ANALYSIS OF GENE TRAPPED EMBRYONIC STEM CELLS AND CHARACTERIZATION OF HOX GENE EXPRESSION PATTERN IN ADDITIONAL SEX COMBS LIKE-1 (ASXL1) MUTANTS

by

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ABSTRACT

Cells differentiate into different cell types through the development process. To maintain cell identity, trithorax and Polycomb proteins, which are responsible for the active state and silent state respectively, play an important role by maintaining the gene expression pattern of a mother cell during cell division, so the gene expression pattern can be passed on to its daughter cell. To date, many of these Polycomb group proteins have been identified in *Drosophila*. We look at a member of Enhancer of trithorax and Polycomb (ETP), called the Additional sex combs (Asx) protein because it is necessary for repression and activation maintenance functions in *Drosophila*. No ETP has been molecularly characterized. Our lab is studying the mammalian homologs of *Asx* in mice, called (*Additional sex combs-like*) *Asxl1*, *Asxl2*, and *Asxl3*. We have successfully created *Asxl1* mutant mice using homologous recombination by Cynthia Fisher, our former PhD student. 1) Using a gene trapped ES cell line, we would like to create another mutant mice, *Asxl2*, by gene trapped method. The characterization of ES cell line shows that this cell line is not ideal to make chimera mice because it has double integration. This will be difficult for future molecular analysis. 2) I also would like to characterize the expression pattern of *Hox* genes of *Asxl1* embryos by mRNA in-situ hybridization since homeotic transformations are observed in these mutants. The analysis of *Hox* genes in *Asxl1* mutant shows that all three *Hox* genes I examined, *Hox a4*, *a7*, and *c8*, show anterior shift of the anterior boundaries; *Hox c8* in the head region is suppressed in *Asxl1* mutants. My results provide evidence that homeotic transformation in *Asxl1* mutants is due to mis-expression of *Hox* genes. Results also support that *Asxl1* is a true ETP because the mutants show both derepression and repression of *Hox* genes expression.
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<table>
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<th>Definition</th>
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<tr>
<td>Antp-C</td>
<td>Antennapaedia complex</td>
</tr>
<tr>
<td>Ash</td>
<td>Abnormal, small and homeotic</td>
</tr>
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<td>BX-C</td>
<td>Bithorax complex</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E(z)</td>
<td>Enhancer of zeste</td>
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<tr>
<td>GAF</td>
<td>GAGA factor</td>
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<td>Mixed lineage leukemia</td>
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<td>Maintenance proteins</td>
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<td>Polycomb group</td>
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<td>Trithorax group</td>
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1 INTRODUCTION

1.1 Maintenance and epigenetic

In mammalian development, a single fertilized cell proliferates and differentiates into about 250 different cell types. Cells acquire their identities by interacting with each other and their environment. These transient events cause changes in their gene expression patterns, which define the identities of cells. Daughter cells must inherit the specific gene expression patterns of their mother cells to maintain their identities. This process is called "maintenance" (Jacobs and van Lohuizen, 2002). Over twenty years ago, Brown proposed that there must be marks for active and silent gene expression that are stable to mitosis that allow the reestablishment of the gene expression (Brown, 1984). Disruption of gene expression patterns can result in developmental defects and in disease (Jacobs and van Lohuizen, 2002; Korber and Horz, 2004; Otte and Kwaks, 2003). Therefore, it is important to understand the mechanisms of maintenance.

The mechanisms of maintenance must be at the epigenetic level because all cells inherit the same genomic DNA, yet gene expression pattern differs (Cavalli, 2002). It is important for maintenance to be stable to mitosis (Brock and Fisher, 2005; Ringrose and Paro, 2004). Epigenetic regulatory mechanisms are common in eukaryotes, and include mating type silencing in yeast, position-effect variegation, gametic imprinting, and X chromosome inactivation. Epigenetic regulatory mechanisms use an overlapping set of common processes including DNA methylation, histone modification, histone variants, nucleosome remodeling, nuclear localization, and establishment of specific chromatin structures (Ringrose and Paro, 2004; Carmo-Fonseca, 2002; Kornberg and Lorch, 2002; Vermaak et al., 2003).

Recently, much attention has been paid to the roles of histone modification in epigenesis. Eukaryotic DNA is wrapped around nucleosomes, composed of four core histones (H2A, H2B, H3, H4) (Klug et al., 1980). Extending from the nucleosomes are tails of undetermined structure,
which can be modified by acetylation, methylation, phosphorylation, or ubiquitination at multiple residues. It is proposed that the modifications of histone act as a histone code which act directly to change the structure of the nucleosomal arrays or indirectly to recruit other proteins required for repressing or activating gene expression (Jenuwein, 2001; Strahl and Allis, 2000). Gene activation is correlated with methylation of lysine 4 (K4), and K79 of H3 and K20 of H4, and acetylation of H3K9 and K14, while gene repression is correlated with methylation of H3K9, H3K27, and absence of acetylation (Wang et al., 2004d). Acetylation and methylation are carried out by histone acetyl transferases and histone methyltransferases (HMTs) respectively. Removal of acetylation and methylation requires histone deacetylases (HDACs) and nuclear amine oxidase homologs respectively (Moazed et al., 2004; Shi et al., 2004; Wang et al., 2004c). It has been proposed that incorporation of histone variants associated with gene activation or repression might provide stable marks or be the normal substrates of histone modification (Brock and Fisher, 2005).

1.2 Organization and regulation of homeotic (Hox) genes

*Hox* genes are the best characterized targets of MPs. In Drosophila, mutations in homeotic (*Hox*) genes cause transformation of one body segment to a homologous segment. Therefore, *Hox* genes determine segmental identities along the anterior-posterior axis. *Hox* genes are expressed in spatially regulated patterns from the anterior to the posterior. The pattern of *Hox* genes expressed in a segment determines segmental identity. Fewer *Hox* genes are expressed in anterior segments, and more *Hox* genes are expressed in posterior segments. Consistent with this observation, loss of function mutations of *Hox* genes cause anterior transformations, whereas ectopic expression of *Hox* genes causes posterior transformations.

The expression patterns of homeotic genes are first initiated by transiently expressed molecules and are then maintained by MPs. However, posteriorly-expressed homeotic genes
repress the expression of more anteriorly-expressed homeotic genes, so Hox genes auto-regulate (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988). The first MP genes were identified because of their homeotic phenotypes, and because the mutations did not map to the homeotic loci. Many PcG proteins were identified based on their posterior homeotic transformations. Conversely, TrxG genes mutants exhibit anterior transformation, which is the phenotype in loss-of-function homeotic gene mutants. In turn, these observations made it possible to deduce that PcG genes repress Hox genes, whereas TrxG genes activate Hox genes.

Hox genes are well conserved throughout evolution from basal Metazoans to mammals (Veraksa et al., 2000). Hox genes in mice specify identity along the anterior-posterior axis, but in addition have acquired many other functions, including roles in the proximal-distal axis in the limb, and in hematopoiesis (Abramovich and Humphries, 2005; Goodman, 2002). Hox genes encode transcription factors with a conserved helix-turn-helix DNA-binding domain, the 180 base-pair homeobox (McGinnis et al., 1984a; McGinnis et al., 1984b; Scott and Weiner, 1984).

In Drosophila, there are eight Hox genes, organized into two clusters, called the Antennapaedia (Antp-C) and bithorax complex (BX-C) (Izpisua-Belmonte and Duboule, 1992). During mammalian evolution, the Hox gene cluster has undergone two large-scale duplication events resulting in four paralogous clusters. They are termed A, B, C, and D, each located on different chromosome (Martinez and Amemiya, 2002). There are a total of 40 Hox genes in mouse. The most 3' homeotic genes are expressed in the most anterior part of the embryo, while the most 5' are expressed posteriorly. This is called the colinearity relationship, and suggests that the organization of Hox genes is functionally important, but the underlying reason for this is not known (Deschamps et al., 1999).

Mice containing a knock-out Hox gene show homeotic transformations (Condie and Capecchi, 1994; Davis and Capecchi, 1994; Gendron-Maguire et al., 1993; Kostic and Capecchi, 1994; Le Mouellic et al., 1992). Single Hox gene mutants show pretty mild phenotypes.
Compound mutants of paralogous Hox genes exhibit more severe phenotypes (Condie and Capecchi, 1994; Horan et al., 1995), suggesting that the functions of paralogous Hox genes are redundant and also that the products of paralogous Hox genes may work synergistically.

1.3 Genetics of maintenance proteins

Proteins which are required for maintaining gene expression patterns are called “maintenance proteins” (MPs). MPs were first discovered in Drosophila (Garcia-Bellido, 1977; Ingham P.W., 1980; Lewis P.H., 1947; Lewis, 1978; Shearn et al., 1971; Slifer, 1942). MPs can be divided into two groups: the Polycomb group (PcG), and the Trithorax group (TrxG), that maintain silencing and activation respectively. It is estimated that there are about 30-40 PcG genes in Drosophila (Jurgen G., 1985; Landecker et al., 1994). About 20 PcG genes and 15 TrxG genes have been cloned and characterized (Brock and Fisher, 2005). Most MPs have homologs in eukaryotes (Brock and Fisher, 2005), and similar function in different species (Mahmoudi and Verrijzer, 2001; Muller et al., 1995; Ross and Zarkower, 2003; Zhang et al., 2003).

Well-studied targeted genes of MPs are homeotic genes, which specify identities of body segments along the anterior-posterior axis (Garcia-Bellido, ; Ingham P.W., 1980; Jurgen G., 1985; Kennison and Tamkun, 1988; Lewis P.H., 1947; Lewis, 1978; Shearn et al., 1971; Slifer, 1942). Many MPs were identified first because they regulate homeotic genes in Drosophila. TrxG mutants exhibit anteriorly directed segmental transformations, which are similar to the loss-of-function mutations in homeotic genes (Kennison, 2004). These results suggest that the function of TrxG proteins is to maintain expression of homeotic genes. On the other hand, PcG mutants exhibit posteriorly directed segmental transformations, whose phenotypes are similar to the gain-of-function mutations of homeotic mutants that cause ectopic expression (Lewis, 1978). These results show that PcG proteins are required to silence homeotic genes. Subsequent
molecular studies have confirmed these conclusions (Breen and Harte, 1993; Mazo et al., 1990). Flies which have mutations in two different PcG genes show more severe posteriorly transformed phenotypes than single mutants (Cheng et al., 1994; Jurgen G., 1985). Similarly, flies that are double mutant for TrxG genes exhibit more severe anteriorly transformed phenotypes than single mutants (Shearn, 1989). These observations suggest that PcG and TrxG genes act combinatorially. Most TrxG genes were discovered because they suppress the posterior transformations of PcG mutations (Kennison and Tamkun, 1988; Shearn, 1989; Kennison J.A., 1987).

The signaling molecules and transcription factors that initiate homeotic gene expression patterns are transiently present in development. Mutants of either PcG or TrxG genes exhibit normal homeotic genes expression pattern initially, suggesting that MPs do not affect initiation of homeotic expression patterns. The homeotic gene expression patterns become abnormal after few hours later in PcG or TrxG mutants (Soto et al., 1995; Struhl and Akam, 1985). Therefore, initiation and maintenance are two distinctive separate mechanisms.

1.4 Molecular biology of MPs

The characterized MPs are chromatin proteins. Unsurprisingly, MPs share domains with other chromatin proteins suggesting that MPs function similarly to other chromatin proteins. The shared domains mostly mediate protein-protein interactions, and include the chromo- and bromodomains that recognize methylated and acetylated histone tails (de, X et al., 2005; Kennison, 1995); the SAM domain (Kyba and Brock, 1998; Peterson et al., 1997), WD40 repeats (Simon et al., 1995); and PHD fingers (O'Connell et al., 2001; Tie et al., 2003; Xia et al., 2003).

A breakthrough in our understanding of MP function has been the discovery that some MPs are histone modifying enzymes. MPs with SET domains including the PcG protein Enhancer of zeste (E(z)) methylates H3K27. The TrxG proteins Abnormal, small and homeotic 1
Ash1) methylates H3K4, H3K9, and H4K20, and Trithorax (Trx) and their mammalian homologs are HMTs (Klymenko and Muller, 2004; Kuzmichev et al., 2005; Shanower et al., 2005; Cao and Zhang, 2004) which methylate H3K4. Ring2 is an E3 ubiquitin ligase specific for histone H2A (Wang et al., 2004a). Grappa, which is required for maintenance, is the H3K79 methylase of *Drosophila* (Shanower et al., 2005). Its yeast homolog DOT1 is required for transcriptional elongation (Krogan et al., 2003a; Ng et al., 2003).

So far two PcG complexes have been purified. The Polycomb Repressive Complex 1 (PRC1) contains Polyhomeotic (Ph), Pc, Posterior sex combs (Psc), Ring/Sex combs extra (Shao et al., 1999), as well as Zeste and general transcription factors. PRC1 is able to inhibit nucleosome remodeling by SWI-SNF complex (Francis et al., 2001). PRC2 contains E(z), Extra sex combs, Suppressor 12 of Zeste, and Polycomblike (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). It has been proposed that histone modification mediated by PRC2 recruits PRC1 (Wang et al., 2004b), even though there is argument against this (Poux et al., 2001). There are very likely multiple complexes that contain PcG proteins (Otte and Kwaks, 2003).

TrxG proteins also exist as multimeric complexes. Brahma, Moira and Osa are members of Brahma-Associated Protein complexes, which are functionally similar to SWI-SNF nucleosome remodeling complexes (Kennison, 2004). Trx is also found in TAC1 complex and human homolog of Trx, Mll, is a component of a complex related to the yeast COMPASS complex (Hughes et al., 2004; Yokoyama et al., 2004).

