-proliferation, was suppressed. Based on this observation, it was hypothesized that *ph*^{505} cells still respond to the ligands they secreted and over-proliferate. On the other hand, *ph*^{del} cells are no longer responsive to these ligands and therefore do not show over-proliferation. Functional results supported this hypothesis, thus providing an explanation on why different *ph* alleles show different patterns of over-proliferation. Furthermore, TU-tagging, a technique that allows the purification of RNA specifically from the mutant cells in mosaic tissues without having to physically isolate the mutant cells, was used in combination with Real-Time PCR, to determine the expression level of JAK/STAT pathway core components and regulators. The results showed that the pathway receptor *domeless* was expressed at a higher level in *ph*^{505} cells than in *ph*^{del} cells, which might explain why *ph*^{505} cells still respond to the Upd ligands. In addition, two JAK/STAT pathway negative regulators have higher expression levels in *ph*^{505} cells than in *ph*^{del} cells, which may represent negative feedback loops, which are common in the JAK/STAT pathway.

ph^{505} cells still respond to Upd ligand, but *ph^{del}* cells do not

I have concluded that *ph^{del}* and *ph^{505}* both induce non-autonomous cell over-proliferation through the same Notch-Upd homologs signaling pathway. Surprisingly, this pathway is also required for *ph^{505}*-induced autonomous cell over-proliferation, as autonomous over-proliferation was suppressed in *ph^{505}*-*Notch* and *ph^{505}*-*$upd^{d1\text{-}3}$* double mutant mosaics (Figure 3-3 H-K). In the case of *ph^{505}*- *Notch* mosaics, this is not completely unexpected, because Notch is a transcription factor that could affect both autonomous and non-autonomous cell proliferation through regulating different downstream targets (Artavanis-Tsakonas & Muskavitch, 2010). But in the case of *ph^{505}*-*upd^{123}* mosaics, the three Upd proteins are secreted factors (Agaisse et al, 2003; Gilbert et al, 2005; Harrison et al, 1998; Hombría et al, 2005) and are not expected to have any direct effect on autonomous cell proliferation. To interpret these observations, I hypothesized that *ph^{505}* mutant cells still responded to Upd ligands secreted by themselves in an autocrine or paracrine manner, and therefore over-proliferated. On the other hand, *ph^{del}* cells were no longer responsive to Upd ligands.

To functionally test this hypothesis, I again applied the double mutant strategy, taking advantage of the fact that the genes *domeless* (*dome*, the only membrane receptor of the *Drosophila* JAK/STAT pathway (Brown et al, 2001)) and *hopscotch* (*hop*, the only *Drosophila* JAK gene (Binari & Perrimon, 1994)) are also on X chromosome as is *ph*. I first recombined *ph^{505}* with two *dome* alleles to generate *ph^{505}*-*dome* double mutant lines. Eye discs mosaic for these double mutant lines were still significantly larger than wild type, but the size of double

mutant clones was dramatically reduced, so that only a tiny portion of the disc was composed of mutant cells. PH3 staining indicated that non-autonomous proliferation level was still high, but autonomous proliferation was largely disappeared (Figure 4-1, A, B). I further examined the adult eyes mosaic for such double mutant lines and found that these eyes were still much larger than wild type, but they generally were not folded as seen in *ph*^{505} mosaic eyes (Figure 4-1, H, J and K).

Next I generated a *ph*^{505}-*hop* double mutant line. I found that autonomous proliferation in the mosaic eye discs of this double mutant was also significantly suppressed, with mutant cells only accounted for a small portion of the whole disc. On the other hand, non-autonomous cell over-proliferation was not affected and the overall size of these discs was still significantly larger than wild type (Figure 4-1, C). Adult eyes mosaic for this double mutant showed similar phenotypes as those of *ph*^{505}-*dome* double mutant mosaic eyes. These eyes were still significantly larger than wild type but they were generally not folded (Figure 4-1, L). Therefore, the removal of either *dome* or *hop* from *ph*^{505} cells only suppressed autonomous over-proliferation and did not affect non-autonomous over-proliferation, causing such double mutant mosaic discs phenotypically similar to *ph*^{del} mosaic discs.

As controls, *ph*^{del}-*dome* and *ph*^{del}-*hop* double mutant lines were also generated using the same *dome* and *hop* alleles. Mosaic analyses on eye discs showed

that the removal of *dome* or *hop* from *ph*^{del} mutant cells did not affect non-autonomous cell over proliferation. It did, however, mildly reduce the mutant clone size (Figure 4-1, D-F), suggesting that the mutant cells might still have a weak response to Upd ligands. Adult eyes mosaic for these double mutant lines were phenotypically indistinguishable from *ph*^{del} mutant eyes (Figure 4-1, I and M-O), consistent with the above observations in mosaic eye discs.

Figure 4-1. – JAK/STAT pathway is involved in autonomous over-proliferation induced by *ph*^{505}.

(A to C) When JAK/STAT pathway components *dome* or *hop* was removed from *ph*^{505} cells, autonomous over-proliferation was completely suppressed, but non-autonomous over-proliferation was unaffected. Mosaic eye discs of wild type allele (A), *ph*^{505}*-dome* (B) and *ph*^{505}*-hop* (C) were stained with PH3 (red), which marks cells in M phase of mitosis. *ey-flp* was used to induce mosaics, and mutant cells were labeled by GFP (green), and DNA was stained with DAPI (blue). To remove *dome* or *hop* from *ph*^{505} cells, *ph*^{505}*-dome* and *ph*^{505}*-hop* double mutant lines were generated and were used for mosaic analyses. (D to F) Eye discs mosaic for wild type allele (D), *ph*^{del} *-dome* (E) and *ph*^{del} *-hop* (F) were stained with PH3 (red) as controls. *ey-flp* was used to induce mosaics, and mutant cells were labeled by GFP (green), and DNA was stained with DAPI (blue). (G to O) When JAK/STAT pathway components *dome* or *hop* was removed from *ph*^{505} or *ph*^{del} cells in mosaic eyes, the eyes were still much larger than wild type. Adult eyes mosaic for wild type allele (G), *ph*^{505} (H), *ph*^{del} (I),two *ph*^{505}*-dome* double mutant lines with different *dome* alleles (J and K), *ph*^{505}*-hop* (L), two *ph*^{del} *-dome* double mutant lines with different *dome* alleles (M and N) and *ph*^{del} *-hop* (O) were analyzed. *ey-flp* was used to induce mosaics.

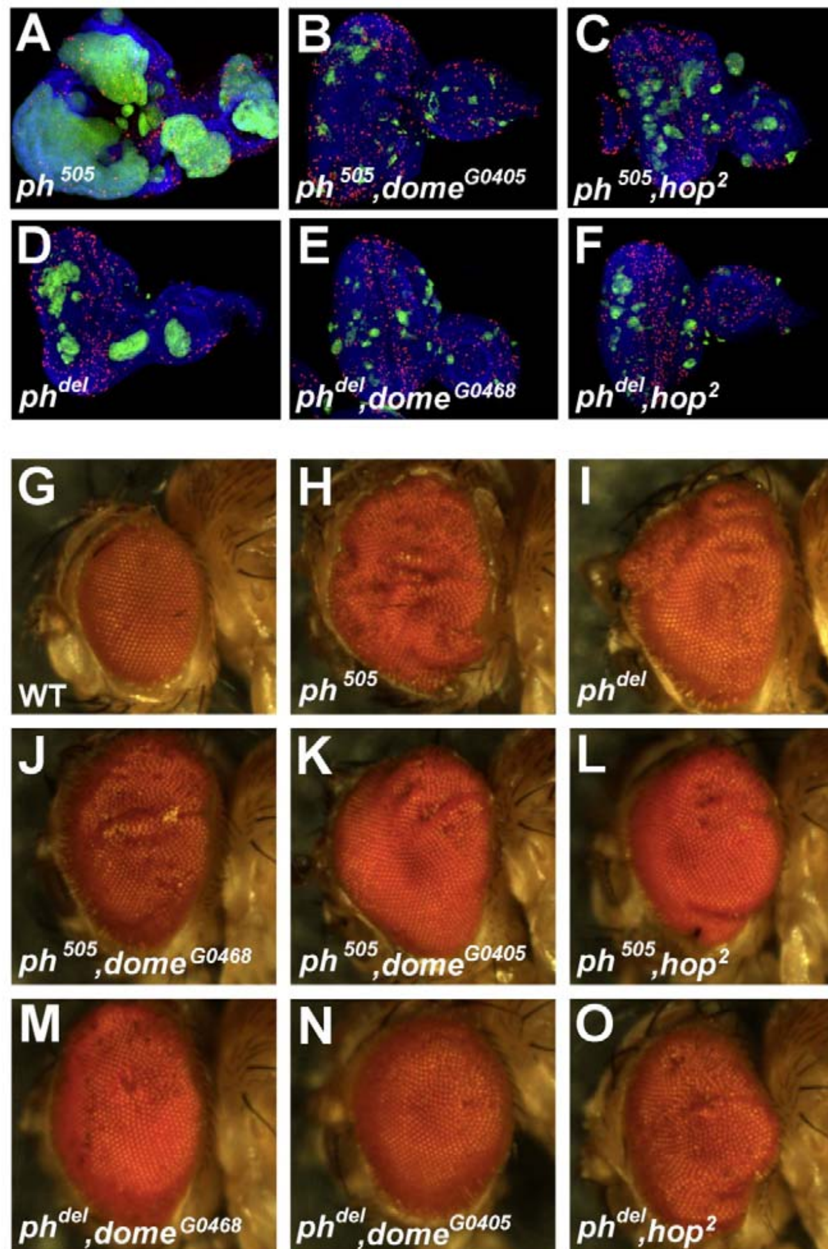

Figure 4-1

Higher level of *dome* may enable ph^{505} cells to respond to Upd ligands

Finally I asked why ph^{del} and ph^{505} cells responds differently to the Upd ligands secreted by themselves. I hypothesized that some of the JAK/STAT pathway modulators might be differentially expressed in ph^{del} and ph^{505} cells. To test this hypothesis, I chose TU-Tagging, a technique that enables the purification of RNA from mutant cells without having to physically isolate such cells (Miller et al, 2009). Briefly, *Drosophila* is unable to synthesize uridine from uracil due to the lack of phosphoribosyltransferase (UPRT). When exogenous UPRT is expressed in mutant cells by MARCM, such cells would acquire the ability to utilize uracil. If these larvae are fed with 4-thiouracil (4-TU), a uracil derivative that contains a thio group, only mutant cells would be able to use 4-TU and eventually incorporate thio- containing uridine into newly synthesized RNA. This treatment has little toxicity, and the thio-labeled RNA can be purified from total RNA using conventional biochemical methods.

I performed TU-tagging to isolate RNA from ph^{del} cells and ph^{505} cells, and used qRT-PCR to examine candidate gene expression (Figure 4-2). The expression of the JAK/STAT pathway receptor *dome* was significantly higher in ph^{505} cells than in ph^{del} cells. A higher receptor expression might sensitize ph^{505} cells to the Upd ligands. The levels of *enok* and *socs42a*, both negative regulators of the JAK/STAT pathway, were also significantly higher in ph^{505} cells compared to ph^{del} cells. This might represent feedback loops that negatively regulate the pathway activity. In fact, several such negative feedback loops, in which elevated pathway

activity upregulates a negative pathway regulator, have been reported in the JAK/STAT pathway (Arbouzova & Zeidler, 2006).

Figure 4-2. Molecular mechanism underlying different responses to Upd ligands of *ph*^{del} and *ph*^{505} cells.

