The Function And Molecular Mechanism Of Asxl2 In The Mammalian Heart

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
A	Amino acid
ADP	Adenosine diphosphate
Ab	Antibody
ATP	Adenosine-5'-triphosphate
BDM	Butanedione monoxime
bp	Base pairs
BSA	Bovine serum albumin
CHD	Congenital heart disease
ChIP	Chromatin immunoprecipitation
Chr	Chromatin fraction
SN	Soluble nuclear fraction
C/SN	Cytosol fraction
cm2	Centimeters squared
DEPC	Diethylpyrocarbonate
diH2O	Deionized water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Embryonic day of development
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
g	Gram
Hox	Homeotic genes
kb	Kilogram base pair(s)
L	Liter
lnc RNA	Long non coding RNAs
M	Molar
mg	Milligram
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
Neo	Neomycin resistance gene
ng	Nanograms
^{o}C	Degree Celsius
PCR	polymerase chain reaction
RA	Retinoic acid
RNA	ribonucleic acid
RNA POLII	RNA polymerase II

LIST OF ABBREVIATIONS

RNA POLII	RNA polymerase II
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHF	Second heart field
TAC	Transverse aortic constriction
TOF	Tetralogy of Fallot
TrxG	Trithorax Group
TSS	Transcriptional start site
U	Units
V	Volt
WT	Wild type
X-gal	5-bromo-4-chloro-3-indoyl β–D-galactoside
β-gal	Beta-galactosidase
PBS	Phosphate buffered saline

SUMMARY

Polycomb Group (PcG) and *Trithorax Group* (TrxG) genes were originally identified in Drosophila as repressors and activators of *Hox* genes, respectively (Schuettengruber et al., 2007). In *PcG* and *TrxG* mutants, the expression of *Hox* genes is mis-regulated, leading to homeotic transformation phenotypes of body segments (Lewis, 1978, 1982). In addition, PcG and TrxG proteins are found to be regulators of numerous genes involved in cell fate decision, stem cell identity and cancer (Aloia et al., 2013; Kohler and Hennig, 2010; Martinez et al., 2006; Sparmann and van Lohuizen, 2006); At the molecular level, PcG and TrxG proteins form multiple complexes that silence and activate chromatin, respectively (Pirrotta, 1998). A hallmark of PcG proteins activity is tri-methylation of histone H3 lysine 27 (H3K27me3), which is associated with a repressed transcriptional state (Cao et al., 2002). In contrast, tri-methylation of histone H3 lysine 4 (H3K27me4) is a product of TrxG activity, and is associated with transcriptional activation (Byrd and Shearn, 2003; Cao et al., 2002).

A class of genes known as *Enhancers of Trithorax and Polycomb (ETP)* genetically interacts with both *PcG* and *TrxG*. *Additional sex combs* (*Asx*) of Drosophila is a member of the *ETP* group because *Asx* mutations enhance both *PcG* and *TrxG* mutant phenotypes (Milne et al., 1999). A recent study has shown that a newly characterized Drosophila PcG protein, Calypso, forms a complex with Asx (Scheuermann et al., 2010). This new PcG complex is named Polycomb Repressive Deubiquitinase (PR-DUB). Biochemical analysis has shown that recombinant Calypso interacts with the Asx-N terminus (aa2-337), and this interaction enhances deubiquitination of nucleosomal H2A by Calypso *in vitro*. The enzyme activity of Calypso is required for repression of the *Hox* gene *Ubx* in Drosophila.

Three mammalian homologs of *Asx,* named *Asx-like 1, 2,* and *3,* have been identified in mice and humans (Fisher et al., 2003; Katoh, 2003, 2004). Like Drosophila Asx and Calypso, mammalian ASXL1 and BAP1, the mammalian homolog of Calypso, form a stable PR-DUB complex *in vitro* (Scheuermann et al., 2010).

We have generated an *Asxl2* $^{-/-}$ mouse line to study *Asxl* in a mammalian system by taking advantage of a gene-trapped ES cell line from the Gene Trap Consortium [www.genetrap.org] (Baskind et al., 2009). The ES cell gene trap line contains a β-geo cassette with a polyadenylation site at the 3' end which is integrated into the first intron of *Asxl2* (Baskind et al., 2009). The truncated mRNA encodes a protein product that is missing all the conserved domains of ASXL2.

Asxl2 is highly expressed in embryonic and adult hearts (Baskind et al., 2009). In collaboration with Dr. David Geenen's laboratory, we have conducted a series of physiological and biochemical assays of *Asxl2*$^{-/-}$ mice in the B6/129 F1 background to assess the role of ASXL2 in heart function. Our results show that *Asxl2*$^{-/-}$ mice can survive to adulthood but gradually develop ventricle dysfunction. Microarray analysis shows more than 753 cardiac genes are mis-regulated in the absence of *Asxl2*. These results suggest that *Asxl2* functions to maintain normal cardiac function and gene expression in postnatal stages.

The axial skeletons of *Asxl2*$^{-/-}$ mice exhibit both posterior and anterior transformations, which are classic *PcG/TrxG* phenotypes, respectively (Baskind et al., 2009). This indicates that *Asxl2* has *ETP* function. Furthermore, *Asxl2* deficiency results in a reduction in the level of bulk H3K27me3, a repressive mark generated by the Polycomb Repressive Complex 2 (PRC2) (Baskind et al., 2009). Our goal is to determine the mechanism by which ASXL2 regulates H3K27me3 level. I show that ASXL2 interacts with PRC2 in the adult heart. The loss of *Asxl2*

results in loss of H3K27me3 as well as loss of PRC2 enrichment surrounding the de-repressed target promoters. The loss of PRC2 and H3K27me3 enrichment at these loci is not due to degradation of PRC2 core components or a failure of these components to form the PRC2 complex in *Asxl2*$^{-/-}$ hearts. In addition, we tested whether PR-DUB function is conserved in ASXL2. Our results show that ASXL2 interacts with BAP1 and is required for mono-deubiquitination of H2A at lysine 119 (H2AK119ub1) in the heart.

In summary, I have made several contributions to further our understanding of ASXL2's biological and functional mechanisms. Specifically, I have shown that (1) ASXL2 is required for normal ventricular function in adult heart; (2) ASXL2 is required for the homeostasis of two important histone marks, H3K27me3 and uH2A; (3) ASXL2 specifically affects the conversion of H3K27me2 to H3K27me3; (4) ASXL2 maintains the repression of select cardiac genes by facilitating the binding of PRC2 to target loci. Taken together, these data show that ASXL2 is a novel epigenetic regulator in the adult heart. In-depth studies of mouse ASXL2 will provide valuable insight on the diagnostic and/or therapeutic value of human ASXL2 in heart disease.

I. GENERAL INTRODUCTION

1.1 The function of mammalian heart

The mammalian adult heart is a four-chambered muscular organ that pumps blood throughout the blood vessels by a sequence of alternating contractions and relaxations, which is essential for nutrient and oxygen supply to the cells of the whole body (Walker and Spinale, 1999).

The basic contractile unit of the heart is the cardiomyocyte, which contains bundles of myofibrils. The myofibrils are organized into repeating segments of sarcomeres. The sarcomere is composed of myosin and actin which are known as thick and thin filaments, respectively. Myosin has a globular head and binds to adenosine-5'-triphosphate (ATP) (Sieck and Regnier, 2001). The myosin head can hydrolyze ATP into adenosine diphosphate (ADP). The ATP hydrolysis subsequently releases the phosphate group which causes contraction. Contraction occurs when the myosin head interacts with actin, causing two filaments to come close to each other. Tropomyosin also attaches to actin filaments. In the relaxing heart muscle, tropomyosin blocks the binding sites of the myosin head from actin, thus preventing contraction. Cardiac contraction is triggered by the elevation of intracellular Ca^{+2} levels. The troponin complex is another important contractility protein that attaches to tropomyosin under the groove of actin filaments. Troponin changes shape when exposed to Ca^{+2}, which in turn changes the confirmation of tropomyosin, exposing the actin filaments to myosin. This network of proteins orchestrates the hearts ability to contract and relax (Solaro et al., 2008).

1.2 Epigenetic regulation in heart development

The formation of the heart is a precisely coordinated process that involves multiple molecular pathways. A network of transcription factors direct cardiac cell fates specification,

differentiation, and proliferation. Mutations in these regulators can result in congenital heart disease (Sachdeva et al., 1964). For example, tetralogy of fallot (TOF) is a type of congenital heart disease (CHD) and a common cause of blue baby syndrome (Bailliard and Anderson, 2009). The causes of TOF were originally linked to environmental factors. Later, mutations in transcription factors such as NKX2.5 (Benson et al., 1999) and GATA4 (Kodo and Yamagishi, 2010) were discovered to be linked to TOF and other CHDs (Clark et al., 2006). The causes of many CHDs and other cardiac diseases are known to be influenced by a mix of epigenetic, environmental, and transcription factors (Benson et al., 1999; Clark et al., 2006; Kodo et al., 2012; McCulley and Black, 2012; Nanda et al., 2012; Wang, 2012).

The importance of epigenetic mechanisms in transcriptional regulation has been gradually recognized. The nucleosome is the basic subunit of chromatin. It consists of an octamer of four core histone proteins (H3, H4, H2A, and H2B) wrapped with DNA. Histones can be covalently modified with a wide range of post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation (Bartova et al., 2008). These modifications occur mostly on the N-terminal tails. A large number of histone modifications have been discovered. Some of them, most notably histone acetylation and histone methylation, have been implicated in regulation of heart development and function.

Besides histone modifiers, chromatin structures can be regulated by ATP-dependent chromatin remodeling (Clapier and Cairns, 2009). Chromatin remodeling locally disrupts or shifts nucleosomes to increase or decrease DNA accessibility from regulatory factors by using the energy from ATP hydrolysis. This leads to either activation or repression of gene expression.

Many studies have begun to uncover the importance of epigenetic processes, such as histone modifications and chromatin remodeling, in cardiogenesis and function. Such factors

activate or repress transcriptional activities at target genomic loci by altering chromatin structure. Many chromatin regulators are involved in the regulation of cardiac differentiation, maturation, and morphogenesis (Bruneau, 2010; El-Osta, 2011; Takeuchi et al., 2011). Studying the function of epigenetic regulators and their targets will provide new insights into the transcriptional regulation networks in the heart. Below I will briefly review several epigenetic processes and the evidence supporting how these processes are important for the development and function of the heart.