In *Drosophila*, MPs bind to cis-regulatory elements of their target loci called Maintenance Elements (MEs) (Brock and Fisher, 2005). The best characterized loci of MEs are those of homeotic genes. Enhancers are responsible for the regulation of spatial expression, while MEs control the maintenance of expression. MEs are made up by intermingled but separable TrxG binding sites, called TrxG Response Elements (TRE), and PcG binding sites.
called PRE (Tillib et al., 1999). MEs are required continuously in development (Busturía et al., 1997). How MPs are recruited to MEs is not clear. The five MPs which have DNA sequence specific binding activity are Pleiohomeotic and Pleiohomeotic-like (Brown et al., 1998; Brown et al., 2003), the GAGA Factor (GAF) which is encoded by *trithoraxlike* (Hodgson et al., 2001) and Pipsqueak (Hodgson et al., 2001) and Dsp1 (Dejardin et al., 2005). Recent studies suggest that multiple factor binding sites are required at MEs (Hodgson et al., 2001; Ringrose et al., 2003). So far, no MEs have been identified in mammals.

1.5 Mechanisms of MPs

Many mechanisms have been proposed for MP function in the past. The original idea was that MPs mediate changes in chromatin structure to make it less (PcG) or more (TrxG) accessible to transcription factors. This idea has been tested with ambiguous results (Schlossherr et al., 1994). Other ideas include the possibilities that MPs regulate nuclear architecture or position of the target locus in the genome, regulate replication timing, or regulate chromosome pairing (Brock and van Lohuizen, 2001). MPs also mediate association with nucleosomes to change chromatin structure (Bantignies et al., 2003; Francis et al., 2004; Lavigne et al., 2004).

More recently attention has been focused on the role of MPs in histone modification at the promoter, and a role in transcriptional initiation or promoter escape. PcG proteins binding to ME might interact with the promoter. PRC1 is associated with TBP Associated Factors (TAFs) (Breiling et al., 2001; Saurin et al., 2001; Wang and Brock, 2003), and PcG proteins bind near promoters (Milne et al., 2002; Nakamura et al., 2002; Orlando et al., 1998). In yeast, the SET1 HMT (homolog of Trx) in the COMPASS complex, has a key role in transcriptional elongation (Dover et al., 2002; Krogan et al., 2003b; Wood et al., 2003). The mammalian MLL complex, contains the homologs of the COMPASS subunits (Hughes et al., 2004; Miller et al., 2001; Yokoyama et al., 2004), and suggests that MLL has a general role in
transcription (Guenther et al., 2005). In Drosophila, Trx is recruited to the hsp70 locus, is required for the heat shock response, and coimmunoprecipitates with RNA Polymerase II that is phosphorylated at Serine 5 of the C Terminal Domain. This modification occurs after transcriptional initiation (Smith et al., 2004). Similarly recent studies suggest that PRC2 acts at the Ubx promoter by phosphorylating histone H3K27 just downstream of the promoter of Ubx (Wang et al., 2004b), and that ubiquitylation of histone H2A by Bmi-1 (Psc homolog) and Ring is a prerequisite for H3K27 methylation by PRC2 at the Hox c13 promoter region. In a test system, PcG mutations allow recruitment of RNA Polymerase II and general transcription factors, but prevent promoter escape (Dellino et al., 2004).

TrxG and PcG proteins might act as antagonists to each other, because PcG/trxG double mutants have nearly normal phenotypes (Klymenko and Muller, 2004). The HMT activity of Ash1 antagonizes Pc binding (Beisel et al., 2002). Conversely, PcG may prevent TrxG function in promoter escape.

1.6 Roles of mammalian MPs

Many mammalian MPs were identified based on sequence homology. Please refer to Table 1.1 for the currently identified mouse PcG proteins. Mice mutant for MPs have been created to understand the functions of the MPs. The results suggest that functions of MPs in anterior-posterior axis formation are conserved in mammals, but that MPs have acquired additional functions.

Mutants of MPs exhibit anterior-posterior transformation and ectopic expression of some Hox genes. The following are phenotypes observed in some MP mutants. Mel18 mutant mice exhibit many posterior transformations: the first cervical (C1) vertebra to the second cervical (C2), C2 vertebra to the third cervical (C3), and fusion of the seventh cervical vertebra (C7) with the first thoracic vertebra (T1) (Akasaka et al., 1996). Also transformations are observed in
thoracic vertebra including T1 to the second thoracic vertebra (T2) and the ninth thoracic vertebra (T9) to the tenth thoracic vertebra (T10), and the thirteenth thoracic vertebra (T13) to the first lumbar vertebra (L1) (Akasaka et al., 1996). The sixth lumbar vertebra (L6) and the fourth sacral (S4) become the sacral vertebra and the first caudal vertebrae respectively (Akasaka et al., 1996).

**Table 1.1: Mouse PcG homolog**

<table>
<thead>
<tr>
<th>Mouse PcG</th>
<th>Drosophila homolog</th>
<th>Conserved domains</th>
<th>Reference</th>
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<tr>
<td>M33</td>
<td>Pc</td>
<td>C-box</td>
<td>(Pearce et al., 1992)</td>
</tr>
<tr>
<td>Pc2</td>
<td>Pc</td>
<td>AT-hook</td>
<td>(Alkema et al., 1997)</td>
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<td>Pc3</td>
<td>Pc</td>
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<td>(Hemenway et al., 2000)</td>
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<tr>
<td>BmiI</td>
<td>Psc</td>
<td>RING zinc finger</td>
<td>(Haupt et al., 1991)(7)</td>
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<td>NsPcI</td>
<td></td>
<td></td>
<td>(Nunes et al., 2001)</td>
</tr>
<tr>
<td>Ring1a</td>
<td>Ring</td>
<td>RING zinc finger</td>
<td>(Schoorlemmer et al., 1997)</td>
</tr>
<tr>
<td>Rnf2(Ring1b)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ScmI1</td>
<td>Scm</td>
<td>SAM/SPM domain</td>
<td>(van, V et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MBT repeats Zinc</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>fingers</td>
<td></td>
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<tr>
<td>EnxI</td>
<td>E(z)</td>
<td>SET domain, C terminal</td>
<td>(Hobert et al., 1996)</td>
</tr>
<tr>
<td>Enx2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eed</td>
<td>Esc</td>
<td>WD40 repeats</td>
<td>(Shumacher et al., 1996)</td>
</tr>
<tr>
<td>AsxI1</td>
<td></td>
<td>ASXH domain</td>
<td>(Fisher et al., 2003)</td>
</tr>
<tr>
<td>AsxI2</td>
<td></td>
<td>NR binding motifs</td>
<td>(Katoh and Katoh, 2003)</td>
</tr>
<tr>
<td>AsxI3</td>
<td></td>
<td></td>
<td>(Katoh and Katoh, 2004)</td>
</tr>
<tr>
<td>EpcI</td>
<td>E(Pc)</td>
<td></td>
<td>(Shimono et al., 2000)</td>
</tr>
<tr>
<td>Epc2</td>
<td></td>
<td></td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>E2F6</td>
<td></td>
<td>DNA binding domain</td>
<td>(Morkel et al., 1997)</td>
</tr>
</tbody>
</table>

*BmiI* null mutants mice were also generated to understand its functions (van der Lugt et al., 1994). Homozygous *BmiI* mutants are able to survive up to birth but will die shortly, about 1-3 days. They have difficulties in growing and are smaller in size compared to the littermates. Like its homologue *Psc*, *BmiI* mutants also suffer posterior transformations. These transformations include extra bone on C1, broadening and splitting of C2, and transformations of C7 to T1, T7 to T8, T13 to L1, and L6 to S1. They have decreased cell density in different layers of the cerebellum. Also *BmiI* mutants have involuted thymus and small spleens.
compared to wild-type. They have abnormal number of hematopoietic cells: high number of immature thymocyte, reduced numbers of B lymphoid and myeloid. Both the immature and mature B lymphoid is reduced. *Hox* genes analysis of *Bmil* null shows that the rostral expression boundaries of *Hox a4, a5, b6, c4, c5, c6, c8,* and *c9* shifts one prevertebra anteriorly (van der Lugt et al., 1996). Overexpression *Bmil* mutants mice are also generated. They suffered the opposite (anterior) transformations as the null. The transformations include C2 to C1, T2 to T1, T3 to T2, S1 to L6, S2 to S1, T8 to T7, and L1 to T13 (Alkema et al., 1995).

*Rae28/mphl* also exhibits various kinds of posterior transformations including C7 to T1, fusion of C1 with C2, the sixth cervical vertebra (C6) to T1, L6 to the first sacral (S1) (Takihara et al., 1997). *M33*, Pc homolog, mutants exhibit both anterior and posterior transformations (Core et al., 1997). *M33* mutants have malformation of the exoccipital bone, are missing the atlas (C1) and exhibit transformations including C2 to C1, the seventh thoracic vertebra (T7) to the eighth thoracic vertebra (T8), and L6 to S1 (Core et al., 1997).

Ring1A, Ring homolog, mutants are not embryonic lethal. But instead of posterior transformation, they exhibit several anterior transformations including the third thoracic vertebra (T3) to the second thoracic vertebra (T2), T8 to T7, and L1 to T13 (del Mar et al., 2000). Unlike other MPs, *Mel18* and *M33* mutants can survive right up to birth and die few days to weeks after birth. Most *Rae28* mutants can survive up to birth and some homozygous mice were born.

The effect of *Eed* is more dramatic. The latest stage for *Eed* mutants to be observed is at 8.5 dpc (Faust et al., 1995). The mutant embryos exhibit abnormal gastrulation (Faust et al., 1995). Some mutants have large amounts of mesoderm located proximally in the embryos and the majority of mutants are still at 6-6.5 dpc egg cylinder-stage (Faust et al., 1995). Some mutants are arrested at gastrulation stage. Similar to the *Eed* mutant phenotypes, *Ezh2* mutants are embryonic lethal. No homozygous *Ezh2* mutant mice are observed in litter dissected at 10.5 dpc. The latest time homozygous embryos are observed is at 7.5 dpc, indicating that *Ezh2* is
required quite early in mouse development (O'Carroll et al., 2001). The 7.5 dpc mutant embryos are abnormal or do not complete gastrulation (O'Carroll et al., 2001). Initiation of gastrulation is detected but most mesoderm cells migrate into extraembryonic regions and form a bulge of cells which pushes on the embryonic portion of the embryos (O'Carroll et al., 2001). This phenotype is also observed in Eed mutants.

Besides PcG mutant mice, TrxG mutant mice are also generated to understand the biological functions of TrxG. Mll, trx homolog, mutant mice are embryonic lethal and viable only up to 10.5 dpc (Yu et al., 1995). They exhibit both anterior and posterior transformation, which are C7 to C6, and T3 to T2, and T13 to L1 and T3 to T2 respectively (Yu et al., 1995). Hox genes expression analysis of Mll +/- embryos have shown that the anterior of Hox c9 has shifted caudally 2-3 segments and Hox a7 is shifted one segment caudally (Yu et al., 1995).

Considering that PcG proteins are members of the PRC1 or PRC2 complexes, the pattern emerges that mutants of members of PRC1 (Mel-18, Bmi1, Rae28, M33) have mild phenotype compared to mutations in PRC2 members (Eed, Ezh2). The exception is the Ring1b mutants, because Ring1b mutants exhibit similar phenotypes to Eed and Ezh2 mutants. No Ring1b mutant embryos can be detected beyond 10.5 dpc (Voncken et al., 2003), and mutant embryos do not go through normal gastrulation. They exhibit mesoderm accumulation in the primitive streak and very little anteriorly expanding embryonic mesoderm and delay in embryonic and extraembryonic development (Voncken et al., 2003).

To determine whether misregulation of Hox genes is the basis for homeotic transformations observed in PcG and trxG mutants, Hox genes expression patterns have been analyzed in some of the MP mutants. Please refer to Table 1.2 for a summary of PcG mutant mouse of their phenotypes and the affected Hox genes. It has been observed that the anterior boundaries of Hoxa5, Hoxa7, Hoxb3, Hoxb4, Hoxb6, Hoxb8, Hoxc8, and Hoxd4, and Hoxa3, Hoxa4, Hoxa5, Hoxb3, Hoxb4, and Hoxd4 are shifted anteriorly in Mel18 and Rae28 mutants.
respectively causing the posterior transformation (Akasaka et al., 1996; Takihara et al., 1997). However, in M33 mutant embryos only Hoxa3 gene is affected among other Hox genes tested (Core et al., 1997). Therefore, M33 may not be critical for maintenance of Hox genes expression. Similarly, Ring1A mutants also exhibit very little effects on Hox genes expression. Only a subtle anterior shift of Hoxc8 anterior border in neuroectoderm and Hoxd4 in mesoderm are observed (del Mar et al., 2000). The penetrance of the later phenotype is low. The mild effects on the MPs mutants Hox genes expression patterns correlate with relatively mild observed phenotypes and support the redundancy functions of MPs in mammals.

### Table 1.2: A summary table of PcG murine mutants with homeotic phenotypes and the affected Hox genes expression pattern

<table>
<thead>
<tr>
<th>Mouse PcG mutant</th>
<th>Phenotypes</th>
<th>Affected Hox genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M33</td>
<td>C2→C1, T7→T8, L6→S1</td>
<td>Hoxa3</td>
<td>(Core et al., 1997)</td>
</tr>
<tr>
<td>Rae28</td>
<td>C7→T1, fusion of C1 with C2, C6→T1, L6→S1</td>
<td>Hox a3, a4, a5, b3, b4, and d4 anterior boundary shifted anteriorly</td>
<td>(Takihara et al., 1997)</td>
</tr>
<tr>
<td>Bmi1</td>
<td>Extra bone on C1, broadening and splitting of C2, C7→T1, T7→T8, T3→L1, L6→S1</td>
<td>Hox a4, a5, b6, c4, c5, c6, c8, and c9 anterior boundary shifted anteriorly</td>
<td>(van der Lugt et al., 1994)</td>
</tr>
<tr>
<td>Mel18</td>
<td>C1→C2, C2→C3, fusion of C7 with T1, T1→T2, T9→T10, T13→L1, L6→S4</td>
<td>Hoxa5, a7, b3, b4, b6, b8, c8, and d4 anterior boundary shifted anteriorly</td>
<td>(Akasaka et al., 1996)</td>
</tr>
<tr>
<td>Ring1a</td>
<td>T3→T2, T8→T7, L1→T13</td>
<td>Minor effect on Hox c8, and d4</td>
<td>(del Mar et al., 2000)</td>
</tr>
<tr>
<td>Ring1b</td>
<td>abnormal gastrulation</td>
<td>-</td>
<td>(Voncken et al., 2003)</td>
</tr>
<tr>
<td>Eed</td>
<td>abnormal gastrulation</td>
<td>-</td>
<td>(Faust et al., 1995)</td>
</tr>
<tr>
<td>Ezh2</td>
<td>abnormal gastrulation</td>
<td>-</td>
<td>(O’Carroll et al., 2001)</td>
</tr>
</tbody>
</table>

Besides maintaining Hox gene expression patterns, MPs are also involved in X chromosome inactivation, genomic imprinting, RNA interference, and stem cell proliferation.
For example: Eed, the Esc homolog, is required for genomic imprinting of paternal gene repression in mice (Mager et al., 2003) and maintenance of inactive state of X chromosome (Wang et al., 2001). Failure of Eed functions causes a subset of imprinted genes to express abnormal parent-of-origin expression alleles, showing that imprinting is impaired in these mutants (Mager et al., 2003). Eed is necessary for maintenance of the inactive state of the X chromosome in mutant mice (Wang et al., 2001). Eed is located in trophoblast stem cells (Mak et al., 2002) and in differentiation embryonic stem cells with the same kinetics as H3K27 trimethylation (Plath et al., 2004; Silva et al., 2003). Pairing sensitive silencing and reciprocal construct silencing in white-Alcohol dehydrogenase (Adh) transgene both are PcG-dependent. RNAi mutations also affect the silencing of these two process (Pal-Bhadra et al., 1997; Pal-Bhadra et al., 2002). Therefore, MPs may interact directly or indirectly with RNAi silencing components to achieve maintenance.