Real-Time PCR revealed differential expression of JAK/STAT pathway components and major regulators. UPRT and GFP were expressed in mutant cells by MARCM with *ey-flp*. Mid-3rd instar Larvae were fed with 4-TU for 10 to 12 hours. Total RNA was extracted from eye discs, labeled and purified according to the published protocols (Miller et al, 2009). Real-Time PCR was performed using purified TU-labeled RNA. Total RNA was also used as the template for Real-Time PCR with primers for *tubulin* and GFP. GFP to *tubulin* ratio was 3 to 6 times higher in purified TU-labeled RNA than in total RNA, indicating that the TU-tagging strategy successfully enriched RNA from the mutant cells.

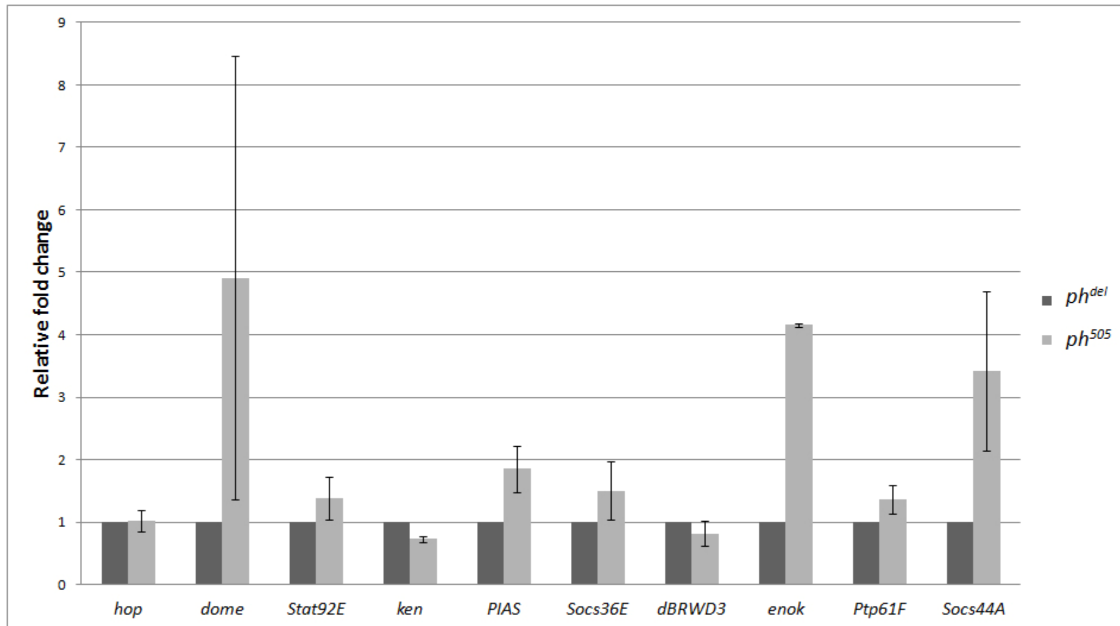

Figure 4-2

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Chapter 5: Conclusions and scientific impacts

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Loss of *ph* causes non-autonomous cell over-proliferation

Drosophila Polycomb group locus *polyhomeotic (ph)* is a complex one, with two duplicated genes that are functionally redundant (Dura et al, 1987). Therefore a loss of function allele can only be obtained by two rounds of independent mutagenesis. All alleles generated this way were not molecularly characterized before my dissertation research, so their molecular nature was not clear. In addition, such alleles are also expected to have many background mutations generated during mutagenesis, which complicates functional analyses. My dissertation research generated the first molecularly defined clean *ph* null allele in *Drosophila*, and also proved that ph^{505} (Dura et al, 1987), one of the "null" alleles generated by two rounds of EMS mutagenesis and a long used classic *ph* "null" allele, was in fact a hypomorph (Feng et al, 2011a).

Using ph^{del}, the true *ph* null allele, I demonstrated that eye discs mosaic for this allele showed dramatic tissue overgrowth. Surprisingly, ph^{del} clones induce over-proliferation in a non-autonomous manner (Feng et al, 2011a). This means that the mutant cells themselves do not over-proliferate, but instead they induce over-proliferation of surrounding cells. This is interesting because mutations at most known tumor suppressors in *Drosophila* cause autonomous over-proliferation, with only a few exceptions. Therefore my dissertation research added a new member to the list of *Drosophila* non-autonomous tumor suppressors.

With the help of Dr. Jianhua Huang, my dissertation research also demonstrated that ph^{del} cells in mosaic eye discs fail to differentiate normally and are eventually eliminated by apoptosis. My data showed that the apoptosis is not induced by

cell competition. Instead, even in the absence of more competitive cells, the *ph*^{del} cells still die (Feng et al, 2011a). This suggests that there is an independent cell death signal that acts downstream of *ph* in a cell autonomous manner. I also showed that *ph*^{del} cells are not dividing at wandering larva stage, even if the clones are in front of the morphogenetic furrow. This suggests that *ph*^{del} cells may enter G0 phase earlier than wild type cells. The early stop of cell proliferation and the onset of cell death signaling may explain why *ph*^{del} cells do not survive to adult stage.

Although *ph*^{del} cells themselves die during development, they are able to induce over-proliferation of surrounding cells, and the resulting tissues are even larger than normal. I demonstrated that *ph* activates *Notch* expression, which causes the over-production of the ligands Upd homologs, which are secreted form mutant cells and activate the JAK/STAT pathway signaling in neighboring cells (Feng et al, 2011a). In addition, I demonstrated that the activation of these signaling pathways is not induced by apoptosis as compensatory proliferation. Rather, it is caused by the absence of Ph protein (Feng et al, 2011a).

Different *ph* alleles cause different patterns of over-proliferation

Although I obtained solid data showing that loss of *ph* causes non-autonomous cell over-proliferation, a recently published paper claimed that *ph* cells in a mosaic tissue cause cell over-proliferation in a cell autonomous manner (Martinez et al, 2009). Although both studies concluded that *ph* mosaic tissues

overgrow, the mechanisms are very different. I was interested in why there was this huge difference.

I noticed that the authors of that paper used *ph*^{505}, a different *ph* allele that was also considered a null allele. Therefore I repeated some experiments I did with *ph*^{del} using *ph*^{505}. My results were identical to what the other study reported. Indeed, *ph*^{505} mosaic eyes are much larger than wild type ones, and *ph*^{505} cells show massive over-proliferation at the wandering larval stage. Nevertheless, my further investigation showed that although *ph*^{505} mutant clones grow to very large sizes during wandering larval stage, they never contribute to the adult eyes (Feng et al, 2011a). In other word, the enlarged adult eyes are the results of non-autonomous over-proliferation.

I then asked why *ph*^{505} causes both autonomous and non-autonomous over-proliferation. *ph*^{505} is an allele that was generated by two rounds of EMS mutagenesis and was long believed to be a null allele based on genetic data. Thus I hypothesized that this allele might not be a null allele, which would potentially explain the discrepancies in the two studies. Indeed, transgene rescue data, embryonic lethality data and sequencing results all proved that *ph*^{505} is actually a hypomorph (Feng et al, 2011a).

_ph_^{del} and _ph_^{505} cells respond differently to JAK/STAT pathway ligands

Data from my functional analyses showed that the same signaling pathway is also responsible for non-autonomous over-proliferation in *ph*^{505} mosaic eye discs,

which is more or less expected. What was surprising was the observation that when the 3 *upd* homologs were all removed from ph^{505} cells, it suppressed not only non-autonomous over-proliferation, but also autonomous over-proliferation (Feng et al, 2011b). Based on this unexpected observation, I hypothesized that autonomous proliferation of ph^{505} cells was due to their response to the Upd ligands secreted by themselves. Functional tests then supported this hypothesis.

Then an interesting question was why ph^{505} cells still respond to Upd ligands, but ph^{del} cells do not. One of the reviewers of my second research paper (Feng et al, 2011b) suggested that some JAK/STAT pathway regulators might be differentially expressed in ph^{del} and ph^{505} cells. Thus I decided to take a candidate gene approach to test this hypothesis. I used TU-tagging to isolate RNA from ph^{del} and ph^{505} cells, and used Real-Time PCR to examine candidate gene expression levels. I found that the differential expression of the JAK/STAT pathway receptor *dome* is likely to underlie the different responses between ph^{del} and ph^{505} cells to the pathway ligands (Feng et al, 2011b). The models of cell proliferation states of different cell populations and underlying signaling pathways in ph^{del} and ph^{505} mosaic discs are given in Figure 5-1A (ph^{del}) and 5-1B (ph^{505}).

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Figure 5-1. Models of cell proliferation and signaling pathways in *ph* mosaic tissues

(A) Model of cell proliferation patterns and signaling pathways in ph^{del} mosaic tissues. *Notch* is activated autonomously and stimulates the expression of all three *upd* homologs. The Upd proteins activate the JAK/STAT pathway and stimulate proliferation of neighboring wild-type cells only, but not the mutant cells themselves, because the mutant cells are insensitive to the ligands. (B) Model of cell proliferation patterns and signaling pathways in ph^{505} mosaic tissues. Similar to the case of ph^{del}, *Notch* is activated in the mutant cells, which activates the expression of the *upd* homologs. In contrary to ph^{del} mosaic discs, in ph^{505} mosaic tissues, both mutant cells and wild type cells are responsive to the Upd ligands. Therefore the JAK/STAT pathway is activated, and over-proliferation is observed in both wild type and mutant cells.