1.3 Role of histone acetylation in heart development

Histone acetylation was the first discovered histone modification and is normally correlated with gene activation (Graff and Tsai, 2013). Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are two main enzymes that regulate acetylation. HAT adds acetyl groups to the histone tails which relaxes nucleosome interactions and allows transcription factor binding. In contrast, HDAC promotes chromatin compaction by removing acetyl groups from histone tails, resulting in gene silencing (Graff and Tsai, 2013). Studies of HAT and HDAC knockout mice and their target genes have revealed important roles of HAT and HDAC in cardiac development.

P300 is a well-studied HAT and a transcriptional activator. Mice homozygous for *P300* knockout die between E9.5 and E11.5. The mutant hearts display thin myocardium and diminished trabeculation. The cardiac defects in *P300* mutant mice might be associated with low expression of actinin, alpha (*Actn*) and myosin, heavy chain 7, cardiac muscle, beta (*Myh7*), which encodes a myosin heavy chain beta in embryonic hearts (Yao et al., 1998).

Mice with double mutations in *Histone deacetylase 1* (*Hdac1*) and *Histone deacetylase 2* (*Hdac2*) die two weeks after birth, their hearts exhibiting dilated cardiomyopathy and cardiac

arrhythmia (Montgomery et al., 2007). *Hyperpolarization-activated cyclic nucleotide-gated channel* (*Hcn*), the *T-type Ca2+ current* (*Cacna1g*), and *actin, alpha skeletal muscle* (*Acta1*) are fetal genes which are only expressed during embryonic stage (Montgomery et al., 2007). ChIP assays show that they are direct targets of HDAC1 and HDAC2. Loss of *Hdac1* and *Hdac2* results in de-repression of these fetal genes in the adult mutant heart. These observations lead to a hypothesis that the abnormalities in double mutation mice may be due to fetal gene re-activation in adulthood (Montgomery et al., 2007).

1.4 Chromatin remodeling in heart development

All known chromatin remodeling ATPases belong to the SNF2 family. Based on sequence and structure analysis, the ATPases can be divided into four subfamilies: SWI/SNF, ISWI, CHD and INO80 (Clapier and Cairns, 2009).

Brahma-associated factor (BAF) complex is one of the complexes of SWI/SNF family. BAF interacts with transcription factors and other chromatin factors to maintain normal cardiac development and function. In mice, the knock down of the BAF subunit *Baf60c* causes multiple cardiac defects, such as a shortened outflow tract, hypoplastic ventricle and abnormal cardiac looping (Lickert et al., 2004). BAF60C interacts with GATA4 and recruits BAP chromatin remodeling complexes to *Nkx2.5* enhancer region during mouse embryonic stages (Takeuchi and Bruneau, 2009). Both BAF and GATA4 are important for the activation of *Nkx2.5*, an essential transcription factor in cardiogenesis.

In mice, a mutation in the BAF subunit *Baf180* results in embryonic death between E12.5 and E15.5 with cardiac defects such as ventricular septal malformation and hypoplastic ventricles (Wang et al., 2004b). Mouse embryos with a mutation in a *retinoid X receptor* gene, a component of the retinoic acid (RA) pathway, have similar cardiac abnormalities as *Baf180*

mutants (Subbarayan et al., 2000). BAF180 is required for the expression of a subset of RA target genes such as *RARβ2* and *CRABPII* (Wang et al., 2004b) suggesting that BAF180 interacts with the RA pathway to regulate cardiac chamber formation.

Overall, many histone modifiers and chromatin modifiers have been implicated in heart formation; however, their roles in cardiac function are largely unknown due to most of their gene knockout mice dying at early embryonic stages, preventing functional analysis of the adult heart.

1.5 Polycomb Group (PcG) and Trithorax Group (TrxG) proteins

Accumulated evidence suggests that Polycomb Group (PcG) and Trithorax Group (TrxG) proteins also have roles in cardiac formation and function (Wang, 2012). PcG and TrxG proteins are key epigenetic activators and repressors, respectively. They regulate developmental gene expression in many tissues and organs. Here, I will first review the general knowledge about PcG/TrxG proteins, then review existing evidence that they are important regulators in the heart.

1.5.1 What are PcG and TrxG proteins?

Homeotic genes (*Hox*) are a set of transcription factors that specify the anterior and posterior axis of the body plan. *PcG* and *TrxG* genes have been discovered to function antagonistically in maintaining the expression patterns of *Hox* genes (Ingham, 1985; Kennison and Tamkun, 1988; Lewis, 1978). Mutations in *PcG* and *TrxG* disrupt specific anterior and posterior body plans and result in homeotic transformations (Lewis, 1978; Lewis, 1982). Posterior and anterior transformations are the hallmark phenotypes of *PcG* and *TrxG* mutations, respectively.

PcG and TrxG proteins regulate gene expression through modulation of target chromatin structures (Zink and Paro, 1989). Biochemical activities of PcG and TrxG proteins are mainly involved in the generation and maintenance of histone modifications of target chromatin (Beisel

et al., 2002; Cao et al., 2002). In addition to histone modifications, it has been shown that PcG proteins promote chromatin compaction (Francis et al., 2004) and TrxG proteins regulate chromatin remodeling (Crosby et al., 1999).

PcG proteins consist of at least four distinct multi-protein complexes that work together. They are known as PhoRC, Polycomb repressive complex 1 and 2 (PRC1 and 2), and Polycomb repressive deubiquitinase (PR-DUB) (Czermin et al., 2002; Scheuermann et al., 2010; Shao et al., 1999). PhoRC contains the DNA binding protein Pho (Fujioka et al., 2008). Initially PhoRC is recruited to target chromatin and then aides in the recruitment of PRC2 (Fujioka et al., 2008; Kuzmichev et al., 2002). EZH2 is a core component of PRC2 that specifically generates mono, di, and trimethylation of Histone H3 at lysine 27 (H3K27me1/2/3) (Cao et al., 2002). H3K27me3 is the hallmark of PcG-dependent gene silencing. H3K27me3 has been associated with promoting compact nucleosomes by recruiting PRC1 which prevents regulatory protein binding (Black et al., 2012). Extra sex comb (ESC) is a subunit of PRC2 (Lu et al., 2013). A knockout study of *esc* in *Drosophila* embryos shows its target genes are de-repressed. The de-repressed genes exhibit H3K27me3 reduction and increased levels of associated RNA polymerase II (RNA POLII) (Chopra et al., 2011). These data are interpreted as H3K27me3 being required for silencing by impeding RNA POLII binding at target promoters.

PRC1 subunit Polycomb (Antipchuk Iu, 1966) specifically recognizes H3K27me3 via its chromo- domain. PRC1 inhibits nucleosome remodeling and promotes chromatin compaction. A subunit of PRC1, Ring1 contains an E3 ligase activity for mono-ubiquitination of histone H2A (uH2A). Depletion of Ring1 results in de-repression of PRC1 targets, suggesting H2A ubiquitination is required for PRC1 mediated silencing (Wang et al., 2004a). However, a recent study suggests that PRC1- mediated chromatin compaction is independent of uH2A activity

(Eskeland et al., 2010). uH2A activity of PRC1-mediated repression is required for genes that are crucial for the maintenance of ESC identity (Endoh et al., 2012) .

A recently discovered PcG complex, Polycomb repressive deubiquitinase (PR-DUB) further complicates the role of H2A ubiquitination in PcG silencing (Scheuermann et al., 2010). Calypso is a Drosophila PcG protein which encodes an H2A deubiquitinating enzyme. PR-DUB consists of Calypso and Additional sex combs (Asx) (Gaytan de Ayala Alonso et al., 2007). *In vitro* experiments demonstrated that PR-DUB removes ubiquitin from nucleosomal H2A, and this catalytic activity is strongly enhanced by the interaction between Asx and Calypso (Scheuermann et al., 2010). Furthermore, deubiquitination of H2A is required for repression of a *Hox* gene (Scheuermann et al., 2010). Genetic studies in Drosophila show that PR-DUB and Ring1 co-localize at select target genes which supports the hypothesis that the balance of ubiquitination and deubiquitination might contribute to PcG silencing. Further investigation is needed to decipher the actual role of de-ubiquitination in gene repression.

TrxG proteins also form several protein complexes, such as SET-1 Like, MLL and BRM. The SET-1 like complex maintains transcriptional activation by H3K4 trimethylation (Rozovskaia et al., 2000). BRM contains an ATP dependent chromatin remodeling factor which mediates nucleosome sliding (Tamkun et al., 1992). The MLL complex generates histone methylation marks on target chromatin and promotes chromatin remodeling activity (Nakamura et al., 2002; Yokoyama et al., 2004).

1.5.2 Recruiting PcG proteins to targets

PcG proteins targeting to specific chromatin loci is required for PcG-mediated repression (Muller and Kassis, 2006). In Drosophila, special regulatory elements have been identified from PcG target genes that are required for PcG mediated repression. These elements are known as

Polycomb Response Elements (PREs) (Muller and Kassis, 2006). PREs are several hundred base pairs in length and often found at distances of ten kilobases or more from the promoters that they regulate. Pleiohomeotic (Pho) protein is a core component of the Pho-RC complex in Drosophila. Pho has DNA binding motifs and binds to PREs (Mohd-Sarip et al., 2002). However, the DNA binding site of Pho alone is not sufficient to repress expression of a *Hox* gene, suggesting more elements are required for PcG targeting (Mohd-Sarip et al., 2002). Other DNA binding proteins such as Dsp1 (Salvaing et al., 2006), GAGA factor (Okada and Hirose, 1998), and Pipsqueak (Schwendemann and Lehmann, 2002) are shown to associate with PREs in Drosophila. However, genome-wide ChIP assays show that the binding profiles of these DNA binding proteins do not completely overlap, suggesting there is no single protein which is necessary for PcG protein recruitment to targets (Schuettengruber et al., 2009).

In mammals, two PRE-like elements, PRE-kr (Sing et al., 2009) and D11.12 (Woo et al., 2010), have been identified. Both elements can repress gene expression through recruiting PcG proteins to target promoter in reporter gene assays. However, PRE-kr and D11.12 do not have consensus sequences (Sing et al., 2009; Woo et al., 2010), suggesting PRE-like elements are not the essential molecular players that recruit PcG proteins to targets.YY1 is the mammalian homolog of Drosophila Pho (Basu and Atchison, 2010). Both PRE-like elements require the presence of the YY1 DNA binding site for repression. YY1 was found to co-localize with PRC2 subunit EED and PRC1 subunit BMI1 to upstream *Hoxc8* and *Hoxa5* (Kim et al., 2006). Genome-wide ChIP assays show that YY1 and PcG proteins co-localize only a subset of genes (Kim et al., 2006), suggesting there are other components required for PcG protein recruitment to targets.