Recently, increasing attention has been paid to the role of PcG genes in cell cycle regulation and in cancer. One important example is the role of MPs in stem cell renewal (Kajiume et al., 2004; Kim et al., 2004; Ohta et al., 2002; Park et al., 2003). Bmil was first identified as a gene mutations in which accelerated the pre-B cell lymphomagenesis (van Lohuizen et al., 1991). Over-expression of Ink4a and Arf, which are the cell-cycle inhibitors, and p53-induced gene Wig1 have been observed in Bmil null mice (Park et al., 2003). Similar observation is also observed in Ring1b mutants embryos. It is found that Ink4a is up-regulated in Ring1b mutants and double mutant of Ring1b;Ink4a can partially rescue Ring1b mutants allowing them to proceed normally through gastrulation until early somite stages. Ring1a mutants exhibit elevated expression of the oncogenes fos and jun (Satijn and Otte, 1999). EZH2 was observed as a target of E2F, which is one of the core effectors of the retinoblastoma pRB pathway. This is a critical pathway in mammalian cell cycle, and it is essential for proliferation and cell growth (Bracken et al., 2003). EZH2 is a target gene of E2F transcription factor which
causes a growth advantage in mouse embryonic fibroblasts (Bracken et al., 2003). However, in W138 cells Ezh2 and Eed are down-regulated in differentiated cells but are expressed at higher levels in growing cells (Bracken et al., 2003). Over-expression of EZH2 is observed in breast cancer (Ding et al., 2006) and metastatic prostate cancer (Varambally et al., 2002; Rhodes et al., 2003). SU(Z)12 is up-regulated in colon tumors (Kirmizis et al., 2003). Even though Bmi1 and Mel18 are both homolog of Psc, it has been shown that Bmi1 is a proto-oncogene up-regulated in mantle cell lymphomas (Bea et al., 2001; Cohen et al., 1996) and Mel18 is a tumor suppressor gene and its expression is decreased in breast cancer cell (Kanno et al., 1995; Matsuo et al., 2002). Absence or hypoplastic of optic cups and bilateral cleft of the secondary palate phenotypes are observed in some of the Rae28 mutants.

MPs have a role in hematopoiesis, because several PeG mice mutants show abnormal hematopoiesis. In competitive repopulation assays (CRU), bone marrow from bmi1-/- and rae28-/- exhibits reduced CRU activity, and reduced myelo- and lymphopoiesis (Kim et al., 2004; Lessard et al., 1999; Lessard and Sauvageau, 2003; Ohta et al., 2002; Park et al., 2003), resulting from a reduction in hematopoietic stem cells (HSC). Rae28 null mutants exhibit severe maturation arrest between the pro-and pre-B lymphocyte stages (Tokimasa et al., 2001). Mll-/- mice do not give rise to B and T lymphocytes, and Mll is essential for fetal liver HSC activity (Ernst et al., 2004). Interestingly, mutants in eed exhibit hyperproliferation in myelopoiesis and B lymphopoiesis (Lessard et al., 1999), the opposite phenotype to rae28 and bmi1 mutants, showing that Eed is a negative regulator of hematopoietic cell proliferation (Lessard et al., 1999). Mel18 null mutants exhibit defects in B- and T-lymphocyte development (Akasaka et al., 1996).

Mutants of some Hox genes also exhibit hematopoietic defects. Mutation of Hoxa9 perturbs early T-cell development, induces apoptosis in primitive thymocytes, myeloid, erythroid, and lymphoid hematopoiesis (Izon et al., 1998; Lawrence et al., 1997). Both Hoxc8 and Hoxb6 mutant mice exhibit abnormal hematopoietic progenitor cells and increased numbers of early
erythrocyte progenitors (Kappen, 2000; Shimamoto et al., 1999). As both PcG and Hox mutants exhibit hematopoietic defects, and PcG is the upstream regulator of Hox genes, one possibility is that the hematopoetic defects of MP mutants arise from misregulation of Hox genes.

1.7 Enhancer of trxG and PcG (ETP)

Recently, a third group of MPs has been postulated. Members of this third group were originally identified as either PcG or trxG genes. In genetic tests, mutations in these genes enhance the homeotic phenotypes of both trxG and PcG genes, suggesting that these genes function in maintenance of both activation and silencing. This third group has been called the Enhancer of trxG and PcG (ETP) (Gildea et al., 2000). Several not mutually exclusive models can explain the dual functions of ETPs. ETPs may be members of complexes required to transcribe both trxG and PcG genes, and thus have an indirect role in silencing. A good candidate for this model is Enhancer of Polycomb (Boudreault et al., 2003; Ceol and Horvitz, 2004; Sinclair et al., 1998a; Stankunas et al., 1998), which is a subunit of the NuA4 HAT complex. Alternatively, ETPs may encode proteins that are required for both activation and silencing, perhaps by making changes in chromatin structure that are a prerequisite for both processes. Possible candidate proteins for this model are encoded by Trithoraxlike, zeste and batman. Trxl encodes GAGA Factor (GAF) which synergizes with ISWI nucleosome remodeling complexes (Tsukiyama et al., 1995) and with FACT which is required for transcription to establish nucleosomes-free region. Zeste is required for activation via recruitment of the Brm complex, and it’s also a member of PRC1 (Mulholland et al., 2003; Saurin et al., 2001). The molecular basis of mechanisms for ETP function is still not clear.

1.8 Biological (In vivo) studies of Asx

As mentioned above, ETPs are a new group of MPs characterized by dual functions in maintenance of homeotic gene expression. The molecular basis has not been determined for any
ETP. Our lab is studying the \textit{Asx} gene, first cloned in our lab, to understand the molecular basis of ETP mechanisms. As typical for ETPs, \textit{Asx} mutants exhibit both anterior and posterior transformations in the same individuals (Sinclair et al., 1992), and \textit{Asx} mutations enhance the homeotic phenotypes of PcG (Campbell et al., 1995; Cheng et al., 1994) and \textit{trx} mutants (Milne et al., 1999). Mutations in \textit{Asx} enhance position-effect variegation, and therefore the wild-type role of \textit{Asx} is to counteract heterochromatin formation (Sinclair et al., 1998b). This function is consistent with a role for \textit{Asx} in activation which is expected to be the role of \textit{trxG} genes.

Molecular evidence supports a role for \textit{Asx} in silencing. Ectopic expression of \textit{Hox} genes is observed in \textit{Asx} mutants (McKeon J. and Brock H.W., 1991; Simon et al., 1992), consistent with a primary role in silencing in embryos. The effects of \textit{Asx} mutations on \textit{Hox} gene expression are tissue-specific (Soto et al., 1995). About 30\% binding sites of \textit{Asx} on polytene chromosome are unique and 70\% binding sites overlap with Pc or Ph (Sinclair et al., 1998b). However, \textit{Asx} has never been identified as a subunit of any PcG complex. Interestingly, recent genetic experiments suggest that \textit{Asx} is an activator of \textit{Psc} expression (Ali and Bender, 2004). If so, the silencing effects of \textit{Asx} could arise because of failure to activate PcG genes, rather than because \textit{Asx} is directly required for silencing.

There is more direct evidence for a role of \textit{Asx} in activation. \textit{Asx} and \textit{Trx} colocalize at many sites on polytene chromosomes. Consistent with this, \textit{Asx} and \textit{Trx} from nuclear extracts of embryos cofractionate on cation exchange columns, and coimmunoprecipitate. In vitro, full length \textit{Asx} interacts with the SET domain of \textit{Trx}. Like \textit{Trx}, \textit{Asx} is recruited to heat shock loci following heat stress, and \textit{Asx} mutations prevent maximal induction of \textit{hsp70} expression, like \textit{trx} mutations. \textit{Asx} and \textit{Trx} recruitment to \textit{hsp70} is coordinate, because mutations in either locus prevent recruitment of the other protein. Finally, both \textit{Asx} and \textit{Trx} coimmunoprecipitate with phosphorylated RNA Polymerases II. This modification occurs only in RNA polymerase II which is carrying out transcriptional elongation, suggesting that both \textit{Asx} and \textit{Trx} have a role in
this process (J. Hodgson, S. Petruk, M. Kyba, B. Bodiu, A. Mazo, and H.W. Brock, manuscript submitted). In RNAi trx knock down experiments, Asx recruitment to polytene chromosomes is abolished (S. Petruk and A. Mazo, unpublished). Even though these observations suggest that Asx has a key role with Trx, so far, no evidence shows the presence of Asx in Trx containing complexes (Carmo-Fonseca, 2002; Kornberg and Lorch, 2002) or in complexes containing the mammalian homolog of Trx, Mll (Felsenfeld and Groudine, 2003; Yokoyama et al., 2004). The interaction between Asx and trx in vivo may be transient, or unstable, or Asx may associate only with a subset of complexes.

1.9 Mammalian homologs of Asx: Additional sex combs-like (Asxl1)

Base on sequence homology, mammalian Asx homologues have been identified and characterized. The homologs are called ASXL1/Asxl1, ASXL2/Asxl2, and ASXL3/Asxl3, in humans and mouse respectively (Fisher et al., 2003; Fisher et al., 2006; Katoh and Katoh, 2003; Katoh and Katoh, 2004). The organization of mammalian Asxl is very similar to each other. All mammalian Asxl have 13 exons, except human ASXL3, and mouse Asxl2 which have 12 and 11 exons respectively. The published cDNAs for Asxl2 and Asxl3 may not be full length. There are two other cDNAs overlapping with the longest cDNA of Asxl2; therefore, Asxl2 at least has three transcripts. Recently, the expression profiles of Asxl, Asxl2, Asxl3 were analyzed in ES cell line and mouse tissues. Three transcripts, 7.6, 6.7, and 4.7 kb, are detected for Asxl1. For Asxl2, six transcripts are detected in adult mouse tissues, brain and kidney, and three are detected in ES cells. Asxl1 and Asxl2 are expressed in the egg, the pre- and post-implantation embryos. Highest expression for Asxl1 is at mid- and late-gestation while Asxl2 is highest at mid-gestation and neonates. Expression for Asxl3 is more restricted. No expression of Asxl3 is detected in embryos, only adult tissues express Asxl3. Asxl1 and Asxl2 have roles in initiation of gene expression while Asxl3 maybe solely on maintenance. (Fisher et al., 2006)
The expression profiles of human \textit{ASXL1}, \textit{ASXL3} are also analyzed. Similar to \textit{Asx1}, human \textit{ASXL1} has three transcripts of 5, 6, and 8 kb. The expression of \textit{ASXL1} is relatively low in many adult tissues. The 5 kb transcript is undetectable in most tissues except in testis. The 6 and 8 kb transcripts of \textit{ASXL1} are detected in tumor cell lines. The tumor cell lines express 6 kb transcript at a higher level than normal tissues. \textit{ASXL3} can be detected in pancreatic islet, testis and in neuroblastoma, head and neck tumor. Since expression level of Asxl homologs varies between tissues; therefore, it is hypothesized that mechanisms/roles of Asx1s may be tissue specific.

1.10 Structural analysis of \textit{ASXL1}, \textit{ASXL2}, \textit{ASXL3} products

Based on the sequence homology with \textit{Asx}, the first characterized mammalian Asx homologue is human \textit{ASXL1} (accession number AJ438952). Using the amino acid sequence of \textit{ASXL1} (Tblastn program), two other mammalian homologues of Asx, \textit{ASXL2/Asxl2} and \textit{ASXL3/Asxl3}, are later identified and characterized (Fisher et al., 2006; Katoh and Katoh, 2003; Katoh and Katoh, 2004). I will collectively call mammalian Asx homologs “Asx1s”. Two domains are conserved in both \textit{Drosophila} Asx, and mammalian Asx1s: ASXH, which is close to amino-terminal, and the plant homeodomain (PHD) at carboxyl terminal. The PHD domain is found in over 400 eukaryotic proteins, and is thought to mediate protein-protein interaction. Other MPs having a PHD domain are Pcl, Trx, ash1, ash2 and their mammalian homologs (Brock and van Lohuizen, 2001). The PHD domain of Pcl mediates interaction with E(z) (O’Connell et al., 2001). More recent studies have suggested that PHD domain is a necessary component for the histone acetyltransferase activity of CBP (Kalkhoven et al., 2002) and in mediating E3 ubiquitin ligase activity of MEKK1 kinase (Lu et al., 2002). There are also two $\phi XX\phi \phi$ motifs within the ASXH domains. The $\phi XX\phi \phi$ sequences are also present in nuclear receptor coregulators and mediate interaction with the ligand binding domain of their
corresponding nuclear receptors (Rosenfeld and Glass, 2001). Domains which are conserved in all mammalian Asxls are $\phi XX\phi \phi$, ASXL-BOX1, which are 100% identical in all Asxls, ASXL-BOX2, and two consecutive $\phi XX\phi \phi$ flanked by prolines. Please refer to figure 1.1 for the relative location of the domains.