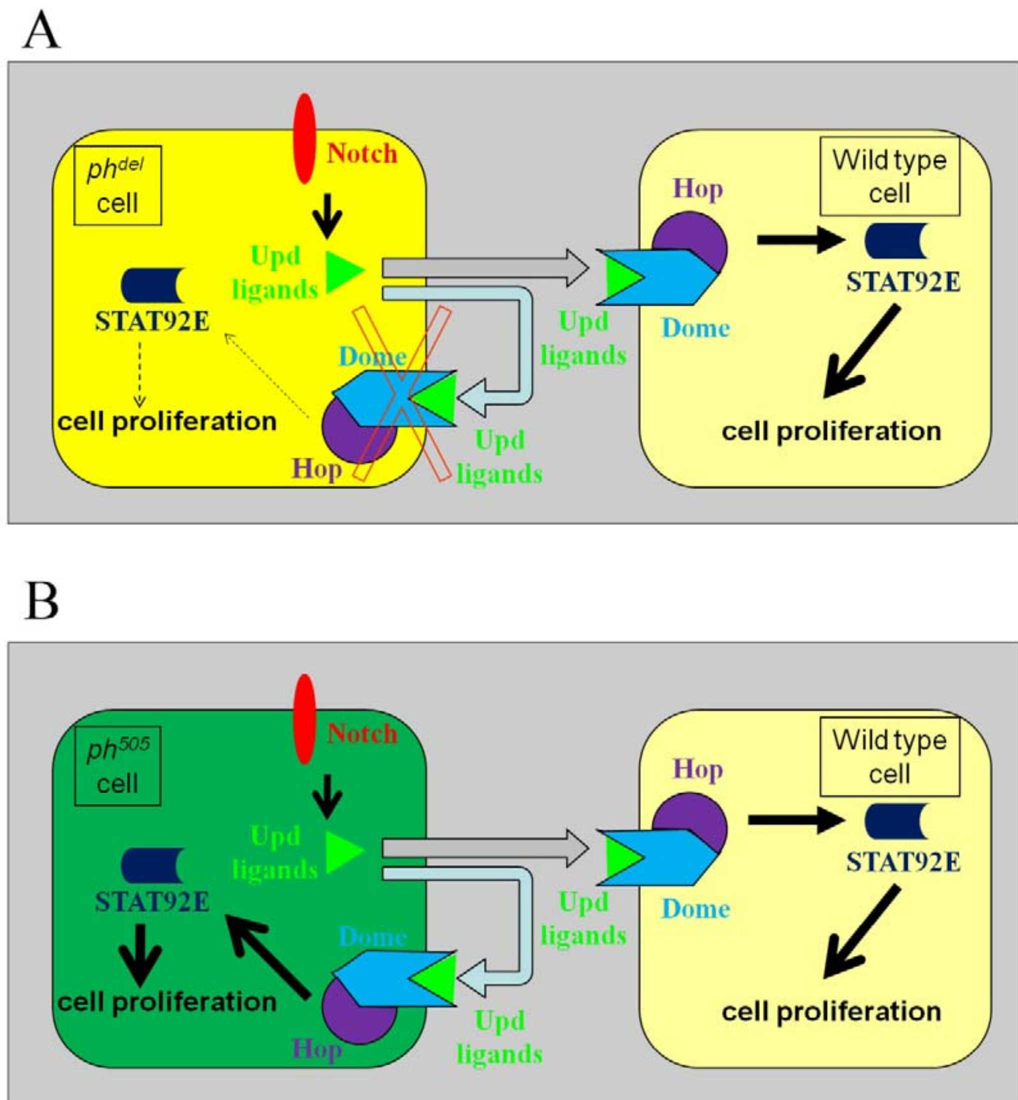

Figure 5-1

The function of wild type Ph protein in maintaining tissue homeostasis

My data have demonstrated that loss of *ph* severely disrupts tissue homeostasis. Although loss of *ph* causes tissue over-growth, *ph* is different from most tumor suppressors not only in the non-autonomousness of *ph* induced over-proliferation, but also in that it is not part of a signal transduction cascade that controls cell proliferation, like most tumor suppressors are. Thus I suggest that *ph* probably should not be called a tumor suppressor, because the name "tumor suppressor" infers that the function of the gene product is to suppress cell proliferation. Certainly Ph protein does not fit this definition.

Ph is a PcG protein, which function in keeping hundreds or even more targets at the right transcription state. Thus an important question is how wild type Ph protein functions in maintaining tissue homeostasis. I have identified *Notch* as a downstream target of Ph, therefore I asked the question if *Notch* is a direct Ph target or not. I searched the modENCODE database, but did not find any ChIP data of Ph. Nevertheless I found ChIP data of Pc, which together with Ph and several other proteins, form the core of PRC1. One clear Pc binding site about 4kb upstream of the *Notch* gene was detected in two independent Pc ChIP experiments in S2 cells (modENCODE_326) and Kc cells (modENCODE_3791), both derived from embryos. However, another Pc ChIP in a cell line derived from the CNS did not detect this binding site (modENCODE_325). In addition to Pc binding site, a Pho binding site was detected within one of the introns of *Notch* (modENCODE_3894). No other PcG binding sites were detected within 50 kb of *Notch*. These results suggest that the PRC1 complex may be able to directly

repress *Notch*, and this repression is differentially regulated in different tissues. However, further functional data are needed to fully justify this statement.

Reevaluate the non-autonomous over-proliferation pathway in *Drosophila*

Compared to the large number of genes whose mutants cause over-proliferation autonomously, there are only a few genes, when mutated, cause over-proliferation in a non-autonomous manner (See Introduction). Interestingly, in all cases where the underlying signaling pathways have been fully determined, it was reported that Notch signaling is autonomously activated, which causes the over-production of the ligand Upd in the mutant cells. Upd then causes a hyperactive JAK/STAT pathway and eventually over-proliferation in surrounding cells. In the case of *ph*, my functional data demonstrated that *Notch* is required in *ph* induced non-autonomous over-proliferation. This is consistent with others' results from over-expression of a dominant-negative form of Notch in *vps25* clones (Herz et al, 2006). Since there is an increase in Notch expression in ph^{del} clones, it is unlikely that *Notch* and *ph* act in parallel pathways. Therefore, I conclude that *Notch* is a downstream target of Ph.

My data however, demonstrated that *upd* is not necessary in *ph* induced non-autonomous over-proliferation, since the removal of *upd* gene from ph^{del} clones has no effects on the phenotype. I then dug one step further and found that the three *upd* homologs work redundantly to cause over-proliferation (Feng et al, 2011a). My results are different from all the other reports that claimed it is Upd that causes non-autonomous over-proliferation. To my knowledge, my result is

the first functional data that address if Upd actually causes over-proliferation. I observed an increase in Upd expression in *ph^del^* cells, which is consistent with others' conclusion that Notch activates the transcription of *upd*. But no one has demonstrated that over-production of Upd really causes the over-proliferation phenotype. Actually in one report, the authors tried to phenocopy the *vps25* phenotype by over-expressing Upd but failed (Herz et al, 2006). My data clearly demonstrated that although Upd expression is elevated in ph^{del} cells, it is not required for over-proliferation in neighboring cells. Rather, the three *upd* homologs work together to cause the phenotype. It would be interesting to study if all three homologs are required or only a subset of them. My results suggest that it is time to rethink the signaling pathways in non-autonomous over-proliferation in *Drosophila*.

ph^{del} cell polarity and ph^{del} clone morphology

In the absence of *ph*, the expression of lots of genes would be expected to change and there would be a chaos in the mutant cells. Therefore it is surprising that ph^{del} cells do not lose their normal cell polarity, but undergo invagination towards basal side of the disc to form unique 3 dimensional structures (Feng et al, 2011a). Such structures look very similar to some secretory glands. Moreover, like normal glands, ph^{del} mutant cells are able to secret proteins (in this case the Upd ligands) through their apical surface into the lumen like space at the center of the structures. The morphological and functional similarities suggest that the ph^{del} mutant clones may be transformed into glands. If this is true, it suggests

that a signaling pathway that is normally activated to induce the formation of a gland may be ectopically turned on by loss of *ph*, and then transforms the ph^{del} clones into functional glands. Given the function of PcG genes in repressing *Hox* gene expression, it is possible that one or more *Hox* genes or other classes of selector genes are derepressed in ph^{del} cells, and the ectopic expression of such genes transforms normal eye discs cells into functional glands. Normally homeotic transformation refers to the transformation of one organ or one body segment to another. What is seen in ph^{del} cells may be an example of homeotic transformation at the tissue level.

Implications to cancer genetics and treatment

The differences between ph^{def} phenotypes and ph^{505} phenotypes underscore the complexity of cancer. My results showed that different alleles of the same locus could cause tumors in very different ways. It adds another level of complexity to the nature of cancer and also another level of difficulty in cancer treatment. Currently, more and more cancer treatments are based on our understanding of the underlying molecular mechanism, with each treatment only effective in patients with certain mutations. For example, the blockbuster monoclonal antibody drug Herceptin is only effective in patients whose HER2 receptor is hyperactive (Patani & Mokbel, 2010). Thus it is often necessary to perform lab tests to determine what genes have mutated in the tumor of a patient and what treatments may be good for the patient. The studies on the *ph* locus imply that merely identifying which genes are mutated may not be enough to determine

which cancer treatments a patient should receive. Instead, digging deeper into the nature of the mutations may be necessary to determine the best treatment. If different alleles of *ph* gene could cause tumors in *Drosophila* in very different ways, there is no reason to think that mutations in some human genes would not do the same. When the same genes are mutated in different ways, the resulting cancers might be very different molecularly and may require different treatments to achieve the best prognosis.

Chapter 6: Materials and methods

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Materials

Drosophila alleles

l(X)MB342 and *p{ph-d⁺}* were previously described (Wang et al, 2006). ph^{505} and ph^{600} were the gift of N. B. Randsholt, and $dome^{G0468}$ was from James Castelli-Gair Hombría. Deficiency lines ph^{del} and $upd^{d1\text{-}3}$ were generated in this study using an FRT-based deletion technique (Parks et al, 2004). *UAS-HA-UPRT3.2* was kindly provided by Chris Doe before it was available at the Bloomington Stock Center. Other alleles used include $Dp(1;2;Y)w^{+}$, $Notch^{55e11}$, upd^{4} (upd^{YM55}), $Wapl^{2}$, csw^{G0170}, $Pgd^{KG08676}$, Pgd^{G0385}, $dome^{G0405}$, hop^{2}, ph^{503} and ph^{602}, and were all obtained from the Bloomington Stock Center. The following alleles were used in the genetic interaction experiments and were all obtained from the Bloomington Stock Center: Dl^{3}, Dl^{6B}, $Ser^{Bd\text{-}1}$, eyg^{1}, $Stat92E^{06346}$, $Stat92E^{EY14209}$, hh^{AC}, hh^{21}, ptc^{9}, ptc^{S12}, $wg^{Sp\text{-}1}$, $wg^{l\text{-}8}$, $wg^{l\text{-}17}$, $wg^{l\text{-}12}$ and dpp^{hr92}.

Antibodies

The following primary antibodies were used: mouse anti-Elav (9F8A9), mouse anti-BrdU (G3G4), mouse anti-Notch extracellular domain (C458.2H), and mouse anti-En (4D9) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-Upd was a gift from D. Harrison. Rat anti-mCD8 (catalog number RM2200) was purchased from Caltag Laboratories, rabbit anti-PH3 (06-570) was from Upstate Biotechnology, and rabbit anti-aPKC (catalog number sc-216) was from Santa Cruz Biotechnology. FITC and Cy3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Alkaline phosphatase conjugated secondary antibody was purchased from Sigma.

Reagents

Geneticin (catalog number 11811-031) was purchased from GIBCO. OsO_4, glutaraldehyde, and HMDS (hexamethyldisilazane) were provided by the EM lab at the University of Maryland, College Park. BrdU (catalog number B5002), 4-thiouracil (4-TU) (catalog number 440736) and 2-Mercaptoethanol (catalog number M3148) were purchased from Sigma. 16% paraformaldehyde was purchased from Electron Microscopy Laboratories (catalog number 15710), and mounting medium with DAPI was purchased from Vector Laboratories (catalog number H-1200). EZ-link Biotin-HDPD (catalog number 21341) was purchased from Pierce, and MPG-Streptavidin beads (catalog number MSTR0502 or MSTR0510) was purchased from Pure Biotech. NBT/BCIP ready-to-use tablets (catalog number 1697471001) were purchased from Roche.

Commercial Kits

The following commercially available kits were used: RNeasy mini kit (catalog number 74106) was purchased from Qiagen. *In Situ* Cell Death Detection Kit, TMR red (catalog number 12156792910), Transcriptor First Strand cDNA Synthesis Kit (catalog number 04379012001) and LightCycler® 480 SYBR Green I Master (catalog number 04707516001) were purchased from Roche.