It has been proposed that PRC2 is recruited to its targets by JARID2 (Li et al., 2010) and PHF1 (Qin et al., 2013) in specific cell lines. JARID2 interacts with PRC2 in embryonic stem cells. ChIP seq data shows that the binding profiles of JARID2 and a PRC2 component, EZH2 are very similar. In the absence of *Jarid2*, PRC2 targets are de-repressed, and there is significant loss of both H3K27me3 and PRC2 binding (Li et al., 2010). PHD finger protein 1 (PHF1) contains a tudor domain and two PHD domains (Qin et al., 2013). *In vitro* assays showed that PHF1 interacts with EZH2 via the PHD domain (Qin et al., 2013). PHF1 co-localizes with EZH2 at PcG target genes such as a *Hoxa* locus and the non-*Hox MYT1* and *WNT1* loci in HeLa cells (Sarma et al., 2008). PHF1 interacts with H3K27me3 via the tudor domain. It has been proposed that the interaction of H3K27me3 with PHF1 can somehow stabilize the interaction of EZH2 and target chromatin (Sarma et al., 2008).

In addition, long non coding RNAs (lnc RNA) have been implicated in the targeting of PcG proteins in mammal. X inactivation is known to be dependent on an lncRNA, *Xist* that initiates inactivation of one of the X chromosomes (Penny et al., 1996). A non-coding RNA within the Xist region, called *RepA*, is required for EZH2 recruitment to inactive X chromosome (Zhao et al., 2008). *RepA* is also essential for *Xist* expression (Zhao et al., 2008). *Kcnq1ot1* is an lncRNA that is transcribed from the imprinted *Kcnq1* gene cluster. *Kcnq1ot1* can be immunoprecipitated with PRC2 and the H3K9 histone methyltransferase. *Kcnq1ot1* is required for recruitment of these histone methyltransferases to generate repressive marks, H3K27me3 and H3K9me3, at target chromatin (Pandey et al., 2008). *HOTAIR* is a 2.2 kb lncRNA transcribed from the *HOXC* locus. Depletion of *HOTAIR* results in *HOXD* de-repression. *HOTAIR* binds to PRC2, which stimulates PRC2 enzymatic activity to generate H3K27me3 marks at the *HOXD*

locus (Rinn et al., 2007). This suggests that *HOTAIR* has a role in PRC2 targeting and its enzymatic activity (Rinn et al., 2007).

PRC1 is well known to be recruited to target chromatin through recognition of the PRC2-mediated H3K27 methylation mark by the chromo domain of Pc (Min et al., 2003). Recently, alternative mechanisms, which are independent of PRC2, have been proposed to be responsible for recruiting PRC1 to targets. A variant PRC1 complex cannot recognize H3K27me3 marks due to the lack of the Pc subunit. This PRC1 variant contains a histone demethylase, KDM2B, which is required for PRC1 recruitment to target genes in pluripotent stem cells (Wu et al., 2013). The transcription factors REST (Dietrich et al., 2012) and RUNX1 (Yu et al., 2012) were also discovered to be required for PRC1 targeting in ES cells. These data suggest that PRC2 is not absolutely required for PRC1 targeting in all contexts.

In summary, no dominant recruitment mechanism has been identified in mammals. One possible reason might be due to the fact that PcG complexes are formed with different subunits depending on the targets and cell contexts. Alternative variants of PcG proteins may be recruited by distinct mechanisms.

1.5.3 Regulating PRC2 enzyme activity

PRC2-mediated H3K27me3 is the hallmark of PcG silencing (Cao et al., 2002). EZH2 is a methyltransferase and catalyzes mono, di, and tri-methylation of H3K27 (Cao et al., 2002). EZH2 enzyme activity can be regulated in several ways. (1) EZH2 is not active without SUZ12 and EED. SUZ12 binds to EZH2 through its C-terminal VEFS domain (Cao and Zhang, 2004), thereby promoting PRC2 assembly. EED is a WD-repeat protein that binds specifically to tri-methylated lysine. When PRC2 binds to H3K27me3 through EED, it stimulates EZH2 methyltransferase activity (Han et al., 2007). (2) Certain genes have been shown to play a role in

PcG complex recruitment and have an effect on EZH2 enzymatic activity, such as *Jarid2* and *PHF1*. PHF1 is required for conversion of H3K27me2 to H3K27me3 (Sarma et al., 2008). (3) It has been shown that PRC2 enzyme activity can be regulated by the phosphorylation of EZH2 at multiple threonine residues. Cyclin-dependent kinase 1 (CDK1) phosphorylates EZH2 at Thr487 (Wu and Zhang, 2011). Phosphorylation of EZH2 disrupts EED and SUZ12 binding, thereby impairing EZH2 methyltransferase activity (Wu and Zhang, 2011).

1.5.4 Enhancer of Trithorax and Polycomb Group (ETP) protein

A group of genes, *Enhancers of Trithorax and Polycomb* (*ETP*), were originally identified as *PcG* genes, but later were discovered to genetically regulate both PcG and TrxG protein activities. Double mutation of an *ETP* with either *PcG* or *TrxG* enhances *PcG* or *TrxG* mutations, respectively (Lopez et al., 2001).

It has been proposed that ETP proteins can regulate PcG and TrxG protein activities in several ways (Brock and van Lohuizen, 2001). (1) ETP proteins are part of PcG and TrxG complexes; (2) ETP proteins transcriptionally regulate *PcG* and *TrxG* gene expression; (3) ETP prtoeins are co-activators/repressors of PcG and TrxG proteins; (4) ETP proteins are required for PcG and TrxG recruitment to targets. Additional Sex Combs (Asx) is an ETP protein. The mammalian homolog of Asx, Additional sex combs-like 1 (ASXL1) co-localizes with PRC2 at select target loci in hematopoietic cell lines (Abdel-Wahab et al., 2012). The loss of *Asxl1* results in compromised PRC2 binding and H3K27me3 enrichment at target promoters, and the corresponding genes are de-repressed. These results suggest that ASXL1 is required for PRC2 binding and H3K27me3 enrichment at target chromatin (Abdel-Wahab et al., 2012). Whether ASXL1 is required for recruitment of PRC2 to target remains uncertain. However, the discovery of ASXL1 function in PRC2 binding supports the ETP protein model (4). There is no direct

evidence in *Drosophila* or mammalian systems to support ETP protein models (1), (2), and (3). However, some ETP proteins are known to interact with PcG proteins (Perry, 2006; Peterson et al., 2004).

Asx genetically interacts with both *PcG* and *TrxG* genes, as *Asx* mutations enhance both *PcG* and *TrxG* mutant phenotypes (Milne et al., 1999). Asx binds to distinct loci on polytene chromosomes; 70% of those loci overlap with the binding sites of the PcG proteins Pc and Ph (Milne et al., 1999). In addition to genetic evidence found with Drosophila Asx, ASXL1 has been shown to interact with PRC2 in a hematopoietic cell line by immunoprecipitation assays (reference). Yet, there is a lack of biochemical evidence supporting the direct interaction of PcG complexes with Asx or other ETP proteins.

1.5.5 PcG and TrxG proteins in heart development and function

Multiple PcG and TrxG proteins are expressed in mammalian hearts (Vallaster et al., 2012). Most constitutive *PcG* or *TrxG* knockout mice succumb to early embryonic lethality. Therefore, conditional knockout mice aid in the discovery of the roles of PcG and TrxG proteins in heart development and function. RAE28 is a subunit of the PRC1 complex. *Rae28* knockout mice develop outflow tract septation defects and aortic valve stenosis during E8.5-9.5 (Shirai et al., 2002). These defects are similar to the phenotypes of *Nkx2.5* knockout mice. This study also shows that RAE28 is required for maintaining *Nkx2.5* expression (Shirai et al., 2002). It is known that PRC1 is a transcriptional repressor. Therefore, RAE28 may indirectly regulate activation of *Nkx2.5* expression. The molecular function(s) of PRC1 in heart development remains to be determined (Koga et al., 2002).

It has been shown that PRC2 has roles in regulation of mammalian heart development and adult heart function. Inactivation of the PRC2 subunit EZH2 in ventricular cardiomyocytes

causes compact myocardial hypoplasia, hypertrabeculation, and ventricular septal defect (He et al., 2012). Another study inactivated *Ezh2* beginning at E7.5 in cardiac progenitors of the second heart field (SHF). Interestingly, these mice did not exhibit defects in cardiac morphogenesis. Instead, the mutant animals survived to adulthood but developed cardiac hypertrophy and fibrosis in the SHF- derived right ventricle.

BRG1 is a TrxG protein that functions in the myocardium to regulate cardiac growth and differentiation (Delgado-Olguin et al., 2012; Hang et al., 2010). Myosin heavy polypeptide 6, cardiac muscle, alpha (*Myh6*) and myosin, heavy chain 7, cardiac muscle, beta (*Myh7*) are the main myosin heavy chain (MHC) isoforms expressed in adult and embryonic heart, respectively. BRG1 forms a complex with HDAC and PARP to repress *Myh6* and activate *Myh7* in the embryonic stage. Loss of *Brg1* in the embryonic stage results in premature cardiomyocyte differentiation due to switching fetal of form (MYH7) to the adult form (MYH6). *Brg1* is normally repressed during adulthood. *Brg1* will be reactivated when the adult heart is stressed. De-repressed *Brg1* induces cardiac hypertrophy, and reactivates fetal gene (*Myh7*) expression in the adult heart. Overexpressed *Brg1* in adult heart is associated with MHC switch and cardiac hypertrophy. These observations suggest that the ATP-dependent chromatin remodeler BRG1 has an important role in both embryonic heart development and in adult heart disease (Hang et al., 2010).

Taken together, many studies have demonstrated the role of epigenetic regulation in cardiac development and function. A full understanding of how they regulate cardiogenesis and function is still limited because only a small number of epigenetic factors been functionally analyzed in the heart. Future studies of epigenetic mechanisms in cardiac development and

function will be important not only for our understanding of the causes of the diseases but also for potential applications to medical strategies.

1.6 Purpose of this study

Three homologs of *Asx*, named *Asx- like1*, *2*, and *3*, have been identified in mouse and human (Fisher et al., 2003; Katoh, 2003, 2004). We have undertaken a study of the mouse *Asxl2* gene to understand the function of ETP proteins. An *Asxl2* mutant mouse line was generated in our laboratory by taking advantage of a gene-trapped ES cell line from the Gene Trap Consortium [www.genetrap.org]. The ES cell gene trap line contains a β-geo cassette with a polyadenylation site at the 3' end which is integrated into the first intron of *Asxl2* (Baskind et al., 2009). This cassette disrupts endogenous splicing, resulting in a truncated mRNA. The truncated mRNA encodes a protein product that is missing all the conserved domains of ASXL2. This *Asxl2* gene trap also contains a *lacZ* reporter under control of the *Asxl2* promoter. *LacZ* expression can be used to check the *Asxl2* expression pattern. Based on X-gal staining, *Asxl2* is highly expressed in the heart at all developmental stages examined, starting in E9.5 dpc and throughout the postnatal stages. Surviving adult *Asxl2*$^{-/-}$ mice have higher heart-to-body weight ratios compared to wild-type (WT) (Baskind et al., 2009).