Recently, a detailed structural analysis of AsxII was made (Fisher et al., 2006). Besides the common domains mentioned above, AsxII has PEST sequences for proteolytic degradation, 4 putative cyclin interaction substrate recognition sites, 2 putative substrate motif for phosphorylation by cyclin-dependent kinases, a putative retinoblastoma protein interaction motif, and a putative sumoylation motif for modification by SUMO-1. In addition to the retinoblastoma protein interaction motif, and a putative sumoylation motif, other features can also be found in hASXL1. These features suggest that Asxls may have multiple functions and involve in numerous mechanisms to serve its maintenance functions.

1.11 AsxII knock-out mice

Our lab was the first to make AsxII knock-out mice (C. Fisher, PhD thesis, University of British Columbia). In the early analysis of AsxII homozygous mutants before extensive outcrossing, AsxII mutants died perinatally, but one third of mutant embryos survived to adulthood. Mutant AsxII mice surviving past the age of weaning are smaller than their littermates, and have enlarged spleens. Some male AsxII mutants are infertile. They also exhibit defects in eye development, and severity varies from small eyes to no eyes. Later backcross generations of AsxII mutants show more dramatic and severe phenotypes. Lethality of AsxII mutants occur between day 14.5 and birth. The embryos show dramatic midline defects, including missing eyes, or single eye in the midline, and forebrain defects. Analysis of hematopoietic lineages of AsxII mutants shows abnormal numbers of B and T cells, macrophage, and megakaryocytes.
Figure 1.1: Domain structure of Drosophila Asx compared to predicted mammalian Asxls.

The boxes represent protein and their representative domains: 

- represents \( \phi \)XX\( \phi \) domain (Nuclear receptor), 
- represents ASXL-BOX1 domain (GTSPACLNAMLH), 
- represents ASXH domain, 
- represents ASXL-BOX2 domain, and 
- represents PHD domain.
*Asxll* mutants exhibit simultaneous anterior and posterior transformation of the axial skeleton. Two anterior transformations, second cervical vertebra (C2) to the first cervical vertebra (C1), and C1 to occipital, are observed in *Asxll* homozygous mutants. The latter phenotype is also observed in Hoxb4, Hoxd3, and Hoxd4 mutants. *Asxll* mutants also exhibit posterior transformations of the seventh cervical vertebra (C7) to the first thoracic vertebra (T1) and T13 to the first lumbar vertebra (L1). These phenotypes are also observed in *Hoxa4, Hoxa5, Hoxa6, Hoxb7, Hoxa7,* and *Hoxb9* (Chen et al., 1998; Horan et al., 1995; Jeannotte et al., 1993; Kostic and Capecchi, 1994) and *Hoxa9, Hoxb8, Hoxb9, Hoxc8, Hoxd8,* and *Hoxd9* null homozygous mice (Chen and Capecchi, 1997; Fromental-Ramain et al., 1996; Le Mouellic et al., 1992; van den et al., 2001) respectively. *Asxll* mutants possess abnormal xiphoid processes.

Compound mutant mice of *Asxll*M33 were made to verify if *Asxll* is a member of the PcG. *M33* is chosen because *Drosophila Pc*, the *M33* homolog, exhibits the strongest genetic interaction with *Asx* (Milne et al., 1999). The *M33* mutant is a C-terminal deletion of the chromodomain. No *Asxll-l-;M33cterm/cterm* mice or embryos were obtained. They are not observed in the set of 10.5dpc-11.0dpc embryos, and therefore, are lethal prior to this stage. Skeletal analysis was made with *Asxll+l-;M33cterm/+* trans-heterozygotes and *Asxll-l-;M33cterm/+*. A higher percentage of trans-heterozygotes exhibit C2 to C1 transformation than single *Asxll +l- or M33cterm/+*, and, increased penetrance of T13 to L1 is observed in trans-heterozygotes. *Asxll-l-;M33cterm/+* mice exhibit novel phenotypes such as reduced or defective ossification of the 5th sternebrae, and reduced or absent ossification centers. The enhancement of skeletal transformation phenotypes strongly support that *Asxll* is a PcG gene because it enhances PcG gene phenotypes. However, the reciprocal experiment, to determine if *Asxll* enhances phenotypes of trxG mutant mice has not been done.
1.12 Thesis aims

The evidence that *Asxl* mutants exhibit both anterior and posterior phenotypes provide strong evidence that *Asxl* is ETP. The picture will be more complete if we can show that *Asxl* mutation will also enhance phenotypes of *trxG* genes. Therefore, one of my project aims is to generally *Mll* mutant mice by gene trap ES cell. *Mll* is chosen among several of *TrxG* member because genetic interaction is observed between *Asx* and *trx*, *Drosophila* homolog of *Mll* (Milne et al., 1999). Also molecular evidence shows that *Asx* interacts with SET domain of *trx* (J. Hodgson, S. Petruk, M. Kyba, B. Bodiu, A. Mazo, and H.W. Brock, manuscript submitted). When *Mll* heterozygous mice are generated, we can use them to generate compound mice and observe if any enhancement in the phenotypes.

I also want to generate *Asxl2* and *Asxl3* knock-out mice. The relatively weak homeotic and hematopoietic phenotypes observed in *Asxl* mutants may occur because *Asxl2* or *Asxl3* rescue the phenotype. We do not know if *Asxl2* and *Asxl3* are ETPs. Consortia have been established to recover targeted insertions in ES cells for all known mouse genes; therefore, making knock-out mice will be less time consuming compared to making knock-out mice by homologous recombination. The first part of my project is to analyze the gene trapped ES cell lines corresponding to *Asxl2*, *Asxl3*, and *Mll*. Details about gene trapped ES cell lines will be discussed in details in Chapter Two.

The second part of my project is to characterize the role of *Asxl1* on Hox genes expression. The anterior and posterior transformation observed in *Asxl1* homozygous mice are also observed in numerous *Hox* genes null mice (see above), suggesting that *Asxl1* positively and negatively regulates Hox genes. Therefore, I examined *Hox* genes expression patterns *Asxl1* mutant mice.
2 USING “GENE TRAP” TO CREATE MUTATED EMBRYONIC STEM (ES) CELLS

2.1 Introduction

To create a mouse with a gene of interest mutated is a time consuming process. The constructs are complex to make because they must contain genomic DNA, and selectable markers. The mouse genome is relatively big compared to other simple animal models, and the rate of homologous mutation is low. Therefore, recovery and characterization of targeted mutations is tedious because many cells must be screened (Joyner, 1991).

To circumvent these problems, consortia have been established to recover targeted insertions in ES cells for all known mouse genes. When complete, this resource will greatly simplify analysis of mutations in mice. Scientists can check if their gene of interest is available in the existing ES cell library, and the consortium will ship the cells to the researcher. After verifying that the targeted insertion is in the correct gene, and will generate the expected mutants, then the ES cells can be injected into blastocysts to generate chimeric mice with germline clones. Lines of mutant mice can be established, and the phenotype of the mice can be studied extensively. This chapter will describe my analysis of ES cells targeted for \( \text{Asxl2, Asxl3, and Mll.} \)

The idea of gene trapped ES cells was inspired by the genetic screening method called enhancer traps in \( \text{Drosophila.} \) An enhancer trap vector contains a minimal promoter and a \text{beta galactosidase (LacZ)} reporter gene within a transposable element. The transposable element is mobilized and is integrated randomly into the \( \text{Drosophila} \) genome. The position or developmental timing of the reporter gene expression depends on insertion near to regulatory elements of endogenous genes (Gonzy-Treboul et al., 1995). Thus the enhancer trap construct screens for previously unknown regulatory elements in the genome.

Similarly, a gene trap vector is designed to create ES cells with defective genes. The vector contains an intron sequence with a 3’ splice acceptor, the \text{LacZ gene}, and a bacterial gene
for neomycin resistance under the regulation of a eukaryotic promoter. This gene trap vector integrates randomly into the mouse genome following transfection into ES cells. ES cells with an integrated vector can be selected with neomycin. After insertion into an intron, the primary transcript will be spliced to create a chimera, using the 5’ splice donor from the endogenous gene, and the 3’ splice acceptor from the vector. If the vector lies in the same orientation of the endogenous gene, after splicing the reading frame of the endogenous gene may be in frame with \( \text{LacZ} \). Only cells in which the vector is inserted into an intron in the correct orientation will express \( \text{lacZ} \), because the vector lacks a promoter to express \( \text{lacZ} \). The chimera will contain 5’ sequences from the endogenous gene fused to \( \text{lacZ} \) and neomycin and the protein transcribed from the disrupted gene will contain part of the endogenous protein and the whole beta galactosidase protein (Figure 2.1). Neomycin-resistant cells can be screened for \( \text{lacZ} \) expression to identify insertion mutants. The expression of this fusion protein will have the same expression pattern as the endogenous protein, so \( \text{LacZ} \) gene can be used to study the expression pattern of the gene of interest without the need to generate an antibody.

To identify which gene has been targeted, 5’ RACE (rapid amplification of cDNA ends) is used to amplify the exon sequence upstream of the insertion (Joyner, 1991). The amplified sequence is then sequenced to determine the location of the vector integrated in the genome. The sequence is called a “sequence tag” and identifies each unique clone of ES cell mutants. The sequence tags are compiled into a library, so that researchers can screen the library with cDNA sequence for any gene of interest to determine which clone of ES cells is suitable for further studies.

Hundreds of clones of ES cells were available from BayGenomics when I started my project. I searched for ES clones containing insertions in \( \text{Asxl2, Asxl3, and Mll} \). Additional sex comb-like 2(\( \text{Asxl-2} \)) and the other is Mixed- lineage leukemia (\( \text{Mll} \)). Two clones of ES cells containing mutations in \( \text{Asxl2} \) and \( \text{Mll} \) were purchased and analyzed further. In this chapter I
Figure 2.1: Diagram of gene trap strategy.

The gene trap vector is shown in the top line, and consists of intron □, lacZ □, neomycin coding gene □, driven by its own promoter □. After insertion of the vector into the genome (2nd line), following splicing (3rd line), a novel transcript containing sequences from the endogenous gene, and also containing lacZ is created. After translation (4th line) a novel fusion protein containing LacZ is created.
will describe my characterization of these ES lines, and show why they were not suitable for further analysis.

2.2 Results and discussion

2.2.1 Identifying and selection ES clones

Three mammalian Asx homologs were identified based on sequence homology. The genomic structure and the product of murine Asxl1, human ASXL1, ASXL2, and ASXL3 are well characterized (Fisher et al., 2003; Fisher et al., 2006; Katoh and Katoh, 2003; Katoh and Katoh, 2004). The cDNAs corresponding to murine Asxl2 and Asxl3 are AK036839 and AK082659 respectively. The complete 5’ and 3’ UTR of these two genes are not yet verified. The genomic structures of murine Asxl2 and Asxl3 described in this section are based on the cDNAs published on the NCBI website. It is possible that the 5’ and 3’ UTR can be extended for these two genes.

I did BLAST searches at NCBI website to see if there are other cDNAs containing overlap sequences of either Asxl2 or Asxl3. I found that there are two other cDNAs whose sequences are partially matched with AK036839, Asxl2 cDNA. Their accession numbers are AK035679 and AK122540. No other cDNAs are found with overlapping sequence with AK082659, Asxl3 cDNA. There is only one transcript for Asxl3. There are possibilities that Asxl2 has three transcripts. The genomic structures of all three transcripts of Asxl2 are shown in Figure 2.2. I used the longest transcript, AK036839 of Asxl2 to blast the database of BayGenomic Company (http://baygenomics.ucsf.edu/). Part of the sequence of the Asxl2 transcript matches the sequence tag of one ES cell clone, whose identification is XB745. The gene trap vector is located in the 3044bp intron between exon 10 and exon 11 of AK036839 or exon 10 and exon 13 of AK122540. The matched sequence is at exon 10 (figure 2.3), a common sequence among the three transcripts.
Figure 2.2: Genomic Structures of genes studied in this chapter.
Each rectangle represents the genomic DNA of the gene of interest. Introns are indicated by white boxes. Exons are indicated by black boxes, and numbered from 5' to 3'.
Figure 2.3: Asxl2 has 3 possible transcripts.
Each rectangle represents the genomic DNA of the gene of interest. Introns are indicated by white boxes. Exons are indicated by black boxes, and numbered from 5' to 3'. Each transcript has accession number on the top, which represents the cDNAs of the transcripts. The bottom number is the size of the genomic DNA in bp.
Therefore, all three transcripts of Asxl2 are disrupted in this ES cell clone. So the mice produced from this ES cell clone may have no functional Asxl2. It would be an ideal clone to produce mutant mice for analysis of Asxl2 function.

Similarly, I also used cDNAs for Asxl3 and Mll using cDNA AK082659 and AK081958 respectively and performed blast searches in the database of BayGenomics. The ES cell lines contain the corresponding sequence tag for Asxl3 and Mll are RRI366 and XE699 respectively. The matched sequence is located at exon 7 for Asxl3 and exon 27 for Mll. The intron between exon 7 and 8 is about 17.5kb. Unfortunately, part of the intron sequence between exon 7 and exon 8 is not completely sequenced. We decided not to order Asxl3 clone because it is impossible to verify the exact location of the vector with the sequence information. It would be hard to genotype mice in the future. For Mll ES cell line, the sequence tag is part of exon 27 and the gene trap vector is located in exon 27. Theoretically, it is easy to verify the exact location for Mll ES cell line.