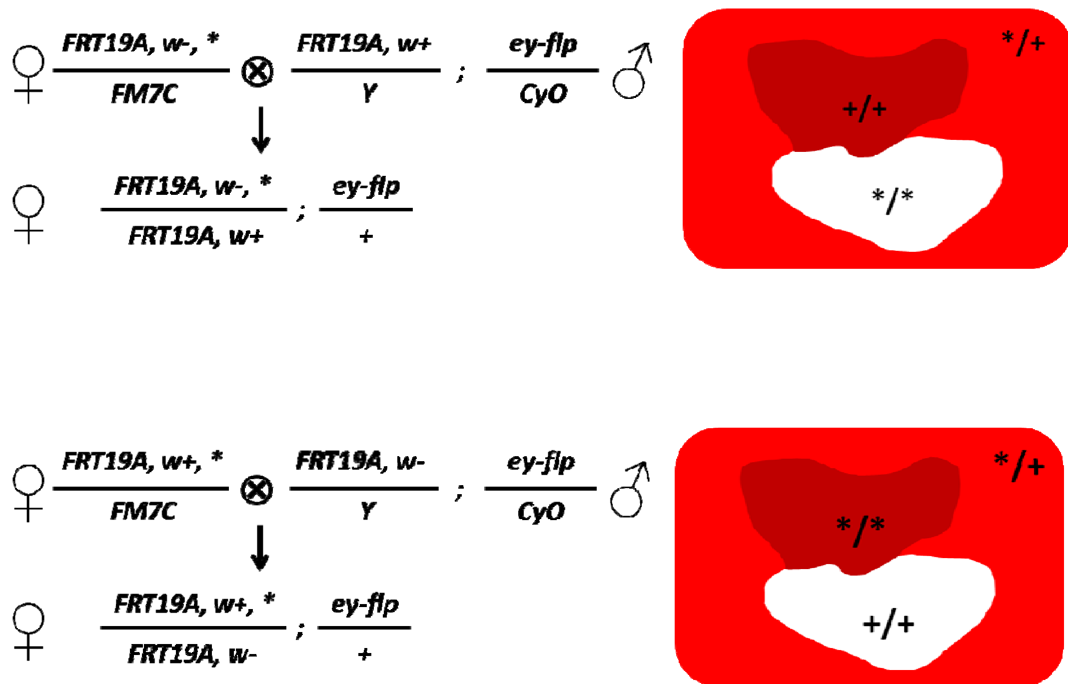
Methods:

Generation of *ph*^{del} allele and *upd*$^{d1\text{-}3}$ allele

FRT lines *PBac{WH}csw*^{f02923} and *P{XP}ph-p*^{d01798} were chosen as the starting lines for generating *ph*^{del}, and FRT lines *PBac{WH}f04435* and *P{XP}d01676* were used as starting lines for generating *upd*$^{d1\text{-}3}$. Crosses and heat shock was performed as described (Parks et al, 2004). *white-* female progeny was individually crossed with *FM7C/Y* males. Stocks in which males are hemizygous lethal were kept. For *ph*^{del}, complementation tests with known *ph* alleles and mutant alleles of surrounding genes were performed to confirm that the deletion disrupted and only disrupted the *ph* locus. Then the deletion breakpoints were confirmed by PCR and sequencing. For *upd*$^{d1\text{-}3}$, PCR was used to confirm the presence of the deletion. *FRT19A* was then recombined to both deficiency lines using conventional genetic techniques. 300mg/L of geneticin in fly food was used to select for larvae with *FRT19A*.

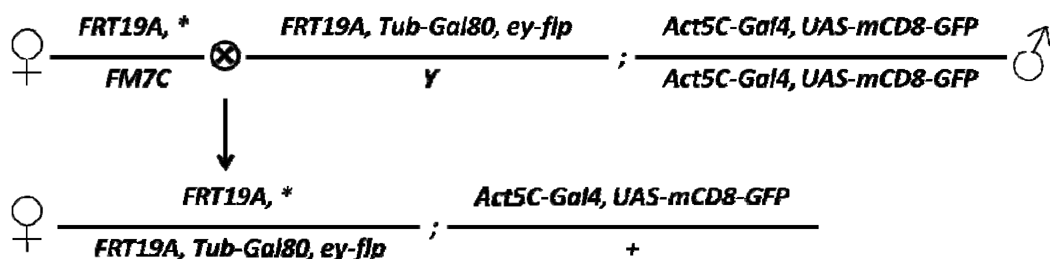
Mosaic analysis of the adult eyes and labeling with *white*

FRT19A and *ey-flp* were used in all mosaic analyses of the adult eyes. In order to use the gene *white* to differentially label different populations of cells, *white* was recombined to *FRT19A, ph*^{del} and *FRT19A, ph*^{505}. Then the following reciprocal crosses were performed, and the effect on colors of different cell populations was shown in the corresponding schematics. * could be wild type, *ph*^{del} or *ph*^{505} in different experiments.



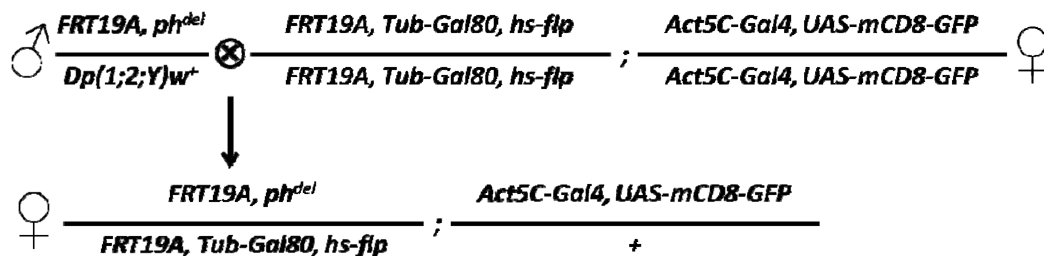
MARCM in imaginal discs

MARCM was performed as described (Lee & Luo, 1999) with the following modifications. *ey-flp* was always used to induce clones in eye discs, and *hs-flp* was used to induce clones in wing discs and leg discs. Heat shock was performed 60 hours after egg laying. *Act5C-Gal4* was always used as the source of Gal4, and *UAS-mCD8-GFP*, a cell membrane localized GFP was used to label mutant clones. The following crosses were performed for MARCM in eye discs. * could be wild type, ph^{del}, ph^{505}, or various double mutant lines.



Third instar larvae with the right genotype were picked up under a GFP dissection scope based on the pattern of GFP experssion. Those larvae that only have sporadic GFP+ dots at their anterior end were picked up for dissection, whereas larvae without GFP or those with GFP throughout the body were discarded.

The following cross was conducted for MARCM in wing and leg discs.



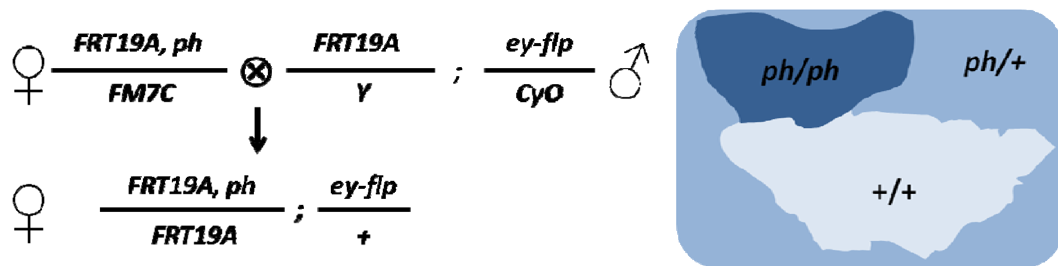
After heat shocking at an earlier stage as indicated above, third instar female larvae were picked for dissection. All female larvae should have the right genotype.

Removal of another gene specifically from *ph* mutant cells

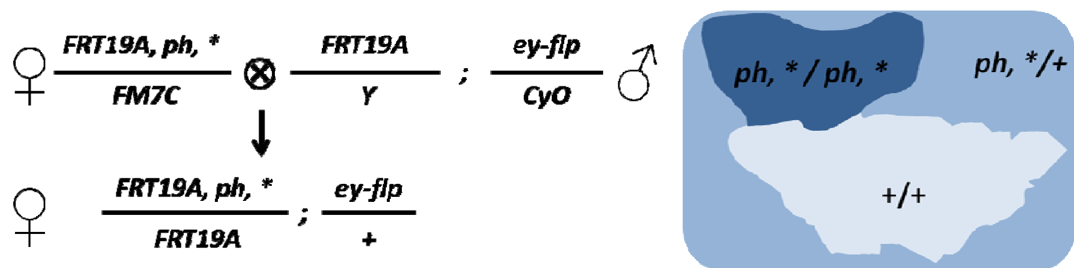
When taking a candidate gene approach to study the signaling pathways involved in *ph* induced over-proliferation in vivo, the candidate gene must be specifically removed only from *ph* mutant cells in a mosaic tissue. When the candidate gene is on the same chromosome (the X chromosome) as *ph*, this could be achieved by inducing mosaics using a double mutant line which lacks both *ph* and the candidate gene (compare the two situations below, schematics show different populations in mosaic tissues). *ph* indicates either ph^{del} or ph^{505} in

different experiments, whereas * indicates the candidate gene to be removed from *ph* cells.

Inducing mosaics using a *ph* mutant allele:



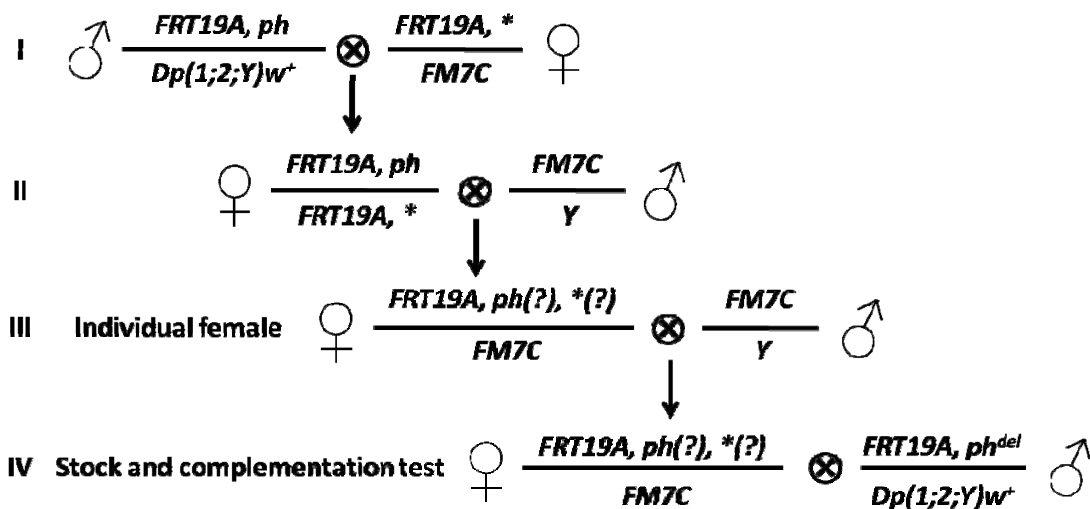
Inducing mosaics with a double mutant line of *ph* and another gene:



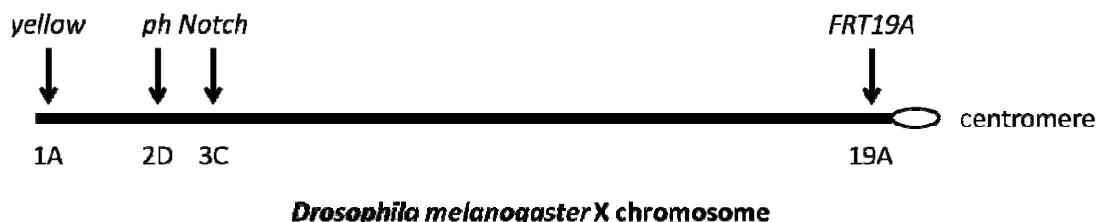
Generation of double mutant lines

Double mutant lines of *ph* and *upd*, upd^{d1-3}, *hop* or *dome* were generated using conventional genetic techniques. *FRT19A* was first recombined with each allele, then two mutant alleles were brought to the same chromosome by another round of meiotic recombination. $Dp(1;2;Y)w^{+}$, a Y chromosome that carries a duplication that covers the *ph* locus, was used in order to use males carrying *ph* alleles. The two *dome* alleles used in this project are both transposon insertion lines, and *mini-white* was used as a dominant marker for the presence of *dome* to simplify the crosses. Complementation tests were performed by crossing putative double mutant females with $ph^{del}/Dp(1;2;Y)w^{+}$. The presence of both *ph*

allele and a second mutant allele could be tested in this single complementation test. If *ph*^{del} failed to complement with the possible double mutant line, it indicated the presence of the *ph* allele. On the other hand, if *Dp(1;2;Y)w*$^{+}$ failed to complement with the possible double mutant line, the second mutation was present.