Baskind *et al*. reported that *Asxl2*$^{-/-}$ axial skeletons showed highly penetrant posterior transformations and less penetrant anterior transformations. As previously mentioned, posterior and anterior transformations of the axial skeletons are classic *PcG*$^{-/-}$ and *TrxG*$^{-/-}$ phenotypes, respectively. This suggests that ASXL2 has conserved ETP protein function. H3K27me3 and H3K4me3 are the hallmarks of PcG and TrxG protein activity, respectively. *Asxl2*$^{-/-}$ hearts have reduced bulk level of H3K27me3 but no observable differences in the level of H3K4me3 when compared to wild-type (WT) (Baskind et al., 2009). This suggests that ASXL2 likely has a

bigger role in the regulation of PcG than TrxG protein activities. The goal of this study was to determine the effect of the *Asxl2* mutation on cardiac function as well as the molecular mechanism through which ASXL2 regulates H3K27me3 levels in the adult heart.

II. MATERIALS AND METHODS

2.1 Animal breeding

All mice used in this study were in the C57BL/6 J×129Sv F1 background because *Asxl2*$^{-/-}$ animals in either C57BL/6 J or 129Sv inbred background die postnatally. *Asxl2*$^{+/-}$ females in the 129Sv inbred background were mated to *Asxl2*$^{+/-}$ males in the C57BL/6 J inbred background to produce *Asxl2*$^{-/-}$ animals and wild-type littermates. The genetic compositions of the experimental and control animals were identical except at the *Asxl2* locus.

2.2 Echocardiography

Transthoracic echocardiography was performed while under isoflurane anesthesia and positive pressure ventilation. Transthoracic two-dimensional targeted M-mode and pulsed-wave Doppler echocardiography was performed with a 30-MHz mechanical transducer attached to a VisualSonics Vevo 770 system (Visual Sonics, Toronto, ON, Canada). Fractional shortening (FS) was calculated by left ventricular internal dimension in diastole (LVIDd) minus left ventricular internal dimension in systole (LVIDs) and all divided by the left ventricular internal dimension in diastole (LVIDd). Ejection fraction (EF) is the amount of blood in the ventricles at the end of diastole that is ejected during each beat. EF was calculated by stroke volume (SV) divided by end-diastolic volume (EDV).

2.3 Hemodynamic measurements

Hemodynamic measurements were performed on 5-month-old wild-type and *Asxl2*$^{-/-}$ mice. Mice were anesthetized with isoflurane (1.5%) and injected with etomidate (10 mg/kg body weight; I.P.) for intubation. Anesthesia was maintained at 1% isoflurane and mice were ventilated with a Harvard Respirator at a rate of 140 breaths per minute and a 250 μm volume. A medial laparotomy exposed the diaphragm and a Millar Pressure/Volume transducer (SPR-839)

was inserted into the left ventricle through an apical puncture. Steady state measurements of pressure/volume loops were recorded and the inferior vena cava was occluded to derive load-independent measurements of the end-systolic pressure/volume relation.

2.4 Myofibril protein preparation

The left ventricle (~ 30-50 mg) was homogenized in 1 ml of SRB-X100 buffer (75mM KCl, 10mM imidazole pH7.2, 2mM $MgCl_2$, 2mM EDTA, 1mM NaN_3) using a 2 ml Dounce homogenizer. The homogenate was spun down for 1 minute at 13,000 rpm at 4 ºC. The supernatant was decanted and the pellet was re-suspended in 1 ml of SRB-X100. The re-suspended pellet was homogenized again using Dounce homogenizer. The homogenate was spun down at 13,000 rpm for 1 minute at 4 ºC. The supernatant was removed and the weight of pellet was determined in a pre-weighted eppendorf tube. The pellet was re-suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) industrial buffer (8M urea, 2M thiourea, 0.05M Tris pH 6.8, 75mM Dithiothreitol (DTT), 3% SDS and 0.05% bromophenol blue) based on a 1:20 (W/V) ratio (1mg = 1µl). The re-suspended pellet was homogenized using Dounce homogenizer at room temperature (RT). The homogenate was sonicated for 10 minutes at 4ºC using a water bath sonicator (Benson 1510). Samples were denatured at 100 ºC for 3 minutes and were spun down at 13,000 rpm for 5 minutes. The supernatant contained myofibril proteins. The concentration of myofibril proteins was determined using Pierce 660 nm Protein Assay (Thermo Scientific) with ionic detergent compatibility reagent (Thermo Scientific). The samples were stored at -80 ºC.

2.5 High resolution SDS-PAGE gel electrophoresis

For separation of myofibril proteins, An SE600 Hoefer gel system (Pharmacia) was used with 0.75-mm gel spacers. Glass plates were 16x18 cm and coated with Rain X to allow

removing the gel from the glass plate easily. 7-10 µg/ lane of myofibril proteins were separated on 12 % SDS-PAGE (resolving gel: 10% acrylamide cross-linked with 0.5% DATD, 0.375M Tris pH8.8, 10% glycerol, 0.1% SDS, 0.064% APS, 0.064% TEMED; stacking gel: 2.95% Acrylamide cross-linked with 15% DATD, 0.125M Tris pH6.8, 10% glycerol, 0.1% SDS, 0.064% APS, 0.064% TEMED) as previously described (Arteaga et al., 2005). The gels were subjected to either Coomassie staining to visualize all proteins or to Pro-Q Diamond staining (Invitrogen) to visualize phosphorylated proteins. Alternatively, separated proteins were transferred to a PVDF membrane and subjected to western blot analysis. For high-resolution SDS-PAGE for separation of MYH6 and MYH7 proteins, an SE400 Vertical Unit Hoefer gel system was used with 1.5-mm gel spacers (GE healthcare). Myofibril samples were loaded on 6% SDS-PAGE (6% acrylamide, 0.5% DATD, 0.375M Tris pH8.8, 10% glycerol, 0.1% SDS, 0.064% APS, 0.064% TEMED; stacking gel: 4% Acrylamide, 15% DATD, 0.125M Tris pH6.8, 10% glycerol, 0.1% SDS, 0.064% APS, 0.064% TEMED), run at 100V until the samples passed the stacking gel and then run at 150V for 8 hours at 4 °C. The acrylamide gel was subjected to silver staining. Proportions of protein of interest were determined using densitometry.

2.6 Silver staining

The gel was soaked in fixing solution (40% methanol, 5% formaldehyde) overnight at room temperature. The gel was washed in diH_2O twice for 5 minutes each and then soaked in 0.02% $Na_2S_2O_3$ for 1 minute. The gel was washed in washed in diH_2O twice for 5 minutes each and then soaked in 0.1% $AgNO_3$ for 10 minutes. Fresh-made developing solution (3% sodium carbonate, 0.0185% formaldehyde, 0.000016% $Na_2S_2O_3$) was added to the gel until protein band intensities were adequate. 2.3M citric acid was added to the developing solution to stop the reaction.

2.7 Blood pressure measurement

Blood pressures of male mice ranging from 1-month to 10-months of age were measured in un-anesthetized mice using an NIBP-8 tail-cuff blood pressure monitor (Columbus Instruments, Columbus, Ohio). The Animals were acclimated to the restrainer and the warming compartment for 30 min/day for at least 3 days. On the day of the experiment, animals were acclimated in the apparatus for 20 min before measurements were taken. The sensor cuff pressure was set at 45 mm Hg and the occlusion cuff pressure was 200 mm Hg. Each data point (for one animal at a specific age) represents the average of 10 or more sequential measurements, spaced at a minimum of one minute intervals.

2.8 Quantitative RT-PCR

RNA was isolated from the adult left ventricle using Trizol (Invitrogen) and followed with DNAase treatment. Quantitative RT-PCRs were performed on an ABI Prism7900HT sequence detection system (Applied Biosystems) using the SuperScript™ III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). The expression level of each gene analyzed was normalized against that of 18S rRNA or β-Actin in the same sample. For each gene, two wild-type and two mutant animals were analyzed. 100 ng of RNA was used for each reaction. The qRT-PCR program was as follows: (1) 50º 30 min (2) 95º 15 min (3) 95° 30 sec. (4) 60° 30 sec. (5) repeat step (3) - (4) for 39 times, (6) melting curve cycle.

Detailed information on primer sequences is given in Appendix A.

2.9 Chromatin immunoprecipitation (ChIP)

The whole heart (~100mg) was minced with scissors and then was formaldehyde-fixed at the final concentration of 1% for 10 minutes at RT. The fixed tissue was homogenized in 1ml Cell Lysis buffer (5mM HEPES; 85mM KCl; 0.5% NP-40; 1X protease inhibitor; 0.1mM

PMSF). The cell lysate was incubated on ice for 30 minutes. Nuclei were pelleted from cell lysate at 10,000 rpm for 5 minutes at 4 ºC. The nuclei were re-suspended in 2 ml Nuclei Lysis buffer (50mM Tris-HCl pH8.0; 10mM EDTA; 1% SDS; 1X protease inhibitor; 0.1mM PMSF) and incubated on ice for 1 hour. Chromatin was sheared by sonication three times in the length of 3 minutes (Biorupter) to an average length of approximately 100-500bp. The sheared chromatin was spun down at 13,500 rpm to remove insoluble chromatin. Soluble chromatin (one heart; 2ml) was pre-cleared by adding 50µl Dynabeads® Protein G beads (the beads were pre-incubated with 1µg of Mock IgG serum and rocked at 4 ºC for 2 hours). Dynabeads® Protein G beads were removed from the chromatin lysate. 300 µl of chromatin lysate was incubated with desired amount of anti-protein of interest (POI) antibody and mock serum, respectively, overnight at 4 ºC. The total input sample was prepared from 300 µl of chromatin lysate. 30 µl of Dynabeads® Protein G beads were added to each chromatin lysate except total input sample. The chromatin samples were rocked at 4ºC for 2 hours. The chromatin bound-beads were washed 30 seconds with 1 ml of RIPA buffer for 3X (50mM; 150mM NaCl; 0.1% SDS; 0.5% Deoycholate; 1% NP-40; 1mM EDTA in water) ; 1 ml of High Salt buffer for 3X (50mM Tris-HCl pH8.0; 0.1% SDS; 0.5% Deoxycholate; 1% NP-40; 1mM EDTA in water); 1 ml of LiCl buffer for 3X (50mM Tris-HCl pH 8.0; 1mM EDTA; 250mM LiCl; 1% NP-40; 0.5% Deoxycholate in water) and 1 ml of TE buffer for 3X (10mM Tris-HCl; 1mM EDTA in water). The chromatin bound-beads were re-suspended in 300 µl of TE buffer. ChIPed and total input samples were incubated at 37 ºC for 30 minutes with 1 µl of RNase A (10mg/mL). Subsequently, each sample was incubated at 55 ºC for 2 hours with 15 µl of 10% SDS. All the samples were reverse cross-linked at 65ºC for overnight. The ChIP-ed DNA was extracted by phenol/chloroform twice using the phase-log DNA purification system (5 Prime) and followed by ethanol precipitation. 1 µl from

each ChIPed-DNA and total input was analyzed by qPCR using primers specific for conserved regions in the gene of interest (GOI) promoter.