2.2.2 Analysis of Asxl2 gene trapped ES cell line

Asxl2 is a large gene of 7.375kb, containing 11 exons (Katoh and Katoh, 2003). Analysis of expressed sequence tag libraries shows that Asxl2 has at least three alternative spliced transcripts (Figure 2.3) (Katoh and Katoh, 2003). The sequence tag comes from exon 10, allowing us to place the gene trap vector in the 3044 bp intron immediately downstream. The region of interest is shown in Figure 2.4. I designed PCR primers to amplify a sequence between the upstream exon and the vector from the Asxl2 ES cells genomic DNA using primer 2 and primer 9 shown in Figure 2.4. The expected possible sizes for the PCR product range from 228bp to 3338bp depending on the insertion point of the vector. I did regular PCR to amplify using Asxl2 ES cell genomic DNA as template. After the first round of PCR, there were six bands amplified of 280bp, 300bp, 700bp, 1000bp, 1300bp, and 1500bp (data not shown). Since
Figure 2.4: Location of gene trap relative to Asxl2 exons.
Introns are indicated by white boxes. Exons are indicated by black boxes, and numbered from 5' to 3'. Arrows indicate the primers locations. • represents Pst1 restriction enzyme sites, and ♦ represents Nco1. The dashed box is the location of the sequence tag. The triangle is the gene trap vector and the solid line along the triangle is the LacZ gene.
the first round of PCR has produced multiple bands, I amplified the PCR products again with the same set of primers. The bands were amplified more clearly and more bands were produced. To reduce non-specific amplification, nested primers were designed. The PCR products were amplified again with primer 3 and primer 8 (Fig. 2.4), and the result for the nested PCR is shown in figure 2.5. A band about 700bp was produced (Fig. 2.5, lane 3).

The band was excised from the gel, DNA was extracted, and sequenced by the NAPS sequencing facility as described in the Materials and Methods. However, the sequence did not match the sequence of the corresponding intron.

In an attempt to make it easier to amplify a genomic/vector fusion fragment from the ES genomic DNA, I designed primers that spanned the intron of the insertion in the hope that I could amplify successfully a shorter fragment. These primers (2 through 7 in Fig. 2.4) should have made it possible to amplify short fragment regardless of the position of the vector relative to the exons. However, this attempt also failed (Fig. 2.6). The result was that again the 700bp band amplified with primer 2 and primer 9 in lane 2. Another clear band of about 300bp was produced by primer 5 and primer 9 in lane 3 of Figure 2.6. The result for other combinations of other primers was either no product or a smear (lanes 4-6, Figure 2.6). The 300bp band was sequenced but again it was not from Asxl2. To eliminate the possibility that PCR conditions were suboptimal, I successfully amplified the Beta-galactosidase gene in the vector (Figure 2.6, lane 7) and a region of the genomic DNA around the insertion region (Fig. 2.4) using primer 1 and primer 7 (Figure 2.5, lane 2) using the exact same PCR conditions.

Therefore, I adopted an alternative strategy using ligation-mediated PCR in an attempt to amplify the genomic sequence next to the vector (Please refer to Fig. 2.7 for a schematic explanation of ligation-mediated PCR). I selected the restriction enzyme PstI which does not digest the vector, but which has convenient genomic sites (Fig. 2.4). After digestion of the
Figure 2.5: PCR amplification of genomic DNA from Asx12 gene trap lines. Length of size standards are indicated to the left in bp. Primers are indicated in Fig.2-4. Lane 1) 100bp MW markers. Lane 2) PCR product with primers 1 and 7. Lane 3) PCR product with primers 3 and 8.
Figure 2.6: PCR amplification of genomic DNA from \textit{Asxl2} gene trap lines.
Length of size standards are indicated to the left in bp. Primers are indicated in Fig. 2-4. Lane 1) 100bp MW markers. Lane 2) PCR product with primers 2 and 9. Lane 3) PCR product with primers 4 and 9. Lane 4) PCR product with primers 4 and 9. Lane 5) PCR product with primers 6 and 9. Lane 6) PCR product with primers. Lane 7)PCR product with LacZ primers. Lane 8) Negative control with no template with LacZ primers.
Isolate genomic DNA from ES cells

Digested with restriction enzyme (matched with the linkers)

Ligate bubble linkers with digested genomic DNA fragments

PCR with a linker primer and a primer specific to the gene of interest

PCR product can be sent for sequencing

Figure 2.7: Diagram of ligation-mediated PCR.
Genomic DNA is isolated from ES cells. Then genomic DNA is digested with a restriction enzyme (Hinf3) and DNA fragments are produced (2nd line). The digested fragments are ligated with bubble linkers at both end (3rd line). Primers complementary to bubble linker and
genomic DNA, restriction fragments were ligated to linkers. The linker chosen was a double-stranded DNA with complementary base pairing at both 5' and 3' ends, but the middle sequence of the linker is not complementary to its opposite strand. The unpaired region creates a bubble structure, which allows PCR primers to bind easily to amplify the DNA sequence, and thus reduces background from hybridization to genomic sequence. After one round of PCR, many fragments were amplified (Figure 2.8, lane 2, 3). Therefore I re-amplified these fragments using nested PCR in the hope of obtaining specific PCR products. As shown in Figure 2.8 lane 4, 5, 6, this approach was unsuccessful.

At this point, I abandoned the PCR approach and used Southern blotting to map the location of the vector. Genomic DNA from Asxl-2 cell lines was digested with PstI. The blot was probed first with genomic DNA amplified by PCR using primer 1 and primer 7 (Figure 2.4). I predicted that I would detect a 5.31 kb fragment corresponding to the endogenous genomic DNA, and a band ranging from 6.986-10.03 kb corresponding to the genomic DNA with the insert. Surprisingly, the autoradiograph exhibited three bands (Figure 2.9A). One was the 5.5 kb wild-type endogenous band. There were other two bands of 6kb and 7.5kb. To confirm that these additional bands corresponded to the genomic DNA containing the vector, I re-probed the blot with a probe for lacZ. The results show that the lacZ probe hybridizes to bands with the same mobility as the two higher molecular weight bands found on the Southern blot probed with genomic DNA (Figure 2.9B).

The possible explanations for an extra band were either restriction site polymorphism, perhaps because the ES line was not clonal, or double integration of the vector. To distinguish these possibilities, another Southern blot with Asxl-2 cell line genomic DNA digested with NcoI. I predicted that if the extra band resulted from PstI site polymorphism, then it would be unlikely for NcoI to also be polymorphic. However, if there was double integration, there should be an extra band regardless of which enzyme is used for digestion. As shown in Figure 2.9, three bands
Figure 2.8: PCR amplification using ligation-mediated method of Asx/2 gene trap cell line DNA.

Length of size standards are indicated to the left in bp. Primers are indicated in Fig. 2-4. Lane 1) 100bp MW markers. Lane 2) PCR product with linker primer and primer 9 using 2μl DNA. Lane 3) PCR product with linker primer and primer 9 using 4μl DNA. Lane 4) Re-amplification of 1μl PCR product from lane2 using linker primer and primer 8. Lane 5) Re-amplification of 1μl PCR product from lane1 using linker primer and primer 8. Lane 6) Re-amplification of 2μl PCR product from lane2 using linker primer and primer 8. Lane 7) 1 kb MW markers.
Figure 2.9: Southern blot results of *Asxl2* (XB 745) ES cell line.

Length of size standards are indicated to the left in kb. A) *Asxl2* ES cell line genomic DNA digested with PstI probed with genomic probe. B) *Asxl2* ES cell line genomic DNA digested with PstI probed with *LacZ* probe. C) *Asxl2* ES cell line genomic DNA digested with NcoI probed with genomic probe. D) *Asxl2* ES cell line genomic DNA digested with NcoI probed with *LacZ* probe.
are present in *Asxl2* genomic DNA digested with NcoI and probed with genomic DNA (Fig. 2.9C) and two bands were observed from the blot probed with *lacZ* DNA (Figure 2.9D). I conclude that the *Asxl*-2 cell line contains two integration sites for the vector. Double integration means that it will be difficult to characterize the molecular structure which underlies the phenotypes of *Asxl*2 mutants mice. The double integrant would also be subject to gene rearrangement by unequal crossing over. For these reasons, I decided not to go to the effort and expense of making an *Asxl*2 mutant mouse line from this ES cell line.

### 2.2.3 Analysis of *Mll* gene trapped ES cell line

In theory, gene traps should only recover insertions into intron DNA. However, my analysis of the sequence tag suggests that the *Mll* gene cell line has the vector inserted into an exon. Figure 2.2 shows the genomic structure of *Mll* and the location of the vector is inserted into exon 27. Figure 2.10 shows the region of interest of *Mll*. Therefore I designed primers to the vector (primer 4) and to the exon 5' to the insertion site (primer 2), and attempted to amplify the genomic DNA using the PCR (Refer to Fig. 2.10). The expected product was 279bp. This attempt was unsuccessful. The first round of amplification was very unclear and produced smear (data not shown). The product from the first round was amplified again and multiple bands were produced (data not shown). No product around 279bp was produced. As a positive control, I used primers 2 and 6 (Figure 2.11) and obtained successful amplification, showing that the quality of the DNA, and general PCR conditions were adequate (data not shown).

Given my experience with *Asxl*2, I decided to switch to Southern blotting. As before, two probes were made for Southern blot. One probe was a genomic probe made by primer 1 and primer 5 (Figure 2.10). This probe will hybridize the wild type DNA without insertion and DNA with insertion. The expected sizes are 1494bp and 5064bp respectively. Another probe was a *lacZ* probe to identify DNA containing the vector. I only expect one band which is around
Figure 2.10: Location of gene trap relative to *Mll* exons.
Introns are indicated by white boxes. Exons are indicated by black boxes, and numbered from 5' to 3'. Arrows indicate the primers locations. ▪ represents PstI restriction enzyme sites, and ▪ represents NcoI. The dashed box is the location of the sequence tag. The triangle is the gene trap vector and the solid line along the triangle is the *LacZ* gene.
Figure 2.11: PCR amplification of genomic DNA from *Mll* gene trap lines. Length of size standards are indicated to the left in bp. Primers are indicated in figure 2-10. Lane 1) 100bp MW markers. Lane 2) – 5) PCR product with primers 2 and 6. Lane 6) Negative control of the PCR with primers 2 and 6 but no template.
5064bp in size. The genomic DNA from the *Mll* ES cell line was digested first PstI. However, hybridization was very weak. PstI was not an ideal restriction enzyme for the genomic probe used because there was only 349bp of overlap between the genomic probe and PstI fragment of interest. I didn’t detect anything when the blot was probed with the genomic probe (data not shown).

Therefore I repeated the Southern blot of the *Mll* cell line with NcoI (Figure 2.12). This time the genomic probe worked well. But I only detected one band for the blot hybridized with genomic probe when I was expecting two bands, which are 6571bp with insert and around 6000bp without insert. A clear band was detected in both the *Mll* cell line (Figure 2.12A, lane 1 and 2) and wild-type control samples (Figure 2.12A, lane 3 and 4). It may be because the band with insert and the band without insert are very close together, so may not be distinguishable on the blot. However, the *lacZ* probe did not hybridize to the same blot. Another blot was prepared separately and hybridized to the *lacZ* probe, and again no hybridization was detected (Figure 2.12B). The same batch of *LacZ* probe was used to hybridize the NcoI digested *Asxl-2* genomic DNA and the *LacZ* bands could be detected (Figure 2.9D); therefore, the there is no doubt about the *LacZ* probe or the blot itself. I concluded that the *Mll* cell line did not have a vector inserted at the site expected from the sequence tag data. This may be a curation error. In any case, I did not proceed with blastocyst injection with this cell line.

In principle, a library of ES cells containing mutations for every mouse gene will be a wonderful resource for the scientific community, and save time and resources. The data base of sequence tags is readily available and searchable, the time from placing an order to receiving the line is short, and the price is reasonable. However, the results presented in this chapter suggest the need for caution when using gene-trap lines from the Bayview Genomics collection. Only two lines were tested, so the sample size is too small to allow general conclusions. Nevertheless, given the cost of each line (about $400), the cost to culture the lines, and the time taken for
Figure 2.12: - Southern blot result of *Mll* (XE699) ES cell line.

Figure A: Length of size standards are indicated to the left in kb. Lane 1) and 2) Genomic DNA from *Mll* gene trap line is digested with NcoI and probed with genomic probe. Lane 3) and 4) C57 genomic DNA digested with NcoI probed with genomic probe. Figure B: Southern blot result of *Mll* (XE699) ES cell line. Length of size standards are indicated to the left in kb. Lane 1) and 2) Genomic DNA from *Mll* gene trap line is digested with NcoI and probed with *LacZ* probe. Lane 3) and 4) C57 genomic DNA digested with NcoI probed with *LacZ* probe.
analysis, time and money was wasted. I recommend that researchers using this resource should obtain multiple ES lines for each gene of interest, test them as soon as possible, and work only with lines that behave as expected.

I expected that PCR-based analysis of integration sites would be the most efficient method of characterizing the ES lines. It is essential to have a PCR-based method of genotyping mice to optimize breeding of mutant lines, and in outcrossing, so it seemed reasonable to develop the assay from the beginning. In practice, I wasted a lot of time trying to optimize PCR reactions based on the assumption that the ES lines had the expected insertion sites. The decision to switch to Southern analysis, which is more labour-intensive and time-consuming was taken with reluctance, yet it yielded much cleaner and more interpretable data. Therefore I recommend that initial analysis be done using Southern blotting, and once the location of the insertion is confirmed, then switch to PCR-based genotyping.
3 MRNA IN-SITU HYBRIDIZATION ANALYSIS OF ASXL1 MUTANT EMBRYOS

3.1 Introduction

ETPs are MPs required to maintain the repressed and active states of homeotic genes. The mechanisms of ETPs are poorly understood. To begin to characterize an ETP, and to understand its mechanisms, we studied Drosophila Asx. Asx mutants exhibit both anterior and posterior transformation (Sinclair et al., 1992). Also mutations in Asx enhance the phenotype of trx and Pc mutations, and suppress position-effect variegation (PEV) (Sinclair et al., 1998a). Other PcG mutations do not have any affects on PEV (Sinclair et al., 1998a). About 70% of Asx binding sites overlap with those Pc, Ph, and Pcl and 30% of binding sites are unique. ETPs may interact with other MPs directly (eg Asx and Trx) and thus act directly in regulation of Hox genes. The evidence supports the idea that Asx and Trx act in transcriptional elongation. One possibility is that Asx is required to activate expression of MP genes, rather than acting directly at Hox loci.