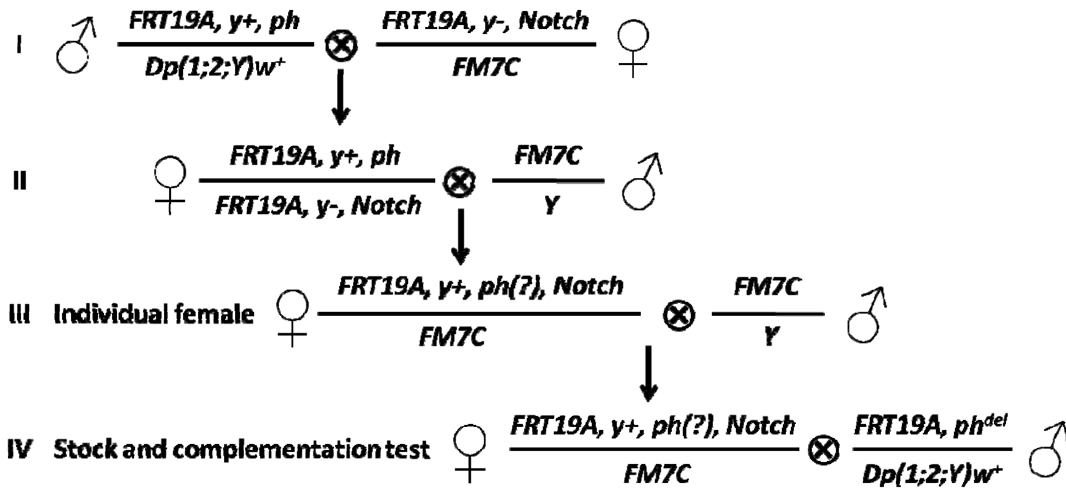


ph (band 2D on cytological map) and *Notch* (band 3C on cytological map) are very close to each other (only about 1 Mb away from each other), therefore a very large number of progenies must be individually screened to obtain the desired *ph, Notch* double mutant line. To overcome this hurdle, the dominant marker *yellow* was used to reduce the number of progenies needed to be screened (see the figure below for the positions of *yellow, ph* and *Notch*).



Drosophila melanogaster **X chromosome**

The following crosses were performed (*ph* could be either ph^{del} or ph^{505}):

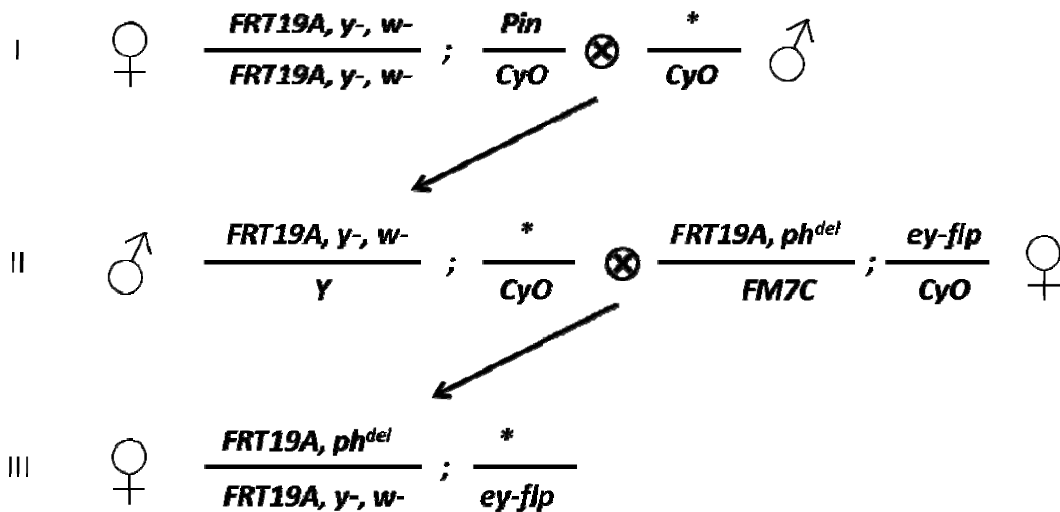


Notch was first recombined with *FRT19A*, and a *yellow-* line was chosen. On the other hand, the *FRT19A, ph* line was *yellow+*. At generation III, only *yellow+, Notch* progenies were picked up to establish individual stocks. Then complementation test with ph^{del} was used to examine if the *ph* allele is present.

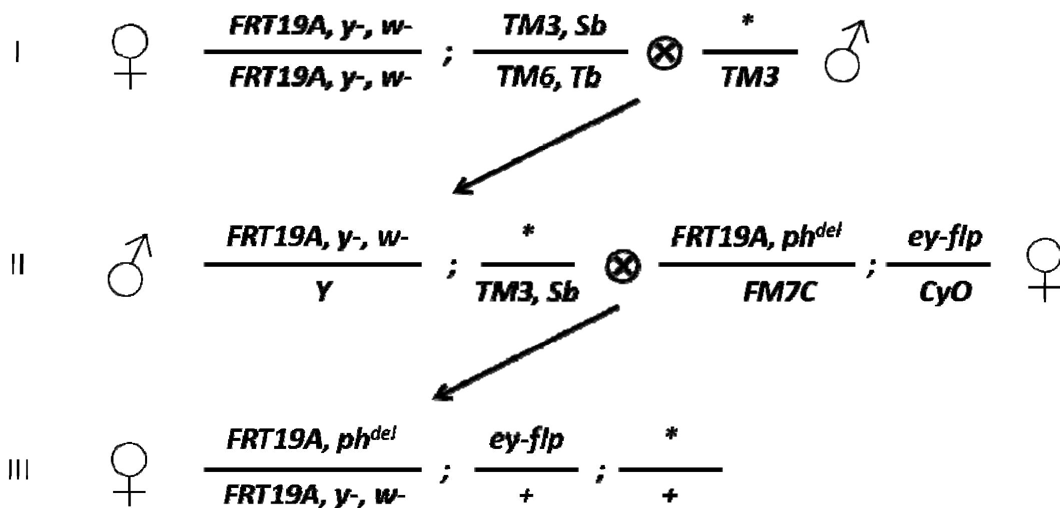
Genetic interaction

The following crosses were performed for the genetic interaction tests in order to remove a copy of a candidate gene. (* indicates a mutant allele of a candidate gene, or a wild type allele as a control.)

Crosses for candidate genes on the second chromosome:



Crosses for candidate genes on the third chromosome:

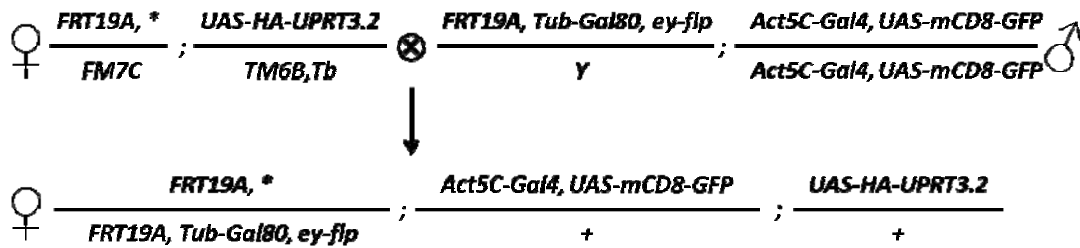


TU-tagging

UAS-HA-UPRT3.2 (Miller et al, 2009) was used in all TU-tagging experiments.

The following crosses were performed to get larvae with desired genotypes.

TM6B, Tb was chosen as the balancer chromosome in order to genotype the larvae.



Third instar larvae about 12 hours ahead of wandering stage and with the right genotype were picked up under a GFP dissection scope, and were fed on mocha cap containing 0.5 mM of 4-TU at dark for about 10 hours. Then their eye discs were dissected with mouth hooks still attached, but salivary glands and fat bodies were carefully removed. Brains were either attached for simplicity (in the case of ph^{505}) or removed to enrich mutant cells (in the case of ph^{del}). About 60 larvae were dissected for each sample. Two independent biological repeats were prepared for both ph^{del} and ph^{505}. Total RNA was purified using Qiagen RNeasy mini kit. TU-tagging, affinity purification of tagged RNA, and elution of purified RNA were modified from published protocols (Miller et al, 2009). Briefly, TU-tagged total RNA was labeled with biotin using EZ-link Biotin-HDPD from Pierce, labeled RNA was then extracted twice with chloroform to remove excess labeling reagent, followed by isopropanol precipitation. The pellet was dissolved in 60 µl of RNase free water. A small volume (5 µl) of sample was kept as a control for unpurified total RNA, and the rest was subjected to affinity purification by streptavidin. Immobilized streptavidin on magnetic beads (MPG-Streptavidin beads) was used to capture biotin labeled RNA. After washing the beads 3 times at room temperature, bound RNA was eluted with 5% BME. Eluted RNA was then precipitated with ethanol aided by linear acrylamide as the carrier, and re-

dissolved in 10 µl of RNase free water. Both unpurified total RNA and eluted RNA were used as template to synthesize cDNA using Transcriptor First Strand cDNA Synthesis Kit from Roche. Real time PCR was then performed as described below. Relative GFP ratio in eluted RNA versus total RNA was used to indicate the fold of enrichment of RNA from the mutant cells. In the two biological repeats for each *ph* allele, only data from the one that gave the higher such ratio were used in data analysis.

Real-Time PCR

RNA was either purified from dissected eye discs using RNeasy mini kit from Qiagen or from purified TU-tagged RNA. cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit from Roche. Real Time PCR was performed using LightCycler® 480 SYBR Green I Master from Roche and Roche LightCycler 480 Real Time PCR machine according to manufacturer's instructions. Multiple biological repeats and multiple PCR repeats of the same sample were performed, and data were analyzed using LightCycler 480 Software that came with the Real Time PCR machine and Microsoft Excel.

Scanning electron microscopy

Adult flies with the desired phenotype were collected and anesthetized. Their heads were cut with a razor blade, and fixed in 2% glutaraldehyde in PBS at 4C overnight. The heads were then washed with PBS for 3 times, 10 minutes each. Then the samples were post fixed with 2% OsO_4 for 1 hour at room temperature, follow by 3 washes with water, 10 minutes each. Next a series steps of washing with shaking were performed to dehydrate the samples. Each step takes 10 minutes at room temperature. The samples were treated once with 75% ethanol,

once with 95% ethanol, and then 3 times with pure ethanol. The samples were then treated mixtures of HMDS (hexamethyldisilazane) and ethanol at different ratios, once with 1:1 mixture, once with 2:1 mixture and once with 3:1 mixture. The samples were then treated with pure HMDS. Then HMDS was removed, and the samples were placed in a vacuum dissicator to let the residue HMDS vaporize. This process takes at least 24 hours. The samples were then removed from the vacuum and mounted on the stubs, followed by coating with gold/palladium. The samples were then pictured with an electron microscope.

Immunohistochemistry of imaginal discs

Antibody staining was performed essentially as the following. Imaginal discs from wandering larvae were dissected, and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. The discs were then washed in PBT for 3 times, with 5 minutes each time, followed by a blocking step with 5% normal goat serum in PBT at room temperature for 30 minutes. The discs were then incubated with the primary antibody at 4C overnight. After washing with PBT for 3 times with 30 minutes each at room temperature, the discs were incubated with the secondary antibody at room temperature for 2 to 4 hours or at 4C overnight. Discs were then washed 3 times in PBT at room temperature, with 30 minutes each, before mounting.