The antibodies, PCR program, and primers sequences are listed in Chapters III, IV, and Appendices A and C.

2.10 Histology and immunofluorescence

Paraffin embedded heart sections were made using standard protocols. H&E stainings were performed on 8 um sections; wheat-germ agglutinin-fluorescein staining was performed on 5 um sections.

2.11 Adult cardiomyocyte size measurement

Cardiomyocyte size was measured for two pairs of 6-month-old *Asxl2*$^{-/-}$ and wild-type hearts. Isolation of adult cardiomyocytes was performed as previously described (Louch et al., 2011). The cardiomyocytes were plated in the presence of butanedione monoxime (BDM), a contraction inhibitor, and allowed to attach for 3 h. Images of live cardiomyocytes were taken. The length, width, and area of the cells were measured using Image J. 124–262 individual cardiomyocytes were measured for each heart.

2.12 Biochemical fractionation of cellular proteins

Whole hearts were cut into pieces and homogenized in Buffer A (10 mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 10% glycerol, 1mM DTT, and protease inhibitors) using a Tissue Master homogenizer (OMNI International). Triton X-100 was added to the homogenate to a final concentration of 0.1% and rocked on ice for 15 minutes. The homogenate was spun down at 1300 g for 5 minutes at 4 ºC to generate the supernatant (S1; cytosolic fraction) and pellet (P1) fraction. S1 was spun down at 20,000 g for 10 minutes to remove insoluble proteins. On the other hand, P1 was washed with Buffer A1 and then spun down at

1300g for 5 minutes. P1 was re-suspended in 1ml of Buffer B1 (3mM EDTA; 0.2mM EGTA, 1mM DTT; 1X proteinase inhibitors) and rocked on ice for 1 hour. The re-suspended pellet was spun down at 1700g for 10 minutes to generate the supernatant (S3; soluble nuclei fraction) and pellet (P2; chromatin fraction) fractions. All the fractions were denatured in 5X SDS sample buffer (Mendez and Stillman, 2000).

2.13 Immunoprecipitation

The whole heart (~100mg) was minced with scissors and then was homogenized in 2 ml of HB buffer (20mM HEPES, 5mM NaF, 10 µM Na_2MoO_4, 0.1mM EDTA, 1X proteinase inhibitors) using a Tissue Master homogenizer (OMNI International). The homogenate was spun down at 13,000 rpm at 4 ºC for 5 minutes to generate the cytosolic and nuclei fractions. The pellet was re-suspended in 1.5 ml of nuclear lysis buffer (50mM HEPES, 300mM NaCl, 10mM NaF, 1mM EDTA, 1% Triton-X, 1mM Na3VO4, 1mM PMSF, 1X proteinase inhibitors) and rocked on ice for 1 hr. 20µl of micrococcal nuclease (NEB) and micrococcal nuclease buffer (NEB, final concentration 1X) was added to nuclei extract, followed by a 37 ºC incubation for 20 minutes. The final concentration of 0.05M EDTA was added to the nuclei extracts to stop the micrococcal nuclease reaction. The nuclei extract was spun down at 13,000 rpm at 4 ºC for 1 minute to remove nuclear debris (pellet). The supernatant fraction (nuclei extract) was pre-cleared with 20 µl of Dynabeads® Protein G beads which were pre-incubated with 1µg of IgG serum, for 1 hour at 4 ºC. The Dynabeads® Protein G beads were removed from nuclei extract. The chromatin lysate was equally divided into 3 parts for immunoprecipitation with anti-POI; mock serum and total input, respectively. 10 µl of Dynabeads® Protein G beads were used to capture 1 µg of the antibody. The protein bound-beads were washed for 30 seconds with 1 ml of RIPA buffer twice (50mM; 150mM NaCl; 0.1% SDS; 0.5% Deoycholate; 1% NP-40; 1mM

EDTA); 1 ml of High Salt buffer twice (50mM Tris-HCl pH8.0; 0.1% SDS; 0.5% Deoxycholate; 1% NP-40; 1mM EDTA); 1 ml of LiCl buffer twice (50mM Tris-HCl pH 8.0; 1mM EDTA; 250mM LiCl; 1% NP-40; 0.5% Deoxycholate) and 1 ml of TE buffer twice (10mM Tris-HCl; 1mM EDTA). Immunoprecipitations were performed using various antibodies. After washing, beads were boiled in 150 µl of 2X Laemmli Buffer and the IPed proteins were analyzed by Western blot with the indicated antibodies.

The information of the antibodies and concentration used is listed in Chapters III, IV, and Appendix E.

2.14 Western blot analysis

The samples were run on a 7-15% SDS-PAGE. The gel was running at 100V in cold buffer (25mM Tris, 0.192mM Glycine, 0.1% SDS). Filter paper (Whatman) and PVDF membrane transfer paper (Millipore) was cut to the gel size. The PVDF membrane was activated by placing it in methanol for a few seconds. The gel was run at 100V for 2 hours to complete the transfer. Next, the PVDF membrane was blocked in 5% non-fat milk for at least 1 hour at RT. The PVDF membrane was then rocked in the chosen antibody at the required dilution with 2.5% non-fat milk in PBST (1x PBS, 0.1% Tween 20) overnight at 4°C. The membrane was then washed with 5 ml of PBST at room temperature for 10 minutes, and this wash was repeated 5 times. Next, the membrane was incubated in the chosen secondary antibody at the required dilution with 2.5% non-fat milk in PBST (1x PBS, 0.1% Tween 20) and rocked at RT for 1 hour. The membrane was then washed with 5 ml of PBST at room temperature for 10 minutes, and this wash was repeated 5 times. The membrane was then transferred to X-ray film for detection. Immobilon Western chemiluminescent HRP substrate (Millipore) was added to the membrane

for 5 minutes before X-ray film detection. Finally, the membrane was covered in plastic wrap and exposed 5-30 seconds before being developed.

The information and condition of the antibodies are listed in Chapters III, IV, and Appendix D.

2.15 Microarray

RNA was isolated from the adult left ventricle using Trizol (Invitrogen) and followed with DNase Treatment. RNA was cleanup using RNeasy mini kit (Qiagen). ~ 2 µg total RNA were converted into first-strand cDNA using T7-Oligo (dT) primers. The second-strand synthesis reaction was performed immediately to generate double-strand cDNA. The cDNA was cleaned up using phenol chloroform extraction. The cDNA pellet was dissolved in 12 µl of DEPC-H_2O. The cDNA were biotin-labeled using GeneChip IVT Labeling Kit. The biotin-labeled cDNA was cleaned up using phenol chloroform extraction. The biotin-labeled cDNA pellet was dissolved in 23 µl of DEPC-H_2O. 10 µl of the biotin-labeled cDNA were proceeded to fragmentation reaction (5x fragmentation buffer: 200 mM Tris-acetate, pH 8.1; 500 mM KOAc; 150 mM Mg $(OAc)_2$• $4H_2O$). The biotin-labeled cDNA fragments were hybridized to Affymetrix Mouse Genome 430 2.0 Array. The microarray results were analyzed using Affymetrix dChIP software.

III. ADDITIONAL SEX COMBS-LIKE 2 IS REQUIRED FOR THE MAINTENANCE OF ADULT CARDIAC FUNCTION

3.1 Abstract

During development and differentiation, cell type-specific chromatin configurations are set up to facilitate cell type-specific gene expression. Defects in the establishment or the maintenance of the correct chromatin configuration have been associated with diseases ranging from leukemia to muscular dystrophy. The heart expresses many chromatin factors, and we are only beginning to understand their roles in heart development and function. We have previously shown that the chromatin regulator *Asxl2* is highly expressed in the murine heart both during development and adulthood. In the absence of *Asxl2*, there is a significant reduction in trimethylation of H3K27, a histone mark associated with lineage-specific silencing of developmental genes.

Here we present evidence that ASXL2 is required for the long-term maintenance of ventricular function and for the maintenance of normal cardiac gene expression. *Asxl2*$^{-/-}$ hearts displayed progressive deterioration of ventricular function. By 10 months of age, there was ~37% reduction in fractional shortening in *Asxl2*$^{-/-}$ hearts when compared to wild-type. Analysis of the expression of myofibril proteins suggests that ASXL2 is required for the repression of *Myh7*, which encodes the fetal form of myosin heavy chain. *Asxl2*$^{-/-}$ hearts did not exhibit hypertrophy, suggesting that the de-repression of *Myh7* was not the result of hypertrophic response. Instead, ASXL2 and the histone methyltansferase EZH2 co-localize *Myh7* promoter, suggesting that ASXL2 directly represses *Myh7*. We propose that chromatin factors like ASXL2 function in the adult heart to regulate cell type- and stage-specific patterns of gene expression,

and the disruption of such regulation may be involved in development of certain forms of mammalian heart disease.

3.2 Introduction

Transcriptional regulation plays critical roles in heart development and function (Bruneau, 2002; Clark et al., 2006; Cripps and Olson, 2002; Hatcher et al., 2003; Nemer and Nemer, 2001; Olson, 2006). During embryonic development, the process of heart morphogenesis requires precise regulation of gene expression. Aberrations in the temporal or spatial pattern of gene expression lie at the root of multiple forms of congenital heart defects (Sachdeva et al., 1964). Postnatally, gene expression is fine-tuned to meet the contractile need of adult life. And the appropriate gene expression pattern has to be maintained for a life time. Mutations in a number of transcription factors or changes in the dosage of transcription factors have been shown to cause cardiac dysfunction in humans or in animal models, highlighting the importance of transcriptional regulation in the adult heart (Aries et al., 2004; Balza and Misra, 2006; Oka et al., 2006; Parlakian et al., 2005; Toko et al., 2002).