The mammalian Asx homologs were identified and characterized in human and mouse using bioinformatics analysis. Functional analysis is needed to confirm if Asx acts as an ETPs. Therefore, murine Asxl1 knock-out mice were first made in our lab by Cynthia Fisher (Fisher et al., 2006). The details about the phenotypes of Asxl1 mutant mice are discussed in the introduction- Asxl1 knock-out mice section. For the discussion of the potential role of Asxl1 as an ETP, the key observation is that Asxl1 mutant mice exhibit both anterior and posterior transformations of the axial skeleton, which can be interpreted as evidence that Asxl1 is needed for both activation and repression of Hox genes. Until this work, this hypothesis had not been tested directly.

In Drosophila, there is a clear connection between loss-of-function mutations of Hox genes and anterior transformations and gain-of-function mutations and posterior transformations
respectively of the anterior-posterior axis. In mice, the distinction is not so clear. Anterior transformations of the axial skeleton near the anterior expression boundaries have been observed in many \textit{Hox} loss-of-function mutants (Condie and Capecchi, 1993; Le Mouellic et al., 1992; Rijli et al., 1993). However, more difficult to interpret changes like loss or malformations of segments, defects at locations different from that anterior expression boundaries, and even posterior transformations have been observed (Chisaka and Capecchi, 1991; Chisaka et al., 1992; Condie and Capecchi, 1993; Davis and Capecchi, 1994; Davis et al., 1995; Jeannotte et al., 1993; Lufkin et al., 1991; Ramirez-Solis et al., 1993; Small and Potter, 1993). In addition, the phenotypes of single \textit{Hox} mutants are often weak compared to those of mice that are double or triple mutants for members of a paralogous group (eg. \textit{Hoxa}4, b4, and d4)(Horan et al., 1995). Relatively few experiments have been performed which examine gain-of-function \textit{Hox} mutations, and these have suffered from the defects of having increased expression in the normal domain (Wolgemuth et al., 1989), or ubiquitous expression (Balleng et al., 1989). Therefore, it is difficult to predict over-expression phenotypes of \textit{Hox} genes that might arise from \textit{AsxII} mutations.

To obtain experimental evidence that anterior-posterior transformation of \textit{AsxII} mutants is caused by mis-expression of \textit{Hox} genes, mRNA in-situ hybridization was used to explore the gene expression pattern of three \textit{Hox} genes, \textit{Hox} a4, \textit{Hox} a7, and \textit{Hox} c8. Chen et al. (1998) have suggested that mutations in \textit{Hox} genes preferentially affect differentiation at boundaries between the main types of vertebrae, namely between the skull and the cervical vertebrae, the cervical and thoracic, the thoracic and lumbar, and the lumbar and sacral. This idea has been corroborated by observations that \textit{Hox} mutations can affect axial identity away from the anterior boundary of expression (Horan et al., 1995). If the hypothesis of Chen et al. (1998) is true, then there might not be a good correspondence between \textit{AsxII} axial skeleton phenotypes and the \textit{Hox} gene responsible. If \textit{AsxII} mutations affect \textit{Hox} regulation, then perhaps it is not surprising that our
lab observed changes in C1, C2, C7, and T13 vertebrae. The complications just mentioned mean that it is difficult to pick the most obvious targets to try with confidence. It is too time-consuming and expensive to test all Hox genes. Therefore I attempted to choose Hox genes for analysis that maximized the likelihood of seeing an effect, based on the criteria below.

AsxII interacts directly with Mll (O'Dor and Brock, unpublished results). As noted in the Introduction, Asx and Trx act as elongation factors in transcription of heat shock loci. Therefore one strategy is to use what is known about Mll regulation of Hox genes in mice, or about direct binding to Hox loci obtained from chromatin immunoprecipitation assays to restrict analysis of potential AsxII targets. In mice, Mll mutations cause abnormal activation of Hox a7, c5, c6, and c8 (Subramanian et al., 1995; Yu et al., 1995). Mll binds directly to Hox. a7, a9, and c8 as shown by chromatin immunoprecipitation (ChIP) assays (Milne et al., 2002; Milne et al., 2005b; Milne et al., 2005a). Our lab has shown that AsxII binds directly to Hox a7 by ChIP, confirming that in at least one case, Mll binding predicts AsxII binding. As Hox a7 is the only gene for which we have direct molecular evidence, it was chosen for analysis. The other strategy is to examine Hox loci that have loss-of-function phenotypes that affect vertebrae affected in AsxII mutants. The transformation of seventh cervical vertebra (C7) to the first thoracic vertebra (T1) of Hoxa4 knock-out mice (4) (posterior transformation) is the same as that observed in AsxII mutant mice, so we chose Hox a4 for analysis. In Hox c8 mutants, the L1 vertebra is transformed towards T13 (anterior transformation) (Le Mouellic et al., 1992), which is the opposite of the T13 to L1 (posterior) transformation seen in AsxII mice. However, as Hox c8 is a known target of Mll as shown by binding and mutational analysis, it too was included in the analysis.
3.2 Results

*In situ* hybridization to mouse embryos is a technically demanding procedure. Success requires high quality probes, care in making and maintaining all solutions RNase free, careful attention to dissection and keeping the embryos intact, and obtaining high quality photographs. My procedures are explained in detail in the Material and Methods.

Further difficulties occur when comparing mutant and wild-type embryos because *AsxI* embryos have retarded growth relative to wild-type litter mates, so it is important to consider stage-specific differences in *Hox* expression patterns when comparing the results. Finally, mutations in MP genes often exhibit phenotypes of variable penetrance and expressivity, so it is important to examine sufficient of embryos to determine the range of possible phenotypes. For these reasons we tried where possible to compare *AsxI*+/+ and *AsxI*-- littermates, to compare embryos from different developmental stages, and to analyze more embryos when the phenotypes were more variable.

As a first step, I carried out in situ hybridization with *Hox c8* to wild-type embryos, and compared my results with those published previously. As shown in Figure 3.1, the expression patterns I obtained are very similar to those published (Kwon et al., 2005). *Hox c8* expression occurs only in neural ectoderm from somite 10 to somite 15 and mesoderm at somite 16 to somite 20 (Le Mouellic et al., 1992). Expression analysis of targeted replacement of *Hox c8* by LacZ shows that *Hox c8* is expressed posterior to the seventh and eighth somites in the dorsal half of the neural tube and to the eighth and ninth somites in its ventral half (Le Mouellic et al., 1992). Expression decreases in the tissue posterior to the hind limb bud, except in the most caudal tip of the embryos (Le Mouellic et al., 1992). LacZ expression shows that expression is absent anterior to the forelimb except for two longitudinal stripes of beta-galactosidase-expressing cells are present up to the cervical region. Notably, and unexpectedly, *Hox c8* is expressed strongly in the brain, especially the mesencephalon, the diencephalon, and the
Figure 3.1: Comparison of the published mRNA in-situ hybridization of *Hoxc8* (Kwon et al., 2005) and my wild-type result.

The one on the left is the published wild-type embryos and the one on the right is the wild-type embryos from my experiment. Red arrow head indicates the anterior boundary of neural ectoderm and blue arrow head indicates the anterior boundary of mesoderm.
telencephalon, and in the region of the cervical flexure. These results gave me confidence that my in-situ techniques are working as expected.

The \textit{Hox a4} expression pattern in wild-type embryos is shown in Figure 3.2. \textit{Hox a4} expression in wild-type embryos was examined using 11.5-12.5 day embryos. In sectioned embryos anterior boundary of \textit{Hox a4} starts at posterior or mid-myelencephalon and the expression extends posteriorly to the cervical spinal cord region (Gaunt et al., 1989; Toth et al., 1987). In whole mount embryos, expression of \textit{Hoxa4} is detected at the upper cervical spinal cord and spinal ganglia and expression level starts decreasing in the more caudal sections and becomes undetectable at the thoracic level (Toth et al., 1987). There is that higher expression in mantle layer of the neural tube than the ependymal layer of both lower myelencephalon and cervical spinal cord (Toth et al., 1987). The expression of \textit{Hox a4} is most abundant in anterior to prevertebra 1 (pv1) in dorsal parts of the spinal cord but posterior to pv5 in ventral parts. The expression level is reduced in posterior to the level of pv6 (Gaunt et al., 1989).

The expression of \textit{Hox a4} in the mid-myelencephalon of my whole mounted wild-type embryos is not as strong as the published data (Toth et al., 1987), Figure 3.2. It may be because I used whole embryos rather than the sectioned embryos. The expression of \textit{Hox a4} in my wild-type embryos is very similar to the published results in mesoderm and ectoderm (Toth et al., 1987). I examined expression pattern of \textit{Hox a4} in 7 \textit{AsxlI/-} embryos. \textit{Hox a4} had variable expression in \textit{AsxlI} mutants. Results are shown in Figure 3.2. High expression is observed in the upper cervical spinal cord, and expression is decreased caudally. High expression is observed at the dorsal side (mantle layer) and lower expression in the ventral side (ependymal layer) at the spinal cord. The expression of \textit{Hox a4} in \textit{AsxlI} mutants is affected in the neural ectoderm and the mesoderm. Both the anterior boundaries of \textit{Hox a4} in neural ectoderm and mesoderm are shifted anteriorly in \textit{AsxlI} mutants. Five of seven mutants exhibit 1-2 somites rostral shift of the mesoderm and neural ectoderm anterior boundary. Among these five mutants,
Figure 3.2: *Hox a4* mRNA in-situ hybridization of wild-type and *Asxl1* mutant.
A, B, and C are the wild-type embryos. D, E, and F are the mutant embryos. B and E are the close-up of the neural ectoderm anterior boundary. C and F are the close-up of the mesoderm anterior boundary. The red arrow head indicate the location of the anterior boundary of *Hox a4* in the neural ectoderm in the wild-type embryos. The blue arrow heads indicate the location of the anterior boundary of *Hox a4* in the mesoderm in the wild-type embryos.
two show expression of *Hox a4* in the mesoderm of the caudal region, whereas no *Hox a4* expression is detected in wild-type (data not shown). Two of seven mutants exhibit mild phenotypes with diffuse anterior boundaries of both neural ectoderm and mesoderm (data not shown). The red and blue arrow heads on the figures indicate the locations of the anterior boundaries of neural ectoderm and mesoderm respectively in wild type embryos. In the *Asxll* mutants, *Hox a4* expression is shifted anteriorly relative to wild-type embryos. Together, these phenotypes show that *Asxll* is required to repress *Hox a4* expression in the neural tube, and in caudal mesoderm. Therefore, in this case, *Asxll* is behaving like a PcG gene.

*Hox a7* is expressed in the neuroectoderm and the mesoderm regions of wild-type mice. The neuroectoderm anterior boundary expression of *Hox a7* starts at the fifth cervical (C5) somite and ends at the fourth sacral (S4) somite. The mesoderm anterior boundary starts at the third thoracic (T3) and ends at the thirteenth (T13) (Dressler and Gruss, 1989; Puschel et al., 1991). My wild-type embryos also have similar pattern Figure 3.3. Similar to *Hox a4*, the neural ectoderm and mesoderm anterior boundaries of *Hox a7* are shifted anteriorly in *Asxll* mutants. Results are shown in Figure 3.3. The red and blue arrows on the figures indicate the location of the anterior boundaries of the neuroectoderm and mesoderm respectively in the wild-type embryos. *Hox a7* expression is detected anterior to the arrows. The green brackets show the distance between the hind limb bud and the anterior boundaries of mesoderm. The distance is about 2 somites for mutants and 3 somites for wild-type showing that the mesoderm anterior boundary is shifted forward in mutants. The data indicates that *Asxll* is required to repress *Hox a7* expression in both neural tube and mesoderm.

I hybridized *Hox c8* probes to three wild-type embryos, and four mutant embryos. From the published data, the expression of *Hoxc8* for wild-type embryos is at somite 10 to somite 15 for neural ectoderm and somite 16 to 20 for paraxial mesoderm and there is expression at the tip of the tail for both mutants and wild-type (Kwon et al., 2005). As for *Hox a4* and *a7*, *Asxll* mutants
Figure 3.3: Hox a7 mRNA in-situ hybridization of wild-type and AsxII mutant.
A and C are the wild-type embryos. B and D are the mutant embryos. C and D are the close-up of Hox a7 anterior boundaries of neural ectoderm and mesoderm. The red arrow heads indicate the location of the anterior boundary of Hox a7 in the neural ectoderm in the wild-type embryos. The blue arrow heads indicate the location of the anterior boundary of Hox a7 in the mesoderm in the wild-type embryos. The green brackets represent the distance between the edge of the hind limb bud and the observed Hox a7 anterior boundary of mesoderm.
exhibit anterior shifts of the anterior boundaries of neural ectoderm and mesoderm. Results are shown in Figure 3.4. Red arrow heads point to the anterior boundary of neural ectoderm and blue for mesoderm. There is a 1 somite anterior shift for both neural ectoderm and mesoderm. The green brackets represent the distance of the edge of the hind limb bud and the mesoderm anterior boundary. The distance for wild-type is 3 somites and 2 somites for mutants showing that mesoderm anterior boundary is shifted forward. Besides the changes of expression in the body segments, expression changes in the head region are also noticed in the mutants. In wild-type embryos, Hox c8 expression is detected in the mesencephalon, diencephalon, and telencephalon. AsxII mutants also exhibit similar Hox c8 expression pattern in the head region but the signal is weaker than wild type. These results show that AsxII is required to activate Hox c8 expression in the brain. In this case, AsxII is acting like a trxG gene. AsxII is acting as a PcG gene in the neural ectoderm and mesoderm tissues repressing Hox gene expression.

3.3 Discussion

Using in-situ hybridization, I observed Hox a4, a7, and c8 genes expression patterns in AsxII mutant embryos. The strong conclusion from the work presented in this chapter is that AsxII is required for the normal regulation of Hox genes in mice. The analysis of Hox genes expression patterns supports the classification of AsxII as an ETP, which maintain both the active and silent states of genes. Hox c8 expression is not activated in the brain of AsxII mutants, showing that AsxII is responsible for maintaining the active state of Hox c8 in the mesencephalon, diencephalon, and telencephalon. However, de-repression of Hox a4 and Hox a7 are observed in AsxII mutant embryos, showing that AsxII is also responsible for maintaining the silent state of Hox a4 and Hox a7 in the neural ectoderm and part of the paraxial mesoderm. The targets of AsxII may be tissue specific because expression of Hox c8 is affected in the head region but not in the neural ectoderm and paraxial mesoderm. Similarly, expression of Hox a4
Figure 3.4: *Hox c8* mRNA in-situ hybridization of wild-type and *AsxII* mutant.
A and B are the wild-type embryos. C and D are the mutant embryos. The red arrow heads indicate the location of the anterior boundary of *Hox c8* in the neural ectoderm in the wild-type embryos. The blue arrow heads indicate the location of the anterior boundary of *Hox c8* in the mesoderm in the wild-type embryos. The green brackets represent the distance between the edge of the hind limb bud and the observed *Hox a4* anterior boundary of mesoderm.
and Hox a7 is derepressed mostly in the anterior boundary of neural ectoderm and the mesoderm. In the case of Hox a4, phenotypes are various between mutants. This finding represents a significant advance on the phenotypic analysis of Asxll that was available previously. However, in this discussion, I will point out some of the problems of interpretation of the data.