BrdU labeling was performed as following. Imaginal discs from wandering larvae were dissected, treated with BrdU for 30 minutes at room temperature, and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. The discs were then washed in PBT for 3 times, with 5 minutes each time. After hydrolyzing

with HCl and neutralizing with Borax, the discs were washed twice with PBT, with 20 minutes each time, followed by a blocking step with 5% normal goat serum in PBT at room temperature for 30 minutes. The discs were then incubated with the primary antibody at 4C overnight. After washing with PBT for 3 times with 30 minutes each at room temperature, the discs were incubated with the secondary antibody at room temperature for 2 to 4 hours or at 4C overnight. Discs were then washed 3 times in PBT at room temperature, with 30 minutes each, before mounting.

TUNEL staining performed using *In Situ* Cell Death Detection Kit (TMR Red) from Roche according to the manufacturer's instructions. Briefly, discs from wandering larvae were dissected fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. The discs were then washed in PBT for 3 times, with 5 minutes each time, followed by a blocking step with 5% normal goat serum in PBT at room temperature for 30 minutes. Then the discs were treated with permeabilization solution, followed by washing with PBT for 3 times, 10 minutes each. The discs were then treated with freshly made TUNEL reaction solution for 1 hour at 37C at dark. The discs were then washed 3 times with PBT, 30 minutes each before mounting.

All images of immunofluorescent staining were collected using Leica SP5 X or Zeiss LSM 710 confocal microscopes and processed with Adobe Photoshop.

Embryo antibody staining

ph^{del} and ph^{505} alleles were balanced with GFP marked FM7C balancer chromosome. Embryos from these stocks were collected overnight, and aged for

another 24 hours. Unhatched embryos were picked, and checked to confirm they and GFP-. These embryos were dechorionated with 50% bleach for 2 minutes, and washed with dH_2O for 3 minutes. Embryos were then treated with fixation buffer (formaldehyde + heptane), and shaken for 15 minutes. Then the lower phase, as well as any embryos in this phase, was removed, and methanol was added, followed by shaking for 15 seconds to remove the vitelline membrane. Embryos sank to the bottom were collected and washed twice with methanol. At this point, embryos could be stored at -20C or -80C for long period of time. When the embryos were ready to be stained by antibody, they were rehydrated by washing 3 times with PBT, 30 minutes each. Then the embryos were treated with the primary antibody in PBT at 4C overnight. After washing 3 times with PBT at room temperature, 30 minutes each, embryos were treated with alkaline phosphatase conjugated secondary antibody in PBT for 2 to 4 hours at room temperature or overnight at 4C. Embryos were then washed 3 times with PBT, 30 minutes each. Ready-to-use NBT/BCIP tablets from Roche were used to prepare staining buffer according to manufacturer's instructions. Staining buffer was then added to the embryos, and the color reaction was stopped by washing with water when appropriate. Embryos were then mounted and pictures were taken using a Leica fluorescent microscope.

References

Agaisse H, Petersen U-M, Boutros M, Mathey-Prevot B, Perrimon N (2003) Signaling Role of Hemocytes in Drosophila JAK/STAT-Dependent Response to Septic Injury. *Developmental Cell* **5:** 441-450

Akam ME (1983) The location of *Ultrabithorax* transcripts in *Drosophila* tissue sections. *EMBO J* **2:** 2075-2084

Akam ME, Martinez-Arias A (1985) The distribution of *Ultrabithorax* transcripts in *Drosophila* embryos. *EMBO J* **4:** 1689-1700

Akam M (1987) The molecular basis for metameric pattern in the Drosophila embryo. *Development* **101:** 1-22

Alkema MJ, Bronk M, Verhoeven E, Otte A, van 't Veer LJ, Berns A, van Lohuizen M (1997) Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex. *Genes & Development* **11:** 226-240

Arbouzova NI, Bach EA, Zeidler MP (2006) Ken & Barbie Selectively Regulates the Expression of a Subset of JAK/STAT Pathway Target Genes. *Current Biology* **16:** 80-88

Arbouzova NI, Zeidler MP (2006) JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. *Development* **133:** 2605-2616

Artavanis-Tsakonas S, Muskavitch MAT (2010) Chapter One - Notch: The Past, the Present, and the Future. In *Current Topics in Developmental Biology*, Raphael K (ed), Vol. Volume 92, pp 1-29. Academic Press

Bach EA, Vincent S, Zeidler MP, Perrimon N (2003) A Sensitized Genetic Screen to Identify Novel Regulators and Components of the Drosophila Janus Kinase/Signal Transducer and Activator of Transcription Pathway. *Genetics* **165:** 1149-1166

Baeg G-H, Zhou R, Perrimon N (2005) Genome-wide RNAi analysis of JAK/STAT signaling components in Drosophila. *Genes & Development* **19:** 1861-1870

Beck S, Faradji F, Brock H, Peronnet F (2010) Maintenance of Hox Gene Expression Patterns
Hox Genes. Deutsch JS (ed), Vol. 689, pp 41-62. Springer New York

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL,

Lander ES (2006) A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* **125:** 315-326

Betz A, Lampen N, Martinek S, Young MW, Darnell JE (2001) A Drosophila PIAS homologue negatively regulates stat92E. *Proceedings of the National Academy of Sciences* **98:** 9563-9568

Binari R, Perrimon N (1994) Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes & Development* **8:** 300-312

Boivin A, Fauvarque MO, Dura JM (1999) One-to-one correspondence between the two genetic units and the tandemly duplicated transcriptional units of the *polyhomeotic* locus of *Drosophila. Molecular and General Genetics MGG* **261:** 196-200

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

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441:** 349-353

Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K (2006) Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes & Development* **20:** 1123-1136

Bracken AP, Helin K (2009) Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat Rev Cancer* **9:** 773-784

Bridges CB, Morgan TH (1923) *The third-chromosome group of mutant characters of Drosophila melanogaster*, Washington DC: The Carnegie Institution.

Brown S, Hu N, Hombrla JC-G (2001) Identification of the first invertebrate interleukin JAK/STAT receptor, the Drosophila gene domeless. *Current Biology* **11:** 1700-1705

Buchenau P, Hodgson J, Strutt H, Arndt-Jovin DJ (1998) The Distribution of Polycomb-Group Proteins During Cell Division and Development in Drosophila Embryos: Impact on Models for Silencing. *The Journal of Cell Biology* **141:** 469-481

Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing. *Science* **298:** 1039-1043

Cao R, Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Current Opinion in Genetics & Development* **14:** 155-164

Capowski EE, Martin P, Garvin C, Strome S (1991) Identification of Grandchildless Loci Whose Products Are Required for Normal Germ-Line Development in the Nematode Caenorhabditis elegans. *Genetics* **129:** 1061-1072

Carbia-Nagashima A, Arzt E (2004) Intracellular Proteins and Mechanisms Involved in the Control of gp130/JAK/STAT Cytokine Signaling. *IUBMB Life* **56:** 83-88

Chen H-W, Chen X, Oh S-W, Marinissen MJ, Gutkind JS, Hou SX (2002) mom identifies a receptor for the Drosophila JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family. *Genes & Development* **16:** 388-398

Chopra VS, Hong J-W, Levine M (2009) Regulation of Hox Gene Activity by Transcriptional Elongation in Drosophila. *Current Biology* **19:** 688-693

Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V (2002) Drosophila Enhancer of Zeste/ESC Complexes Have a Histone H3 Methyltransferase Activity that Marks Chromosomal Polycomb Sites. *Cell* **111:** 185-196

de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, Koseki H, Brockdorff N (2004) Polycomb Group Proteins Ring1A/B Link Ubiquitylation of Histone H2A to Heritable Gene Silencing and X Inactivation. *Developmental Cell* **7:** 663-676

Deatrick J, Daly M, Randsholt NB, Brock HW (1991) The complex genetic locus polyhomeotic in Drosophila melanogaster potentially encodes two homologous zinc-finger proteins. *Gene* **105:** 185-195

DeCamillis M, Cheng NS, Pierre D, Brock HW (1992) The polyhomeotic gene of Drosophila encodes a chromatin protein that shares polytene chromosome-binding sites with Polycomb. *Genes & Development* **6:** 223-232

Dejardin J, Rappailles A, Cuvier O, Grimaud C, Decoville M, Locker D, Cavalli G (2005) Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. *Nature* **434:** 533-538

Delaval K, Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. *Current Opinion in Genetics & Development* **14:** 188-195

Denell RE (1978) Homoeosis in *Drosophila*. II. Genetic analysis of *Polycomb Genetics* **90:** 277-289

Desplan C, Theis J, O'Farrell PH (1988) The sequence specificity of homeodomain-DNA interaction. *Cell* **54:** 1081-1090

Duncan IM (1982) *Polycomblike*: a gene that appears to be required for the normal expression of the *Bithorax* and *Antennapedia* gene complexes of *Drosophila melanogaster*. *Genetics* **102:** 49-70

Dura J-M, Brock HW, Santamaria P (1985) *Polyhomeotic*: A gene of *Drosophila melanogaster* required for correct expression of segmental identity. *Molecular and General Genetics MGG* **198:** 213-220

Dura J-M, Randsholt NB, Deatrick J, Erk I, Santamaria P, Freeman JD, Freeman SJ, Weddell D, Brock HW (1987) A complex genetic locus, polyhomeotic, is required for segmental specification and epidermal development in D. melanogaster. *Cell* **51:** 829-839

Dura JM, Ingham P (1988) Tissue- and stage-specific control of homeotic and segmentation gene expression in Drosophila embryos by the polyhomeotic gene. *Development* **103:** 733-741

Edith H (2005) Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. *Current Opinion in Genetics & Development* **15:** 482-489

Enderle D, Beisel C, Stadler MB, Gerstung M, Athri P, Paro R (2011) Polycomb preferentially targets stalled promoters of coding and noncoding transcripts. *Genome Research* **21:** 216-226

Feng S, Huang J, Wang J (2011a) Loss of the Polycomb group gene *polyhomeotic* induces non-autonomous cell overproliferation. *EMBO Rep* **12:** 157-163

Feng S, Thomas S, Wang J (2011b) Diverse tumor pathology due to distinctive patterns of JAK/STAT pathway activation caused by different *Drosophila polyhomeotic* alleles. *Genetics* **in press**

Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, Khorasanizadeh S (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes & Development* **17:** 1870-1881

Fong Y, Bender L, Wang W, Strome S (2002) Regulation of the Different Chromatin States of Autosomes and X Chromosomes in the Germ Line of C. elegans. *Science* **296:** 2235-2238

Forquignon F (1981) A maternal effect mutation leading to deficiencies of organs and homeotic transformations in the adults of *Drosophila*. *Development Genes and Evolution* **190:** 132-138

Francis NJ, Saurin AJ, Shao Z, Kingston RE (2001) Reconstitution of a Functional Core Polycomb Repressive Complex. *Molecular cell* **8:** 545-556

Franke A, DeCamillis M, Zink D, Cheng N, Brock HW, Paro R (1992) Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. *EMBO J* **11:** 2941-2950

Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE (1992) The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proceedings of the National Academy of Sciences* **89:** 7840-7843

Gambetta MC, Oktaba K, Müller J (2009) Essential Role of the Glycosyltransferase Sxc/Ogt in Polycomb Repression. *Science* **325:** 93-96

Gandille P, Narbonne-Reveau K, Boissonneau E, Randsholt N, Busson D, Pret A-M (2010) Mutations in the *Polycomb* Group Gene *polyhomeotic* Lead to Epithelial Instability in both the Ovary and Wing Imaginal Disc in *Drosophila*. *PLoS ONE* **5:** e13946

Garcia-Bellido A (1975) Genetic control of wing disc development in *Drosophila*. In *Ciba Foundation Symposium 29 - Cell Patterning*, pp 161-182. John Wiley & Sons, Ltd.

Gehring WJ (1998) *Master Control Genes in Development and Evolution: The Homeobox Story*.