In recent years, chromatin has emerged as an important layer of transcriptional regulation. Many chromatin-associated proteins have been identified, and studies have shed light on how these chromatin factors can modify chromatin configuration to either facilitate or inhibit transcription. Accumulating evidence suggests that a substantial amount of transcriptional regulation in the heart takes place at the chromatin level (Bingham et al., 2007; Bovill et al., 2008; Chan et al., 2003; Hang et al., 2010; Koga et al., 2002; Lee et al., 2000; Shirai et al., 2002; Takihara et al., 1997; Zhang et al., 2002) . However, much remains to be learned about which chromatin factors are involved, which genes they regulate, what functional mechanisms are used and whether/how deregulation contributes to heart diseases. Polycomb Group (PcG) and

Trithorax Group (TrxG) proteins are two highly conserved protein families that regulate transcription by modifying chromatin structure (Levine et al., 2004; Orlando, 2003; Ringrose and Paro, 2007) . PcG proteins form several complexes to create repressive chromatin structure and maintain long-term silencing of target genes. For example, PRC2 generates H3K27me3, a histone mark of silent chromatin. PRC1 has chromatin compaction activity. TrxG proteins also form multiple complexes but create active chromatin structure and antagonize PcG-mediated repression. Components of the PcG/TrxG system are expressed in the heart both during development and in the adult.

Several pieces of evidence have implicated the PcG/TrxG system in transcriptional regulation in both embryonic and postnatal hearts. For example, studies of mice mutant for *Rae28* suggest that proper heart morphogenesis requires PcG protein activity. *Rae28* mutant mice display severe defects in an early and important step of cardiac morphogenesis, cardiac looping, which takes place between E8.5 and E9.5 (Takihara et al., 1997). Postnatal overexpression of *Rae28* causes dilated cardiomyopathy, cardiomyocyte apoptosis, abnormal myofibrils, and severe heart failure (Koga et al., 2002).

The TrxG protein BRG1 promotes cardiomyocyte proliferation and regulates the activity of multiple cardiac transcription factors in a dosage-dependent manner during heart development (Hang et al., 2010; Takihara et al., 1997). In adult cardiomyocytes, BRG1 is required for stress-induced hypertrophy and the pathological myosin heavy chain alpha (MYH6) to myosin heavy chain beta (MYH7) shift (Hang et al., 2010).

We have previously generated a mutant mouse model for the chromatin factor ASXL2. We showed that ASXL2 is an enhancer of PcG activity and that *Asxl2* deficiency has a significant impact on the level of bulk H3K27me3 (Baskind et al., 2009). *Asxl2* is highly

expressed in the heart throughout development and during adult life. To better understand the role of ASXL2 in the heart, we carried out a longitudinal study of *Asxl2*$^{-/-}$ mice. Our data indicate that ASXL2 is required for the long-term maintenance of ventricular function and for repression of *Myh7*. ASXL2 is likely a direct regulator of *Myh7* and this regulation may involve the PcG protein EZH2.

3.3 Materials and Methods

3.3.1 SDS-PAGE gel electrophoresis

5% of total myofibril proteins/lane was loaded on 15% SDS-PAGE (resolving gel: 15% acrylamide with 0.4% bis, 0.37M Tris pH8.8, 0.1% SDS, 0.05% APS, 0.067% TEMED; stacking gel: 4% acrylamide with 0.173% bis, 0.37M Tris pH8.8, 0.1% SDS, 0.05% APS, 0.067% TEMED). The proteins were transfered to a 0.2 µm PVDF (Bio-Rad) at 100V for 1 hour. The membrane was subjected to western blot analysis as described in 2.14. The primary antibodies which were used in this study are anti-phosphorylated TNNI3^{ser23}, which recognizes cardiac muscle Troponin I (1:1000; ~26KDa; anti-rabbit HRP 1:40,000); anti-ACTIN (1:3000; ~40KDa; anti-mouse HRP 1:50,000); anti-ASXL2 (KC17; 1:500; ~170KDa; anti-rabbit HRP 1:10,000); anti-total TNNI3 (1:5000; ~25KDa; anti-mouse HRP 1:50,000); anti-phosphorylated PLB^{ser16} (1:2000; ~21KDa; anti-mouse HRP 1:10,000); anti-GAPDH (1:2000; ~35KDa; anti-mouse HRP 1:10,000); anti-total PLB (1:5000; ~20KDa; anti-mouse HRP 1:20,000).

3.3.2 Chromatin immunoprecipitation

The experimental procedure was followed as previously described in 2.9. The antibodies which were used in this study are: anti-ASXL2 (8 µl per 300µl chromatin); anti-EZH2 (BD transduction laboratories; 8 µl per 300µl chromatin); rabbit IgG (Invitrogen; 2 µg per 300µl chromatin); mouse IgG (Invitrogen; 2 µg per 300µl chromatin). ChIPed DNAs were subjected to

PCR analysis. The PCR program used was as follows; (1) 95° 30 sec. (2) 95° 30 sec. (3) 57-60° 30 sec. (4) 72° 1 min. (5) go to step 2, (6) repeat steps 2-5 31 times (7) 72° 5 min

3.4 Results

3.4.1 *Asxl2*$^{-/-}$ mice in B6/129 F1 background are partially lethal

We have previously shown that in B6/129 mixed genetic background, only half of the expected number of *Asxl2*$^{-/-}$ pups was recovered at weaning (Baskind et al., 2009). The relative contribution of B6 versus 129 background in those animals differed from mouse to mouse. Thus, whether *Asxl2*$^{-/-}$ animals survive or not may depend on the combination of genetic modifiers that it inherited. Since then, we have backcrossed the *Asxl2*$^{-}$ allele into B6 and 129 backgrounds to create B6-*Asxl2*$^{+/-}$ and 129-*Asxl2*$^{+/-}$ congenic mice, respectively. When B6-*Asxl2*$^{+/-}$ congenic mice are mated to 129-*Asxl2*$^{+/-}$ mice, the genetic composition of the F1 animals is uniform: each mouse is 50% B6 and 50% 129. Nevertheless, B6/129 F1 *Asxl2*$^{-/-}$ animals exhibited inter-individual variation in survival: approximately half of the expected number died before weaning (Table I). This suggests that the cause of partial lethality in *Asxl2*$^{-/-}$ animals is not due to genetically unbalanced mice but results from the effect of loss *Asxl2*.

TABLE I: GENOTYPE COMPOSITION OF B6/129 F1 ANIMALS AT WEANING

Genotype	+/+	+/-	-/-
Observed	48	88	25
Expected	48	96	48
Degree of freedom (df)		1	1
Chi-square (X^2)		0.67	11.02
Chi-square test (*p*)		0.4142[1]	0.0009[2]

[1] Our study has found that the distribution of +/+ to +/- is 48 and 96 at the 0.05 significance level.
H_O: The distribution of +/+ to +/- mice is 48 and 96
H_A: The distribution of +/+ to +/- mice is not 48 and 96

[2] Our study has found that the distribution of +/+ to -/- is not 48 and 48 at the 0.05 significance level.
H_O: The distribution of +/+ to +/- mice is 48 and 48
H_A: The distribution of +/+ to +/- mice is not 48 and 48

3.4.2 ASXL2 is required for the maintenance of ventricular function

Ventricular systolic function was measured by fractional shortening (Hofsteen et al., 2013) and ejection fraction (EF) at 2 months, 4 months and 10 months of age (Fig. 1).While FS and EF of *Asxl2*$^{-/-}$ hearts were comparable to that in wild-type littermates at 2 months, both parameters deteriorated over time. We observed reduced FS in *Asxl2*$^{-/-}$ mice at 4 months, and by 10 months FS was ~38% lower in *Asxl2*$^{-/-}$ mice compared to wild-type littermates (Fig. 1C). EF followed a similar trend and was ~30% lower in *Asxl2*$^{-/-}$ mice compared to wild-type littermates by 10 months (Fig. 1D). The progressive deterioration of contractility in *Asxl2*$^{-/-}$ mice suggests that ASXL2 is required for long-term maintenance of ventricular function.

Ventricular dysfunction in older *Asxl2*$^{-/-}$ mice was confirmed by hemodynamic measurements. At 5 months, two *Asxl2*$^{-/-}$ mice exhibited decreased left ventricular pressure (61 and 81 mm Hg) and maximum dP/dt (3368 and 4751 mmgHg/s) compared to a wild-type littermate (108 mm Hg and 6399 mm Hg/s). Furthermore, contractile performance as measured by the end-systolic pressure volume relation (Rao et al., 2013) and time varying maximal elastance (Emax) were markedly lower in the two *Asxl2*$^{-/-}$ mice (Ees: 10.96 and 4.11 mm Hg/µl; and Emax:13.73 and 6.01 mm Hg/µl) compared to the wild-type littermates (Ees: 14.8 mm Hg/µl and Emax: 25.4 mm Hg/µl). These data demonstrate that by 5 months of age, *Asxl2*$^{-/-}$ mice were already exhibiting depressed ventricular function, independent of loading conditions.

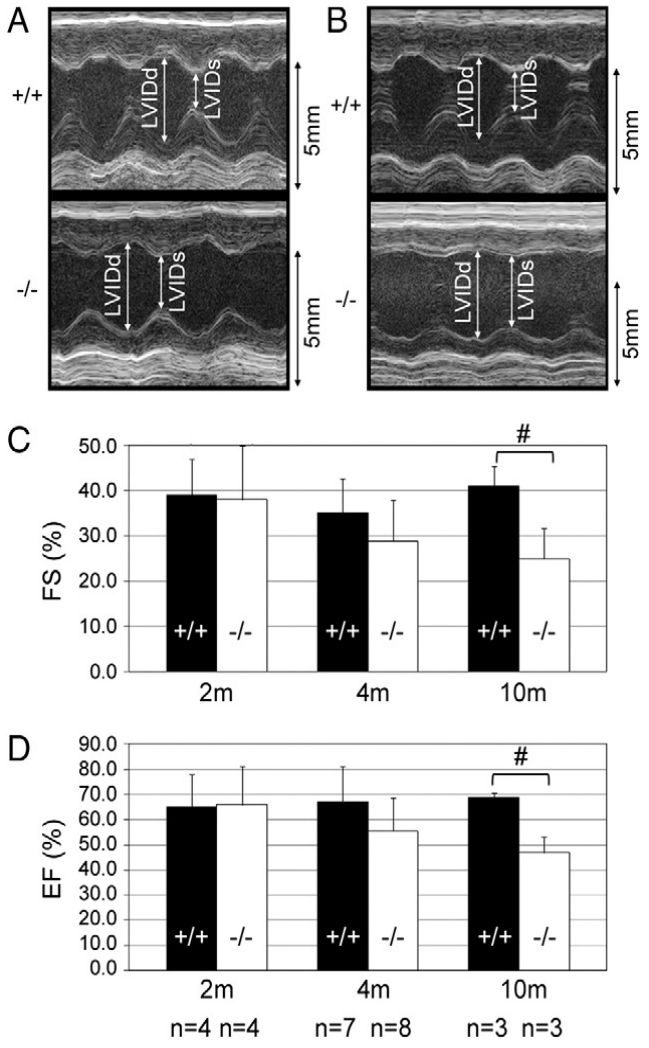

Figure 1: Evaluation of systolic function in *Asxl2*$^{-/-}$ mice and wild-type littermates.