The effects of Asxll on gene expression patterns of Hox a4, a7, and c8 are reproducible, but relatively subtle. The change in Hox c8 gene expression is the most dramatic among the three and the penetrance of effect is high, because all Asxll-/- embryos exhibit nearly complete absence expression in the brain. Hox a4 and a7 mutations cause the anterior boundaries of these two genes in the neuroectoderm to shift only one to two somites anteriorly (rostrally). The penetrance of the phenotypes is high, because all embryos exhibit these changes. However, for Hox a4, only one mutant of seven observed exhibits anterior shifts of greater than two somites. The mild phenotypes of Hox genes expression pattern are consistent with the relatively mild axial phenotypes observed by Cynthia Fisher (Fisher 2005). The most likely explanation is that the existence of the other two Asxll2 and Asxll3 which may have redundant functions that compensate the loss of Asxll1. The apparent tissue-specificity of Asxll may in fact be a reflection of tissue specificity of Asxll2 or Asxll3.

The most likely gene to have redundant function is Asxll2 because its expression patterns completely overlap those of Asxll1 whereas Asxll3 has more limited expression (Fisher et al., 2006). We foresaw the likelihood of redundancy of Asxll2 and Asxll3 with Asxll1 at the commencement of the thesis, which was an important reason for the work undertaken in Chapter 2. These studies are still worth pursuing, so that we can test the possibility that Asxll1, 2 and 3 have overlapping functions. If we can make Asxll2 and Asxll3 mutant mice, we can cross them to create double heterozygous and interbreed the double heterozygous mice to create double mutant embryos with different combinations: Asxll1/Asxll2, Asxll1/Asxll3, Asxll2/Asxll3. I predict that the anterior-posterior transformations of the axial skeleton and the changes in Hox a4, a7, and c8
expression patterns will be much more severe in double mutants compared to single mutants if there is redundancy of function among Asxl homologs.

For the reasons explained in the introduction to this chapter, it is not straightforward to predict what the expected expression pattern of a given Hox gene should be in an Asxl/- embryo should be. Loss of function mutations of Hox a4 have the same phenotype as Asxl mutations, namely posterior transformation of C7 towards T1, so I would expect that the expression boundary of Hox a4 would shift posteriorly in Asxl/- embryos. I observed the opposite, namely that Hox a4 expression is shifted anteriorly in Asxl/- embryos. Loss of function Hox a7 mutants are phenotypically wild-type (Chen et al., 1998), but Hox a7 is derepressed in Hox a7 mutants. In this case, the in situ hybridization result is consistent with the ChIP analysis which implies that Hox a7 is a direct target of Asxl. Finally, one would predict based on the phenotype of Hox c8 knockout mice that one would observe a derepression of Hox c8 expression in the anterior-posterior axis in Asxl mutants. No change was detectable. Unexpectedly, we observed that Asxl is required to activate Hox c8 expression in the brain. Overall, the difficulties that others in the field have had predicting the effect of Hox mutations on phenotype, or predicting the Hox mutation responsible given a phenotype, was true for me as well. The difficulty to predict changes in Hox a4, a7, c8 expression patterns in Asxl-/- may result from altered or compensatory cross-regulatory interactions between genes within clusters, or between homeotic clusters in MP mutants (Chen et al., 1998; Kennison, 2004). Nevertheless, the difficulties in prediction do not detract from the main result, which is that Hox expression patterns are abnormal in Asxl mutants.

Maintenance proteins are categorized as PcG, trxG, or ETP based on their homeotic phenotypes in mutants, which reflect the main functions of the MPs either maintaining the active or silent states of genes. Nevertheless, the phenotypes observed do not always follow a simple rule. For example, Drosophila Pc mutants exhibit minor anterior transformations in caudal
abdominal segments (Denell and Frederick, 1983). This phenotype is not expected because derepression of *Abdominal-B* (*Abd-B*) should cause posterior transformations. These ambiguous phenotypes are observed more often in mammals. Mutants in *M33* and *Mll*, which are homologues of *Pc* and *trx* respectively, exhibit both anterior and posterior transformations in mice (Core et al., 1997) as described in chapter 1. The compound mutants of *Mll* and *Bmil* do show antagonistic effects on differentiation of the anteroposterior axis (Hanson et al., 1999) as expected for compound TrxG and PcG gene mutations. However, compound null homozygous mouse mutants for *Mell8* and *Bmil* show reduction in expression of *Hoxb6* and *Evxl* while showing ectopic expression of other *Hox* genes (Akasaka et al., 2001). This observation suggests that these PcG genes are needed for maintaining the active and silent states of genes.

*Drosophila Ring* mutants exhibit strong posterior transformation (Breen and Duncan, 1986; Campbell et al., 1995; Fritsch et al., 2003) as expected for PcG. Interestingly, in murine both LOF and GOF *Ringla* mutants exhibit anterior transformation (de Laat and Grosveld, 2003).

The unexpected phenotypes of mutations in *Hox* genes, or the effects of MP mutations on *Hox* expression can be explained by indirect effects. One possibility is that PcG and trxG genes cross-regulate themselves since PcG proteins bind on polytene chromosomes to regions containing PcG genes (DeCamillis et al., 1992; Zink and Paro, 1989). Strutt and Paro showed that a gene near the inverted locus was regulated by PcG genes (Strutt and Paro, 1997); this gene was subsequently identified as *toutatis*, a member of the trxG (Fauvarque et al., 2001). Evidence for cross-regulatory interactions of MPs comes from a study in *Drosophila* examining expression of PcG genes in PcG mutant embryos. It was found that *ph, Pc*, and *Psc*, negatively regulate transcript levels of *Psc* and *Suppressor 2 of zeste* (*Su(z)2*) and perhaps *Ring/Sce*, whereas *Additional sex combs* (*Asx*), *Polycomblike* (*Pcl*), and *Enhancer of Polycomb* (*E(Pc)*) positively regulate *Pc* and *Psc* (Ali and Bender, 2004). If *Asxl1* is required to activate PcG genes, then it may have a primary molecular function as an activator, and thus have anterior transformations in
the axial skeleton, but also have phenotypes typical of loss-of-function mutations of repressors caused by failure to activate PcG genes.

Recent studies have shown that Mll1 binds extensively to the Hox A cluster (Guenther et al., 2005) and Mll is associated with Hox c8. Mll ChIP data has suggested that the association of Mll with Hox a4 and Hox c8. More importantly, co-immunoprecipitation shows that Asxl1 is associated with Mll (unpublished data). Our mRNA in-situ hybridization results are consistent with the idea that Asxl1 binds Hox a4, a7, and c8 since Asxl1 has effects on the expression of all three genes. We also have ChIP data shows that Asxl1 is also associated with Hox a7. It is crucial to perform further experiments to verify if Asxl1 binds directly to Hox a4 and c8 or not to determine if the effects I observed of Asxl1 mutations on Hox expression patterns are direct or not.

It could be that the Hox genes we examined may not be the main targets of Asxl1. I only examined three Hox genes out of thirty-eight Hox genes in mouse, so potentially more severe affects of Asxl1 mutations on Hox expression may have been missed. To test this possibility, the experiments begun here could be extended to examine expression of more Hox genes using mRNA in-hybridization in Asxl1 mutant embryos. More work are needs to be done to determine the target specificity of Asxl1 for Hox genes. The obvious experiment to do prior to commencing further studies using in situ hybridization is to use large scale ChIP studies on the Hox clusters, using a combination of ChIP and microarray or CHIP (Guenther et al., 2005) and selected amplification of sequence tags (cite Ivan Sadowski’s paper).

Since Asxl1 has biological effects on Hox genes in A and C clusters, these genes make attractive loci to investigate the molecular function of Asxl1. The next step would be to determine where Asxl1 binds Hox genes using the ChIP studies described in the previous paragraph. I predict that Asxl1 will bind downstream of the promoter. In Drosophila Asx associates with Pol II phosphorylated at Serine 2, and Asx is associated with Trx (unpublished
data). Both these genes are necessary for elongation of \textit{hsp70} together with TAC1 (Smith et al., 2004). Therefore, it is likely that Asxl1 also is required for transcriptional elongation. \textit{Mll} regulates the \textit{Hox A} and \textit{C} clusters. If Asxl1 and Mll always act together, one would predict that Asxl1 mutations should not affect the \textit{Hox B} and \textit{D} clusters. If Asxl1 has effects on \textit{Hox} expression of \textit{B} and \textit{D} clusters independent from \textit{Mll}, this suggests that Asxl1 acts independently of \textit{Mll}.

Recently, a genome-wide analysis identified over 500 genes co-occupied by PRC1 and PRC2 in murine ES cells, and all co-occupied genes contained tri-methylated Lys 27 on histone H3, a classical code for repressed genes (Bracken et al., 2006). Many of these genes encode transcription factors (Bracken et al., 2006). Even though \textit{Hox} genes are the classic targets for MPs, it would be worth awhile to explore if these genes co-occupied genes are also regulated by Asxl1.

It would be worth identifying proteins that interact with Asxl1 \textit{in vivo}. So far, multiple PcG complexes have been identified in \textit{Drosophila} and mammalians, but none contain Asx or Asxl1. Therefore, Asxl1 can be used as the starting point for co-immunoprecipitation assay using cell or embryonic extracts. Since Asxl1 is an ETP, by identifying proteins associated with Asxl1 will help elucidate the mechanisms of ETPs.
4 METHODS AND MATERIALS

4.1 Extraction of Embryonic Stem (ES) Cell DNA

ES cells genomic DNA was extracted by using DNAzol (Invitrogen, Cat. no. 10503-027). 1mL DNAzol was added to lyse 3x10⁷ cells. I mixed the lysate by inversion and by pipetting when necessary. To precipitate DNA, 0.5mL 100% ethanol was added to the lysate and mixed by inversion. The samples were left at room temperature for 5 minutes until cloudy DNA could be seen. I used the pipette tips to swirl the DNA from the samples. DNA was held against the bottom of the tube and the supernatant was removed carefully. The DNA pellet was washed twice with 70% ethanol and air dried for 5-10 minutes. I dissolved the DNA pellet in 100μl Ultra Pure Distilled Water DNase, RNase free (Gibco, Cat. No. 10977-015). The amount of DNA was quantified by measuring the OD₂₆₀.

4.2 PCR conditions for Amplifying ES cell Genomic DNA

For each 25μl PCR reaction I used: 10X PCR buffer (Invitrogen) 2.5 μl, 50mM MgCl₂ 0.75μl, 10mM dNTPs 0.5μl, 10μM forward primer 1μl, 10μM reverser primer 1μl, genomic DNA isolated from ES cell line 60-80ng, Platinum Taq DNA polymerase 0.25μl (1.25Unit), and distilled water to 25μl.

The program for the PCR reaction to amplify Asxl2 ES cells DNA was the following, denature one cycle of 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 54°C for 30seconds, 72°C 3 minutes 30 seconds, and one cycle of 72°C for 2 minutes. Only the elongation time was varied in a scale of 1kb/minute. The sequences for all the primers for Asxl2 and Mll as described in figure 2.4, and figure 2.10 respectively are listed in the following table.
Table 4.1: Table of primers’ sequences used for gene trapped ES cell lines analysis

<table>
<thead>
<tr>
<th>Primer’s name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsxII primer 1</td>
<td>5’GCTGTGTGTGCAGGGGTTTCT3’</td>
</tr>
<tr>
<td>AsxII primer 2</td>
<td>5’GATGCCACCCAGCACTGTTA3’</td>
</tr>
<tr>
<td>AsxII primer 3</td>
<td>5’CCACCAGTGACTGGGAGAA3’</td>
</tr>
<tr>
<td>AsxII primer 4</td>
<td>5’GCTATGGTGACACAGCCTT3’</td>
</tr>
<tr>
<td>AsxII primer 5</td>
<td>5’CCATTCCACGCAGGACTTAA3’</td>
</tr>
<tr>
<td>AsxII primer 6</td>
<td>5’TACAGAAATCTGGGCTGCTCT3’</td>
</tr>
<tr>
<td>AsxII primer 7</td>
<td>5’TTGAGAGGTGGGACTTTTCC3’</td>
</tr>
<tr>
<td>AsxII primer 8</td>
<td>5’CGGTTCTTTGCTTCTCCATGAA3’</td>
</tr>
<tr>
<td>AsxII primer 9</td>
<td>5’CCATTCAACCTCCGCAAACTC3’</td>
</tr>
<tr>
<td>AsxII primer 10</td>
<td>5’ACCCCATATCAAGGTCATGC3’</td>
</tr>
<tr>
<td>AsxII primer 11</td>
<td>5’ACTGGGCTTTTGTGGATA3’</td>
</tr>
<tr>
<td>MII primer 1</td>
<td>5’CCACCAGACCTACATTC3’</td>
</tr>
<tr>
<td>MII primer 2</td>
<td>5’CATCCTCTTGACAGCAGTCA3’</td>
</tr>
<tr>
<td>MII primer 3</td>
<td>5’CTAGAGCGACAGGAGCAG3’</td>
</tr>
<tr>
<td>MII primer 4</td>
<td>5’CACCTCCAACCTCCGCAAACTC3’</td>
</tr>
<tr>
<td>MII primer 5</td>
<td>5’TGTCTGGTGATTGGAATGT3’</td>
</tr>
<tr>
<td>MII primer 6</td>
<td>5’TCCCTGTTCGCTTAGGATG3’</td>
</tr>
<tr>
<td>MII primer 7</td>
<td>5’TGCATTTTCTGGCTTAGG3’</td>
</tr>
<tr>
<td>Beta-galactosidase gene (forward)</td>
<td>5’TTATCGATGAGCGTTGTTATG3’</td>
</tr>
<tr>
<td>Beta-galactosidase gene (reverse)</td>
<td>5’GCGCGTACACATCGGCAATAATATC3’</td>
</tr>
</tbody>
</table>

4.3 Gel extraction and sequencing

I extracted bands of interest from agarose gels using the Qiagen gel extraction kit according to the manufacturer’s directions. The amount of DNA was quantified by measuring its OD

1.260.