Gilbert MM, Weaver BK, Gergen JP, Reich NC (2005) A novel functional activator of the Drosophila JAK/STAT pathway, unpaired2, is revealed by an in vivo reporter of pathway activation. *Mechanisms of Development* **122:** 939-948

González I, Simón R, Busturia A (2009) The Polyhomeotic protein induces hyperplastic tissue overgrowth through the activation of the JAK/STAT pathway. *Cell Cycle* **8:** 4103-4111

Gunster M, Satijn D, Hamer K, den Blaauwen J, de Bruijn D, Alkema M, van Lohuizen M, van Driel R, Otte A (1997) Identification and characterization of

interactions between the vertebrate polycomb-group protein BMI1 and human homologs of polyhomeotic. *Mol Cell Biol* **17:** 2326-2335

Hannah-Alava A (1958) DEVELOPMENTAL GENETICS OF THE POSTERIOR LEGS IN DROSOPHILA MELANOGASTER. *Genetics* **43:** 878-905

Hariharan IK, Bilder D (2006) Regulation of Imaginal Disc Growth by Tumor-Suppressor Genes in Drosophila. *Annual Review of Genetics* **40:** 335-361

Harrison DA, McCoon PE, Binari R, Gilman M, Perrimon N (1998) Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes & Development* **12:** 3252-3263

Haupt Y, Alexander WS, Barri G, Peter Klinken S, Adams JM (1991) Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in Eμ-myc transgenic mice. *Cell* **65:** 753-763

Hemenway CS, Halligan BW, Levy LS (1998) The Bmi-1 oncoprotein interacts with dinG and MPh2: the role of RING finger domains. *Oncogene* **16:** 2541-2547

Hershko A, Ciechanover A (1998) THE UBIQUITIN SYSTEM. *Annual Review of Biochemistry* **67:** 425-479

Herz H-M, Chen Z, Scherr H, Lackey M, Bolduc C, Bergmann A (2006) vps25 mosaics display non-autonomous cell survival and overgrowth, and autonomous apoptosis. *Development* **133:** 1871-1880

Hodgson JW, Cheng NN, Sinclair DAR, Kyba M, Randsholt NB, Brock HW (1997) The polyhomeotic locus of Drosophila melanogaster is transcriptionally and post-transcriptionally regulated during embryogenesis. *Mechanisms of Development* **66:** 69-81

Hoey T, Warrior R, Manak J, Levine M (1988) DNA-binding activities of the Drosophila melanogaster even-skipped protein are mediated by its homeo domain and influenced by protein context. *Mol Cell Biol* **8:** 4598-4607

Hombría JC-G, Brown S, Häder S, Zeidler MP (2005) Characterisation of Upd2, a Drosophila JAK/STAT pathway ligand. *Developmental Biology* **288:** 420-433

Hou SX, Zheng Z, Chen X, Perrimon N (2002) The JAK/STAT Pathway in Model Organisms: Emerging Roles in Cell Movement. *Developmental Cell* **3:** 765-778

Hou XS, Melnick MB, Perrimon N (1996) marelle Acts Downstream of the Drosophila HOP/JAK Kinase and Encodes a Protein Similar to the Mammalian STATs. *Cell* **84:** 411-419

Ingham P (1985) The regulation of the *bithorax* complex. *Trends in Genetics* **1:** 112-116

Ingham P, Whittle R (1980) *Trithorax*: A new homoeotic mutation of *Drosophila melanogaster* causing transformations of abdominal and thoracic imaginal segments. *Molecular and General Genetics MGG* **179:** 607-614

Ingham PW (1983) Differential expression of *bithorax complex* genes in the absence of the *extra sex combs* and *trithorax* genes. *Nature* **306:** 591-593

Isono K-i, Fujimura Y-i, Shinga J, Yamaki M, O-Wang J, Takihara Y, Murahashi Y, Takada Y, Mizutani-Koseki Y, Koseki H (2005) Mammalian Polyhomeotic Homologues Phc2 and Phc1 Act in Synergy To Mediate Polycomb Repression of Hox Genes. *Mol Cell Biol* **25:** 6694-6706

Iwata S, Takenobu H, Kageyama H, Koseki H, Ishii T, Nakazawa A, Tatezaki S-i, Nakagawara A, Kamijo T (2010) Polycomb group molecule PHC3 regulates polycomb complex composition and prognosis of osteosarcoma. *Cancer Science* **101:** 1646-1652

Jenuwein T, Allis CD (2001) Translating the Histone Code. *Science* **293:** 1074-1080

Jurgens G (1985) A group of genes controlling the spatial expression of the *bithorax* complex in *Drosophila*. *Nature* **316:** 153-155

Kaufman TC, Lewis R, Wakimoto B (1980) Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: The homoeotic gene complex in polytene chromosome interval 84A-B. *Genetics* **94:** 115-133

Kawamura A, Yamada K, Fujimori K, Higashinakagawa T (2002) Alternative Transcripts of a polyhomeotic Gene Homolog Are Expressed in Distinct Regions of Somites during Segmentation of Zebrafish Embryos. *Biochemical and Biophysical Research Communications* **291:** 245-254

Kennison JA (1993) Transcriptional activation of Drosphila homeotic genes form distant regulatory elements. *Trends in genetics* **9:** 75-79

Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW (2002) Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* **285:** 1-24

Köhler C, Villar CBR (2008) Programming of gene expression by Polycomb group proteins. *Trends in Cell Biology* **18:** 236-243

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes & Development* **16:** 2893-2905

Kyba M, Brock HW (1998) The SAM domain of polyhomeotic, RAE28, and Scm mediates specific interactions through conserved residues. *Developmental Genetics* **22:** 74-84

Lechtenberg BC, Allen MD, Rutherford TJ, Freund SMV, Bycroft M (2009) Solution structure of the FCS zinc finger domain of the human polycomb group protein L(3)mbt-like 2. *Protein Science* **18:** 657-661

Lee JD, Amanai K, Shearn A, Treisman JE (2002) The ubiquitin ligase Hyperplastic discs negatively regulates hedgehog and decapentaplegic expression by independent mechanisms. *Development* **129:** 5697-5706

Lee MG, Villa R, Trojer P, Norman J, Yan K-P, Reinberg D, Di Croce L, Shiekhattar R (2007) Demethylation of H3K27 Regulates Polycomb Recruitment and H2A Ubiquitination. *Science* **318:** 447-450

Lee T, Luo L (1999) Mosaic Analysis with a Repressible Cell Marker for Studies of Gene Function in Neuronal Morphogenesis. *Neuron* **22:** 451-461

Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K-i, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA (2006) Control of Developmental Regulators by Polycomb in Human Embryonic Stem Cells. *Cell* **125:** 301-313

Lee TV, Ding T, Chen Z, Rajendran V, Scherr H, Lackey M, Bolduc C, Bergmann A (2008) The E1 ubiquitin-activating enzyme Uba1 in Drosophila controls apoptosis autonomously and tissue growth non-autonomously. *Development* **135:** 43-52

Lemons D, McGinnis W (2006) Genomic Evolution of Hox Gene Clusters. *Science* **313:** 1918-1922

Lessard J, Baban S, Sauvageau G (1998) Stage-Specific Expression of Polycomb Group Genes in Human Bone Marrow Cells. *Blood* **91:** 1216-1224

Levine M, Hafen E, Garber RL, Gehring WJ (1983) Spatial distribution of *Antennapedia* transcripts during *Drosophila development*. *EMBO J* **2:** 2037-2046

Levine SS, Weiss A, Erdjument-Bromage H, Shao Z, Tempst P, Kingston RE (2002) The Core of the Polycomb Repressive Complex Is Compositionally and Functionally Conserved in Flies and Humans. *Mol Cell Biol* **22:** 6070-6078

Lewis EB (1978) A gene complex controlling segmentation in *Drosophila*. *Nature* **276:** 565-570

Lewis P (1947) *Pc*: Polycomb. In *Drosophila Information Service* Vol. 21, p 69.

Locke J, Kotarski MA, Tartof KD (1988) Dosage-Dependent Modifiers of Position Effect Variegation in Drosophila and a Mass Action Model That Explains Their Effect. *Genetics* **120:** 181-198

Maeda RK, Karch F (2006) The ABC of the BX-C: the bithorax complex explained. *Development* **133:** 1413-1422

Maeda RK, Karch F (2010) *Cis*-Regulation in the *Drosophila* Bithorax Complex. In *Hox Genes*, Deutsch JS (ed), Vol. 689, pp 17-40. Springer New York

Mager J, Montgomery ND, de Villena FP-M, Magnuson T (2003) Genome imprinting regulated by the mouse Polycomb group protein Eed. *Nat Genet* **33:** 502-507

Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, Dynlacht BD, Reinberg D (2008) Ezh1 and Ezh2 Maintain Repressive Chromatin through Different Mechanisms. *Molecular cell* **32:** 503-518

Martinez A-M, Cavalli G (2006) The role of Polycomb Group Proteins in Cell Cycle Regulation During Development. *Cell Cycle* **5:** 1189-1197

Martinez A-M, Colomb S, Déjardin J, Bantignies F, Cavalli G (2006) Polycomb group-dependent Cyclin A repression in Drosophila. *Genes & Development* **20:** 501-513

Martinez A-M, Schuettengruber B, Sakr S, Janic A, Gonzalez C, Cavalli G (2009) Polyhomeotic has a tumor suppressor activity mediated by repression of Notch signaling. *Nat Genet* **41:** 1076-1082

McCall K, Bender M (1996) Probes of chromatin accessibility in the Drosophila bithorax complex respond differently to Polycomb-mediated repression. *EMBO J* **15:** 569-580

McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring WJ (1984) A conserved DNA sequence in homoeotic genes of the *Drosophila Antennapedia* and *bithorax* complexes. *Nature* **308:** 428-433

Miller MR, Robinson KJ, Cleary MD, Doe CQ (2009) TU-tagging: cell type-specific RNA isolation from intact complex tissues. *Nat Meth* **6:** 439-441

Min J, Zhang Y, Xu R-M (2003) Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes & Development* **17:** 1823-1828

Moberg KH, Schelble S, Burdick SK, Hariharan IK (2005) Mutations in erupted, the Drosophila Ortholog of Mammalian Tumor Susceptibility Gene 101, Elicit Non-Cell-Autonomous Overgrowth. *Developmental Cell* **9:** 699-710

Mohd-Sarip A, Venturini F, Chalkley GE, Verrijzer CP (2002) Pleiohomeotic Can Link Polycomb to DNA and Mediate Transcriptional Repression. *Mol Cell Biol* **22:** 7473-7483

Mohr SE, Boswell RE (1999) Zimp encodes a homologue of mouse Miz1 and PIAS3 and is an essential gene in Drosophila melanogaster. *Gene* **229:** 109-116

Motaleb MA, Takihara Y, Nomura M, Matsuda Y, Higashinakagawa T, Shimada K (1996) Structural Organization of the rae28 Gene, a Putative Murine Homologue of the Drosophila polyhomeotic Gene. *Journal of Biochemistry* **120:** 797-802

Mujtaba S, Manzur KL, Gurnon JR, Kang M, Van Etten JL, Zhou M-M (2008) Epigenetic transcriptional repression of cellular genes by a viral SET protein. *Nat Cell Biol* **10:** 1114-1122

Muller HJ (1932) Further studies on the nature and causes of gene mutations. *Proceedings of the 6th International Congress of Genetics***:** 231-255

Müller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA (2002) Histone Methyltransferase Activity of a Drosophila Polycomb Group Repressor Complex. *Cell* **111:** 197-208

Muller M, Affolter M, Leupin W, Otting G, Wuthrich K, Gehring WJ (1988) Isolation and sequence-specific DNA binding of the Antennapedia homeodomain. *EMBO J* **7:** 4299-4304

Müller P, Boutros M, Zeidler MP (2008) Identification of JAK/STAT pathway regulators--Insights from RNAi screens. *Seminars in Cell & Developmental Biology* **19:** 360-369