(A, B) Representative M-mode images of wild-type and *Asxl2*$^{-/-}$ hearts at 4 months (A) and 10 months (B), respectively. Left ventricular internal dimension in diastole (LVIDd); left ventricular internal dimension in systole (LVIDs). (C, D) Fractional shortening (C) and ejection fraction (D) in wild-type and *Asxl2*$^{-/-}$ hearts at 2 months, 4 months and 10 months. The numbers (n) of animals examined for each genotype at each time point are indicated at the bottom. The error bars represent standard deviations. #$p < 0.05$.

3.4.3 *Asxl2*$^{-/-}$ mice have low arterial blood pressure at older age

Because hypertension is often associated with systolic dysfunction (McDonagh et al., 1997), we asked if the *Asxl2*$^{-/-}$ mice had hypertension. Arterial blood pressure was measured in un-anesthetized mice at six time points from 1 month to 10 months. Both systolic and diastolic arterial pressures were comparable between *Asxl2*$^{-/-}$ mice and wild-type littermates up through 6 months of age. Systolic blood pressure (SBP) in *Asxl2*$^{-/-}$ mice decreases sharply between 6 months and 8 months and was significantly lower than that of wild-type littermates at 8 months ($p=0.0083$) and 10 months ($p=0.0281$) (Fig. 2A). Diastolic blood pressure (DBP) was also lower in *Asxl2*$^{-/-}$ mice at 8 months and 10 months, and the difference with wild-type DBP reached statistical significance at 10 months ($p=0.0385$) (Fig. 2B). Thus, *Asxl2*$^{-/-}$ mice are not hypertensive, and older *Asxl2*$^{-/-}$ mice exhibited a significant reduction in arterial blood pressure, which may be a secondary effect of systolic function impairment.

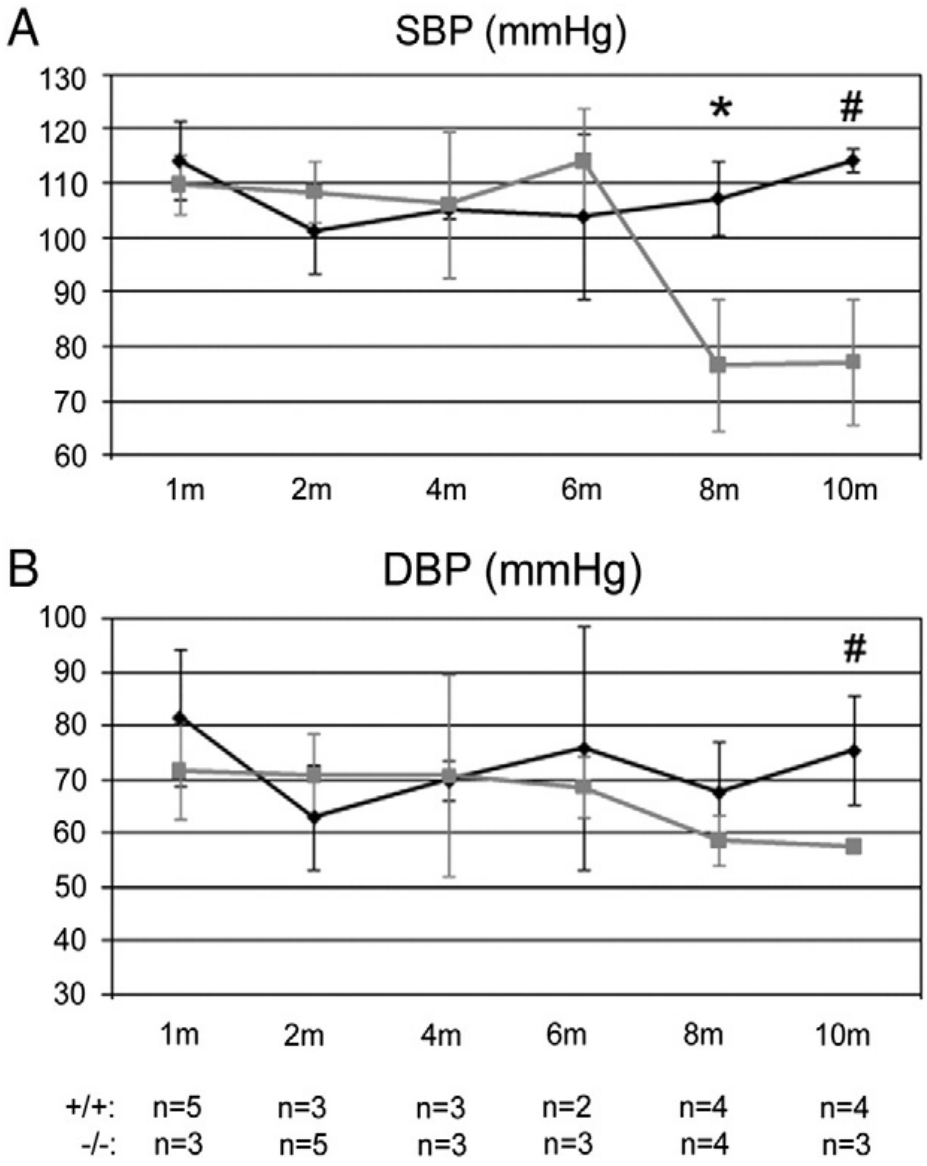

Figure 2: Blood pressure in wild-type (black lines) and *Asxl2*$^{-/-}$ mice (gray lines).

(A) Systolic blood pressure. (B) Diastolic blood pressure. The error bars represent standard deviations. * p <0.01; # p < 0.05.

3.4.4 *Asxl2*$^{-/-}$ hearts exhibit increased PKA signaling

To determine the molecular basis for ventricular dysfunction in *Asxl2*$^{-/-}$ mice, we compared the expression and phosphorylation of myofibril proteins in *Asxl2*$^{-/-}$ and wild-type hearts. We observed an increase in the level of phosphorylated troponin I, cardiac 3 (TNNI3) in 4-month-old *Asxl2*$^{-/-}$ hearts on Pro-Q Diamond stained gels (Fig. 3). TNNI3 contains multiple phosphorylation sites. Of particular importance is phosphorylation at Ser23/Ser24 by PKA, which increases the kinetics of Ca^{2+} exchange with troponin C, cardiac/slow skeletal (TNNC1) (Solaro and van der Velden, 2010). To examine the level of PKA-phosphorylated TNNI3, we performed Western blot analysis using an antibody specific to TNNI3 phosphorylated at Ser23/Ser24. The levels of both total TNNI3 and PKA-phosphorylated TNNI3 in *Asxl2*$^{-/-}$ hearts were comparable to wild-type littermates at 2 months (Figs. 4A, B). However, the level of PKA-phosphorylated TNNI3 was significantly higher in *Asxl2*$^{-/-}$ hearts at 4 months (Figs. 4C, D). Western blot analysis of phospholamban (PLB), another PKA substrate, corroborated the TNNI3 result. PLB binds to the Ca^{2+} pump SERCA and inhibits SERCA function. Phosphorylation of PLB by PKA relieves this inhibition and activates SERCA Ca^{2+} ATPase. At 2 months, we observed an increase in both total and phosphorylated PLB in *Asxl2*$^{-/-}$ hearts (Figs. 4E–G). At 4 months, the level of total PLB is decreased in *Asxl2*$^{-/-}$ heart but the level of phosphorylated PLB is increased (Figs. 4H–J).

Taken together, these results showed that PKA signaling was enhanced in *Asxl2*$^{-/-}$ hearts. This enhancement was observed at a young age (2 months), when systolic function and blood pressure were both normal in *Asxl2*$^{-/-}$ mice. Therefore, it is likely a primary phenotype associated with the loss of *Asxl2*, instead of a secondary result of sympathetic activity stimulation via baroreflex.

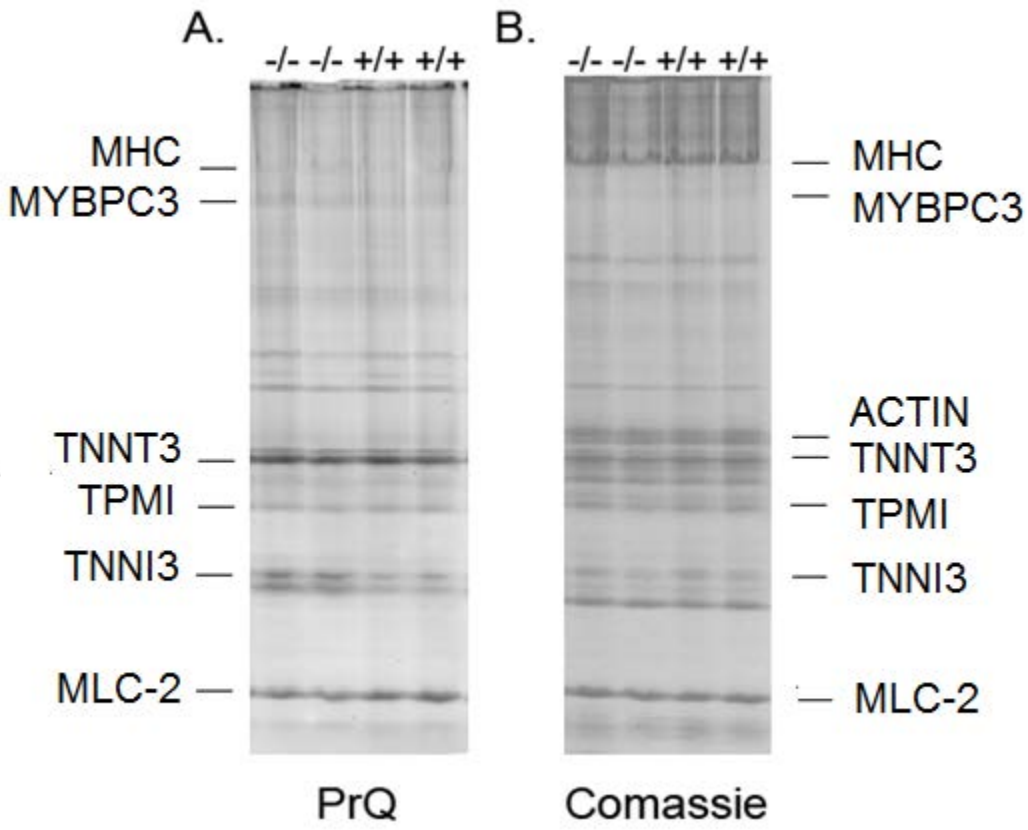

Figure 3: Expression of phosphorylated myofibril proteins in wild-type and *Asxl2*$^{-/-}$ hearts. (A) ProQ Diamond-stained gel of phosphorylated myofibril proteins at 4-month-old wild-type and *Asxl2*$^{-/-}$ hearts (B) Coomassie-stained gel of total proteins at 4-month-old wild-type and *Asxl2*$^{-/-}$ hearts. MHC: myosin heavy chain; MYBPC3: cardiac myosin protein C; TNNT3: cardiac troponin T3; TNNI3: cardiac Troponin I; MLC-2: myosin light chain 2.