I sent the samples to Nucleic Acid Protein Services Unit (NAPS) at UBC for sequencing. The concentration for PCR products I sent for sequencing was 10-15ng/µl and the primer concentration was 5pmol/µl. NAPS requires 15ng PCR product and 5pmol primer for each sequencing reaction.

4.4 Ligation-mediated PCR

I isolated ES cell genomic DNA using DNAzol as described above and digested 1µg DNA with 2µl 20U/mL Hinfl restriction enzyme in a 50µl reaction for three hours at 37°C. I
ligated the digested DNA overnight at room temperature to a double-stranded bubble linker. The following is the ligation reaction recipe: 50μl digested DNA, linkers (10μM) 10μl, 10X ligation buffer 10μl, T4 ligase 1μl, 29μl distilled water to make the volume up to 100μl. One tenth of the ligation mixture was used as a template for PCR reaction. The PCR reaction recipe was described as above. Table 4-2 contains the sequence of the linkers and linkers’ primers. I used the following program to run the PCR: 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C 30 seconds, and one cycle of 72°C for 2 minutes.

Table 4.2: Table of linkers/primers’ sequences for ligation-mediated PCR.

<table>
<thead>
<tr>
<th>Linker/primer’s name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linker 1</td>
<td>5’CTCTCCCTTCTCGAATCGTAACCGTTCGTAACCGTTCGTACGAGAA TCGCTGTCCTCTCCTTG3’</td>
</tr>
<tr>
<td>Linker 2</td>
<td>5’ACTCAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGAGAGAAGGAGAG3’</td>
</tr>
<tr>
<td>Linker primer 1</td>
<td>5’CGAATCGTAACCGTTCGTACGAGGAATCGCT3’</td>
</tr>
<tr>
<td>Linker primer 2</td>
<td>5’CACTTCAACCTCCGCAAACCTC3’</td>
</tr>
</tbody>
</table>

4.5 Southern blot

4.5.1 Restriction enzyme digestion

I isolated genomic DNA from ES cells using the method described above. I digested 5-10μg genomic DNA overnight in a 40μl reaction with 4μl restriction enzyme at the manufacturer’s recommended temperature using the manufacturer’s buffer. The restriction enzymes used in this experiment were PstI and NcoI, both were from New England Biolabs.

4.5.2 Agarose gel electrophoresis

After overnight digestion, I took out 2μl of the reaction samples and separated them by electrophoresis on an agarose mini-gel to check if the digestion was successful. The remainder of the sample was used for Southern blotting. I used a 0.8% agarose gel (11x14cm) to run the rest of the samples overnight at 20 volts.
4.5.3 Transferring DNA from agarose gel to zetaprobe membrane

After electrophoresis, the gel was photographed with a fluorescent ruler to provide scale. Then, I depurinated the gel in 0.1M HCl for eight minutes. After eight minutes depurination, I rinsed the gel with deionized water thoroughly. I denatured the gel with 0.5M NaOH for thirty to forty minutes with gentle shaking. After denaturation, I rinsed the gel with deionized water. Meantime, I prepared five blotting papers and one Zetaprobe membrane, which were cut in the same size as the gel. I placed a blotting paper wick over a glass plate over a 10X SSC reservoir with two ends of paper hanging in the solution. Then, I placed the denatured gel on the blotting paper wick and laid the Zetaprobe membrane on the gel carefully without the creation of bubbles between layers. I laid two wet blotting paper on top of the Zetaprobe membrane and then another three dry blotting papers on top. I put another 3 inches paper towels on the stack and two pieces of thin glass plates for weights. I allowed the transfer to occur overnight.

4.5.4 Hybridization and probe making

I prepared the hybridization solution with the following formula: 17mL deionized water, 0.2g skim milk powder, 2.0g dextran sulfate, 6mL 20X SSC, 2.0mL formamide, 1.0mL 20% SDS, 80μl 500mM EDTA pH 8.0. I pre-warmed this solution at 65 °C and then I added 1mL salmon sperm DNA (10mg/mL in water) that had been boiled for five minutes and sheared with a 21G needle. The membrane was removed carefully from the transfer and wet it with 2X SSC. Then, with a mesh underneath I rolled the mesh and membrane together and put them into a hybridization bottle. I pre-hybridized the membrane with hybridization solution at 65°C for one to two hours. During the pre-hybridization process, I prepared the reaction for making the radioactive probe as follows.
I amplified a probe from genomic DNA using the conditions as described above. I added the amplified DNA fragment about 20ng, together with 1μl 200ng/μg random hexanucleotides, and distilled water to 14μl. I denatured the DNA at 95°C for three minutes. After three minutes boiling, I put the mixture on ice for one minute. Then I added 2μl 10X HLB, 2μl dAGT mix (2.5μM for each nucleotide). 10X HLB contains 500mM HEPES pH 6.9, 100mM MgCl₂, 60mM 2-mercaptoethanol. I brought the reaction mixture to the hood destined for handling radioactive materials, and added 2μl ³²PdCTP into the mixture. After addition of ³²PdCTP, I added the large fragment DNA polymerase. I left the reaction mixture in the hood for one to two hours at room temperature. I prepared a column for cleaning up the probe by adding Sephadex G50 swollen in TE into the 1mL syringe plugged with glass wool. I put the syringe filled with Sephadex G50/TE into the 15mL falcon tube and centrifuged the syringe for three minutes at 1500 rpm. I discard the flow-through and washed the column once by adding 100μl TE and centrifuged one more time at 1500 rpm for three minutes. After the incubation time for making probe, I added 80μl TE to the reaction to make the volume to 100μl. I added the entire 100μl sample to the column and centrifuged as before and collected the sample in an eppendorf tube placed at the bottom of a 15mL falcon tube. I denatured the probe by adding 4μl 10M NaOH to the 100μl sample. I allowed the probe to sit for 1min and then added the probe into the hybridization solution in the hybridization tube. I swirled the tube to mix the probe in the hybridization solution. I allowed the hybridization to go overnight in the chamber at 65°C.

4.5.5 Blot washing and exposure

After overnight hybridization, I washed the membrane with washing solution containing 0.3% SSC, 0.1% SDS, 0.1% tetra-sodium pyrophosphate. The washing solution was pre-warmed to 65°C. I rinsed the blot with about 100mL washing solution and then filled about half of the tube with washing solution. I washed the blot for three times for thirty minutes each at
65°C. I exposed the blot with a phosphorimaging screen overnight. The next day, I scanned the phosphorimaging screen using the phosphorimaging scanner to view the image.

4.6 mRNA in-situ hybridization

4.6.1 Materials and Stock solutions

All solutions for in situ hybridization used water treated with diethylpyrocarbonate (DEPC). I added 2mL of DEPC to 2L distilled water and stirred the solution overnight to make 0.1% DEPC water. 10X PBS contains 80g NaCl, 2g KCl, 14.4g Na$_2$HPO$_4$, 2.4g KH$_2$PO$_4$ at pH 7.4 dissolved in distilled water to 1 litre, and was autoclaved before use. PBSw is 1XPBS made to 0.1% Tween-20, and was filtered before use. 20X SSC contains 175.3g NaCl, 88.2g sodium citrate at pH 7.0 in 1 litre, and was autoclaved before use.

Hybridization Solution contained 10g Boehringer block, 500ml formamide, 250mL 20X SSC, adjusted to pH 4.5 by adding critic acid, and was heated at 65°C for one hour to dissolve the Boehringer block. Then I added 120ml DEPC water, 100mL Torula RNA (10mg/ml in water, filtered), 2mL Heparin (50mg/mL in 1X SSC, filtered), 5mL 20% Tween-20 (filtered), 10mL 10% CHAPS (filtered), and 10mL 0.5M EDTA.

MAB contains 100mM Maleic acid, 150mM NaCl at pH 7.5. Antibody buffer contains 10% heat inactivated goat serum, 1% Boehringer block in PBSw. AP1 buffer contains 0.1M NaCl, 0.1M Tris pH 9.5, 50mM MgCl$_2$. I used BM Purple AP (Alkaline Phosphatase) substrate from Roche to stain the embryos. This stain contains substrate for alkaline phosphatase and develop a permanent, dark purple spots at AP binding site.

For in-vitro transcription reaction for making RNA probe, I used 10x transcription buffer from Roche, DIG (digoxigenin) RNA labeling mix from Roche, RNAsse OUT from Invitrogen, and T3 or T7 polymerase from Invitrogen.
4.6.2 Embryo fixation

Our lab technician, Christy Brookes, maintained our mouse colonies at the Joint Animal Facility of the Terry Fox Laboratory. She set up the Asxl-1 heterozygous mating pairs to create the homozygous Asxl-1 embryos for my studies, checked vaginal plugs to ensure the mice had mated, sacrificed the mothers of the appropriate age, dissected the embryos from the mothers, dissected the embryo from their placentas, and submerged in 4% paraformaldehyde in PBS at 4°C overnight or until they were used.

All steps below are performed on ice with the exception of the Proteinase K digestion step which was performed at room temperature. After fixing, I washed the embryos with 0.1% Tween in PBS (PBSw) three times for five minutes each. I digested the embryos with 2mL 10μg/mL Proteinase K in PBSw at room temperature for thirteen minutes. Afterwards, I placed the embryos back on ice and then replaced the Proteinase K with 2ml freshly prepared 2mg/ml glycine in PBSw to stop the reaction. Next, I did a fast wash with PBSw and washed the embryos two times for 5 min each with PBSw. I re-fixed each embryo with 2ml of 4% paraformaldehyde/0.2% glutaraldehyde in PBSw (freshly prepared) for fifteen minutes. After refixing, I did a fast wash with PBSw and washed three times with PBSw, five minutes each. I did a wash with 50%PBSw/50% hybridization solution for three minutes, and then washed again with hybridization solution for three minutes. At this point, the embryos were stored in the hybridization solution at -20°C until needed.

4.6.3 Making DIG-labeled RNA probes

Plasmids containing cDNAs of genes of interest (10μg) were digested with 2μl of the appropriate restriction enzyme in a 20μl digestion reaction according to the manufacturer’s directions for two hours to linearize the plasmid. All digested samples were separated electrophoretically on an agarose gel and linear plasmid was recovered using Qiagen “QIAquick
Gel Extraction” kit according to the manufacturer’s direction. The amount of DNA was quantified by determining the optical density. The transcription reaction contained 300ng-500ng linearized construct, 2µl 10X transcription buffer (Roche), 2µl DIG labeling mix, 1µl RNase OUT, 1µl RNA polymerase T3 or T7 depending on the construct, and added distilled water up to 20µl. I added RNase OUT before adding the labeling mix, and added RNA polymerase last, and incubated the reaction for two hours at 37°C. During the two hour transcription reaction, I pre-hybridized the embryos with fresh 900µl hybridization buffer at 65°C for one and half to two hours in round bottom 15mL tubes.

After the transcription reaction was complete, I took 2µl from the mixture and ran an agarose gel 200V to ensure that the probe had the correct size distribution and quantity. I added 32µl distilled water to the rest of the reaction to make the volume to 50µl. Unincorporated nucleotides were removed by passing the transcription reaction through a spin column (Amersham Biosciences) according to the manufacturer’s directions. The probe from one transcription reaction is sufficient for 2 embryos. I added 25µl of purified probe to each of two tubes, and then added 100µl hybridization buffer. I denatured the probe in a PCR machine at 95°C for five minutes, and then added the denatured probe directly into the tube containing the embryos and the prehybridization buffer. I hybridized the embryos at 70°C overnight. During the first hour of 70°C overnight incubation, I gently agitated the tubes every fifteen minutes to ensure the probe distributed evenly in the hybridization solution. Then, I allowed the tubes to incubate in the 70°C water bath overnight.

4.6.4 Washing embryos & antibody binding

The next day, I prepared 2.5 ml of antibody buffer for each tube (see above). I removed the probe/hybridization solution mix and replaced it with 800µl hybridization solution. I incubated the tube at 70°C for five minutes. Then, I added 400µl 2X SSC pH 4.5 to each tube
and incubated them again for five minutes at 70°C. I added another 400μl 2X SSC pH 4.5 for total of three times five minutes each at 70°C without pouring out the old solution. Then I washed the embryos two times with 2X SSC pH 7.0, 0.1% CHAPS for thirty minutes at 70°C. Embryos were then washed with twice with MAB at room temperature for ten minutes each, two times with MAB at 70°C for thirty minutes each, followed with two times PBS at room temperature for ten minutes each, ending with a single wash in PBSw at room temperature for five minutes. I incubated the embryos with 1ml antibody buffer with rocking at 4°C for two hours. Meanwhile, I pre-blocked the anti-DIG-alkaline phosphatase antibody with the antibody buffer at 1:10,000 dilution (1.5ml for each vial) with rocking at 4°C for two hours. After two hours incubation, I replaced the antibody buffer without antibody with antibody buffer and incubated the embryos with rocking overnight at 4°C.

4.6.5 Washing embryos & staining

The next day, I washed embryos with 0.1% BSA in PBSw once, followed by five one hour washes in the same buffer at room temperature. I then washed the embryos two times for ten minutes each washes with AP1. After the final wash, I replaced AP1 with 1ml BM purple AP substrate (Roche, described in Materials section), wrapped the tubes with aluminum foil and stained at room temperature for four hours. I viewed the embryos in a Petri dish with PBSw under a light microscope.

4.6.6 Photography

I used a light microscope (Lefebvre Lab), which are connected to a digital camera, to view and take pictures of the embryos. The program allows me to save images in electronic format. The images are saved as “tif” files, which can be used for publishing proposes.
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