Muller P, Kuttenkeuler D, Gesellchen V, Zeidler MP, Boutros M (2005) Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature* **436:** 871-875

Nègre N, Hennetin J, Sun LV, Lavrov S, Bellis M, White KP, Cavalli G (2006) Chromosomal Distribution of PcG Proteins during *Drosophila* Development. *PLoS Biol* **4:** e170

Ng J, Hart CM, Morgan K, Simon JA (2000) A Drosophila ESC-E(Z) Protein Complex Is Distinct from Other Polycomb Group Complexes and Contains Covalently Modified ESC. *Mol Cell Biol* **20:** 3069-3078

Nomura M, Takihara Y, Shimada K (1994) Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: One of the early inducible clones encodes a novel protein sharing several highly homologous regions with a Drosophila Polyhomeotic protein. *Differentiation* **57:** 39-50

Nusslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287:** 795-801

Ohta H, Sawada A, Kim JY, Tokimasa S, Nishiguchi S, Humphries RK, Hara J, Takihara Y (2002) Polycomb Group Gene rae28 Is Required for Sustaining Activity of Hematopoietic Stem Cells. *The Journal of Experimental Medicine* **195:** 759-770

Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS, Edgar KA, Howie E, Killpack K, Milash B, Norton A, Thao D, Whittaker K, Winner MA, Friedman L, Margolis J, Singer MA, Kopczynski C, Curtis D, Kaufman TC, Plowman GD, Duyk G, Francis-Lang HL (2004) Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat Genet* **36:** 288-292

Paro R, Hogness DS (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of Drosophila. *Proceedings of the National Academy of Sciences* **88:** 263-267

Pasini D, Hansen KH, Christensen J, Agger K, Cloos PAC, Helin K (2008) Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and Polycomb-Repressive Complex 2. *Genes & Development* **22:** 1345-1355

Patani N, Mokbel K (2010) Herceptin and breast cancer: An overview for surgeons. *Surgical Oncology* **19:** e11-e21

Peterson A, Kyba M, Bornemann D, Morgan K, Brock H, Simon J (1997) A domain shared by the Polycomb group proteins Scm and ph mediates heterotypic and homotypic interactions. *Mol Cell Biol* **17:** 6683-6692

Pietersen AM, van Lohuizen M (2008) Stem cell regulation by polycomb repressors: postponing commitment. *Current Opinion in Cell Biology* **20:** 201-207

Ponting CP (1995) SAM: A novel motif in yeast sterile and drosophila polyhomeotic proteins. *Protein Science* **4:** 1928-1930

Puro J, NygrÉN T (1975) Mode of action of a homoeotic gene in Drosophila melanogaster. *Hereditas* **81:** 237-247

Rawlings J, Rennebeck G, Harrison S, Xi R, Harrison D (2004) Two Drosophila suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities. *BMC Cell Biology* **5:** 38

Reynolds-Kenneally J, Mlodzik M (2005) Notch signaling controls proliferation through cell-autonomous and non-autonomous mechanisms in the Drosophila eye. *Developmental Biology* **285:** 38-48

Ringrose L (2007) Polycomb comes of age: genome-wide profiling of target sites. *Current Opinion in Cell Biology* **19:** 290-297

Ringrose L, Paro R (2007) Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development* **134:** 223-232

Ringrose L, Rehmsmeier M, Dura J-M, Paro R (2003) Genome-Wide Prediction of Polycomb/Trithorax Response Elements in Drosophila melanogaster. *Developmental Cell* **5:** 759-771

Sato T, Hayes PH, Denell RE (1983) Homoeosis in *Drosophila*: Maternal effect of the *enhancer of Polycomb* locus and its interaction with *Polycomb* and related loci. *Developmental Genetics* **4:** 185-198

Saurin AJ, Shao Z, Erdjument-Bromage H, Tempst P, Kingston RE (2001) A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. *Nature* **412:** 655-660

Schindler C, Fu XY, Improta T, Aebersold R, Darnell JE (1992) Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proceedings of the National Academy of Sciences* **89:** 7836-7839

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

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

Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G (2007) Genome Regulation by Polycomb and Trithorax Proteins. *Cell* **128:** 735-745

Schwartz YB, Kahn TG, Nix DA, Li X-Y, Bourgon R, Biggin M, Pirrotta V (2006) Genome-wide analysis of Polycomb targets in Drosophila melanogaster. *Nat Genet* **38:** 700-705

Schwartz YB, Pirrotta V (2007) Polycomb silencing mechanisms and the management of genomic programmes. *Nat Rev Genet* **8:** 9-22

Scott MP, Weiner AJ (1984) Structural relationships among genes that control development: sequence homology between the *Antennapedia, Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. *Proceedings of the National Academy of Sciences* **81:** 4115-4119

Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu C-t, Bender W, Kingston RE (1999) Stabilization of Chromatin Structure by PRC1, a Polycomb Complex. *Cell* **98:** 37-46

Shearn A (1989) The ash-1, ash-2 and trithorax Genes of Drosophila melanogaster Are Functionally Related. *Genetics* **121:** 517-525

Shevelyov YY, Nurminsk DI (2011) The Nuclear Lamina as a Gene-silencing Hub. *Current Issues in Molecular Biology* **14:** 27-38

Shirai M, Osugi T, Koga H, Kaji Y, Takimoto E, Komuro I, Hara J, Miwa T, Yamauchi-Takihara K, Takihara Y (2002) The Polycomb-group gene Rae28 sustains Nkx2.5/Csx expression and is essential for cardiac morphogenesis. *The Journal of Clinical Investigation* **110:** 177-184

Simon J, Chiang A, Bender W, Shimell MJ, O'Connor M (1993) Elements of the Drosophila Bithorax Complex That Mediate Repression by Polycomb Group Products. *Developmental Biology* **158:** 131-144

Simon JA, Kingston RE (2009) Mechanisms of Polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* **10:** 697-708

Sing A, Pannell D, Karaiskakis A, Sturgeon K, Djabali M, Ellis J, Lipshitz HD, Cordes SP (2009) A Vertebrate Polycomb Response Element Governs Segmentation of the Posterior Hindbrain. *Cell* **138:** 885-897

Smouse D, Goodman C, Mahowald A, Perrimon N (1988) polyhomeotic: a gene required for the embryonic development of axon pathways in the central nervous system of Drosophila. *Genes & Development* **2:** 830-842

Smouse D, Perrimon N (1990) Genetic dissection of a complex neurological mutant, polyhomeotic, in Drosophila. *Developmental Biology* **139:** 169-185

Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* **6:** 846-856

Stock JK, Giadrossi S, Casanova M, Brookes E, Vidal M, Koseki H, Brockdorff N, Fisher AG, Pombo A (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* **9:** 1428-1435

Strahl BD, Allis CD (2000) The language of covalent histone modifications. *Nature* **403:** 41-45

Struhl G (1981) A gene product required for correct initiation of segmental determination in *Drosophila*. *Nature* **293:** 36-41

Struhl G, Akam M (1985) Altered distributions of *Ultrabithorax* transcripts in *extra sex combs* mutant embryos of *Drosophila*. *EMBO J* **4:** 3259-3264

Strutt H, Paro R (1997) The polycomb group protein complex of Drosophila melanogaster has different compositions at different target genes. *Mol Cell Biol* **17:** 6773-6783

Takihara Y, Tomotsune D, Shirai M, Katoh-Fukui Y, Nishii K, Motaleb MA, Nomura M, Tsuchiya R, Fujita Y, Shibata Y, Higashinakagawa T, Shimada K (1997) Targeted disruption of the mouse homologue of the Drosophila polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. *Development* **124:** 3673-3682

Thompson BJ, Mathieu J, Sung H-H, Loeser E, Rørth P, Cohen SM (2005) Tumor Suppressor Properties of the ESCRT-II Complex Component Vps25 in Drosophila. *Developmental Cell* **9:** 711-720

Tokimasa S, Ohta H, Sawada A, Matsuda Y, Kim JY, Nishiguchi S, Hara J, Takihara Y (2001) Lack of the Polycomb-group gene rae28 causes maturation arrest at the early B-cell developmental stage. *Experimental Hematology* **29:** 93-103

Tolhuis B, Muijrers I, de Wit E, Teunissen H, Talhout W, van Steensel B, van Lohuizen M (2006) Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. *Nat Genet* **38:** 694-699

Tomotsune D, Takihara Y, Berger J, Duhl D, Joo S, Kyba M, Shirai M, Ohta H, Matsuda Y, Honda BM, Simon J, Shimada K, Brock HW, Randazzo F (1999) A novel member of murine Polycomb-group proteins, Sex comb on midleg homolog protein, is highly conserved, and interacts with RAE28/mph1 in vitro. *Differentiation* **65:** 229-239

Uhlirova M, Jasper H, Bohmann D (2005) Non-cell-autonomous induction of tissue overgrowth by JNK/Ras cooperation in a Drosophila tumor model. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 13123-13128

Vaccari T, Bilder D (2005) The Drosophila Tumor Suppressor vps25 Prevents Nonautonomous Overproliferation by Regulating Notch Trafficking. *Developmental Cell* **9:** 687-698

van Lohuizen M, Verbeek S, Scheljen B, Wientjens E, van der Guidon H, Berns A (1991) Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. *Cell* **65:** 737-752

Wang GG, Allis CD, Chi P (2007) Chromatin remodeling and cancer, part I: covalent histone modifications. *Trends in Molecular Medicine* **13:** 363-372

Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, Jones RS, Zhang Y (2004a) Role of histone H2A ubiquitination in Polycomb silencing. *Nature* **431:** 873-878

Wang J, Lee C-HJ, Lin S, Lee T (2006) Steroid hormone-dependent transformation of polyhomeotic mutant neurons in the Drosophila brain. *Development* **133:** 1231-1240

Wang J, Mager J, Chen Y, Schneider E, Cross JC, Nagy A, Magnuson T (2001) Imprinted X inactivation maintained by a mouse Polycomb group gene. *Nat Genet* **28:** 371-375

Wang L, Brown JL, Cao R, Zhang Y, Kassis JA, Jones RS (2004b) Hierarchical Recruitment of Polycomb Group Silencing Complexes. *Molecular cell* **14:** 637-646

Wang R, Ilangovan U, Leal BZ, Robinson AK, Amann BT, Tong CV, Berg JM, Hinck AP, Kim CA (2011) Identification of Nucleic Acid Binding Residues in the FCS Domain of the Polycomb Group Protein Polyhomeotic. *Biochemistry* **50:** 4998-5007

Wedeen C, Harding K, Levine M (1986) Spatial regulation of *antennapedia* and *bithorax* gene expression by the *Polycomb* locus in *Drosophila*. *Cell* **44:** 739-748

Wodarz A, Ramrath A, Grimm A, Knust E (2000) Drosophila Atypical Protein Kinase C Associates with Bazooka and Controls Polarity of Epithelia and Neuroblasts. *The Journal of Cell Biology* **150:** 1361-1374

Woo CJ, Kharchenko PV, Daheron L, Park PJ, Kingston RE (2010) A Region of the Human HOXD Cluster that Confers Polycomb-Group Responsiveness. *Cell* **140:** 99-110

Yamaki M, Isono K, Takada Y, Abe K, Akasaka T, Tanzawa H, Koseki H (2002) The mouse Edr2 (Mph2) gene has two forms of mRNA encoding 90- and 36-kDa polypeptides. *Gene* **288:** 103-110

Yan R, Small S, Desplan C, Dearolf CR, Darnell JE (1996) Identification of a Stat Gene That Functions in Drosophila Development. *Cell* **84:** 421-430

Zink B, Paro R (1989) In vivo binding pattern of a trans-regulator of homoeotic genes in Drosophila melanogaster. *Nature* **337:** 468-471