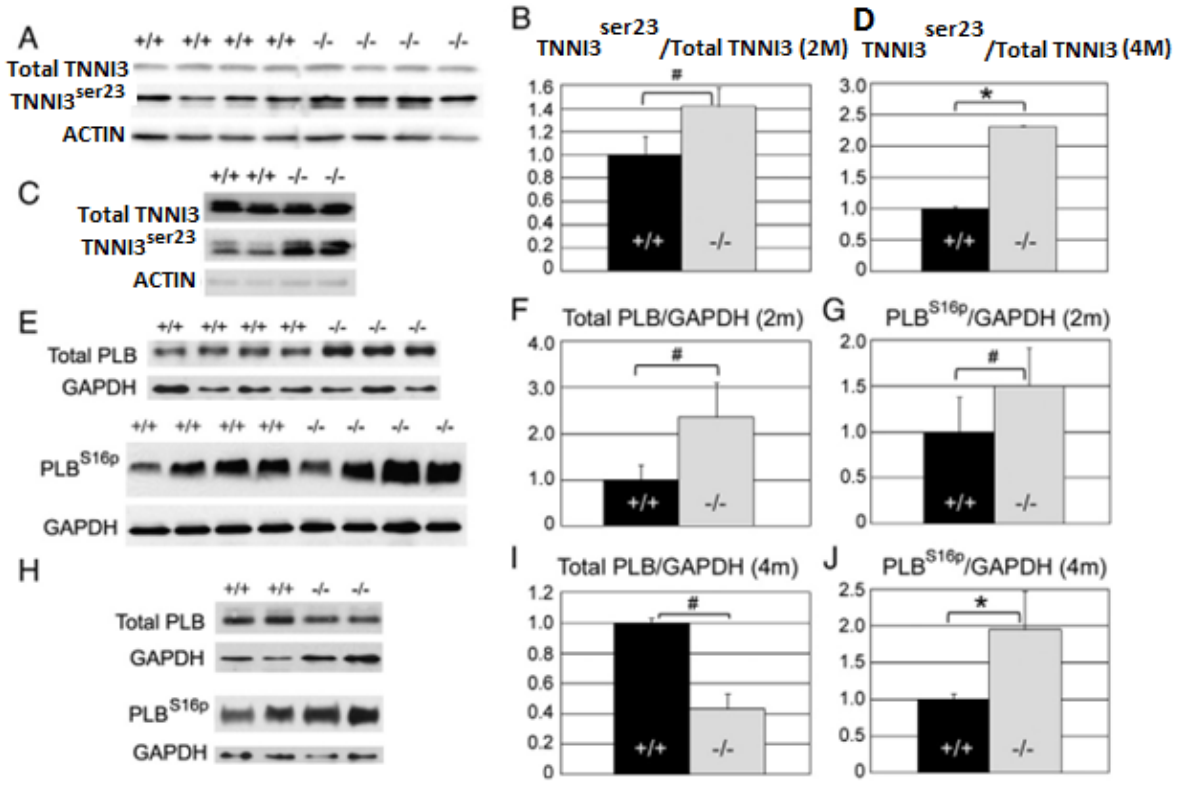

Figure 4: Expression and phosphorylation of TNNI3 and PLB in wild-type and *Asxl2*$^{-/-}$ hearts.

(A–D) Western blot analysis of total and PKA-phosphorylated TNNI3^{ser23} at 2 months (A, B) and 4 months (C, D). The ratio of PKA-phosphorylated TNNI3ser23 to total TNNI3 was quantified by densitometry and shown in C (2 m) and D (4 m). (E–J) Western blot analysis of total and phosphorylated PLB at 2 months (E–G) and 4 months (H–J). F and G show the levels of total PLB and PLB^{S16p} normalized to that of GAPDH at 2 months. I and J show the levels of total PLB and PLB^{S16p} normalized to that of GAPDH at 4 months. * $p < 0.01$; # $p < 0.05$.

3.4.5 *Asxl2*$^{-/-}$ hearts exhibit de-repression of *Myh7*

A recent study showed that BRG1, a chromatin remodeling ATPase and the mammalian homolog of the Drosophila TrxG protein BRM, is required for re-expression of *Myh7* during hypertrophic response (Hang et al., 2010). The normal adult mouse heart only expresses *Myh6*, which has a higher Ca^{2+} ATPase activity than *Myh7*. Myosin fibers composed entirely of MYH6 have higher actin filament sliding velocity than those composed of a mixture of MYH6 and 7. Transgenic studies have shown that even a slight re-expression of *Myh7* in the adult mouse heart can result in physiologically significant changes in cardiac contractility (Tardiff et al., 2000).

Because PcG and TrxG proteins often act antagonistically on the same set of target genes, we reasoned that ASXL2, which is required for PcG activity, might have a role in the repression of *Myh7* in the adult heart. We first used quantitative RT-PCR to examine the transcript level of *Myh7* in *Asxl2*$^{-/-}$ and wild-type hearts. One-month-old *Asxl2*$^{-/-}$ heart exhibited ~3 fold de-repression of *Myh7* in comparison to wild-type (Fig. 5A). The degree of de-repression increased with age, and the level of *Myh7* transcript in 8-month-old *Asxl2*$^{-/-}$ heart was ~19 fold of that in the wild-type heart (Fig. 5A). Quantitative RT-PCR analysis of *Myh6* showed that there was a ~40% decrease in *Asxl2*$^{-/-}$ heart at 1 month and ~50% increase at 8 months (Fig. 5B).

We then examined the level of MYH7 protein with high-resolution SDS-PAGE. Expression of MYH7 could be consistently detected in protein extract from 4-month-old *Asxl2*$^{-/-}$ hearts but never in the wild-type control hearts (Fig. 5D). We could not detect MYH7 on SDS-PAGE in protein extract from 2-month-old *Asxl2*$^{-/-}$ hearts, despite quantitative RT-PCR data suggesting that *Myh7* was de-repressed as early as 1 month (Fig. 5C).While this may be due to different sensitivity of silver staining and quantitative RT-PCR, both protein and transcript data suggest that *Myh7* de-repression in *Asxl2*$^{-/-}$ heart become more pronounced with age.

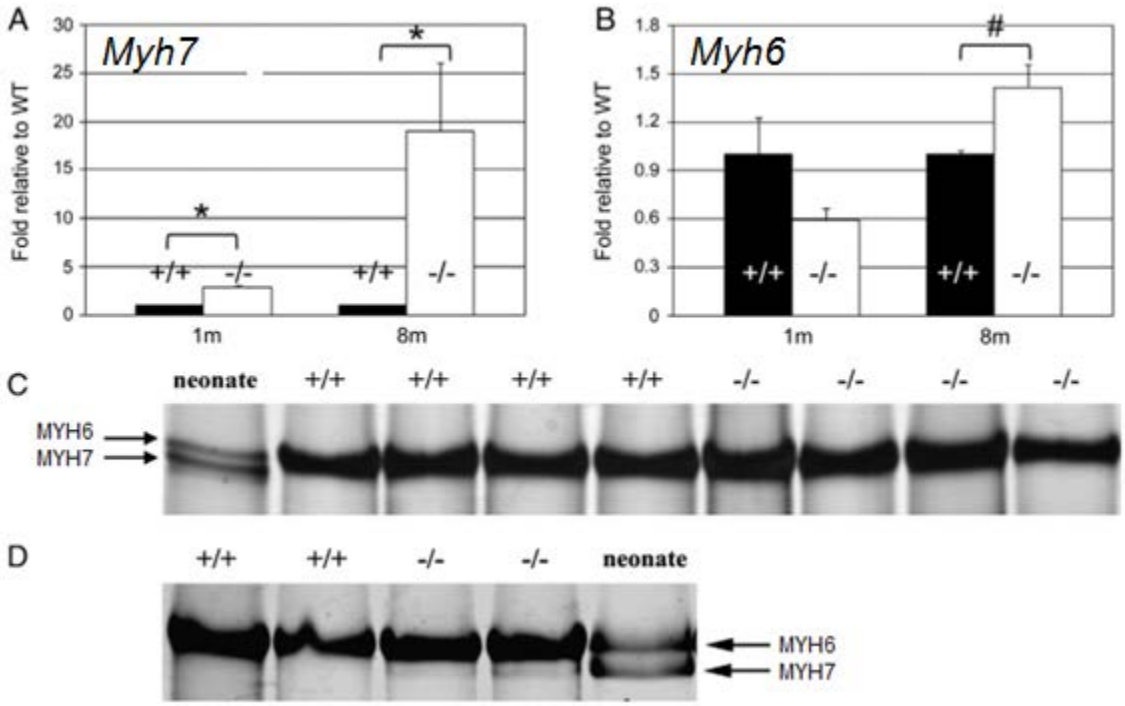

Figure 5: *Asxl2*$^{-/-}$ heart exhibited progressive de-repression of *Myh7*.

(A) Quantitative RT-PCR analysis of *Myh7* transcripts in wild-type and *Asxl2*$^{-/-}$ hearts. De-repression of *Myh7* became more pronounced with age. * $p < 0.01$. (B) Quantitative RT-PCR analysis of *Myh6* transcripts in wild-type and *Asxl2*$^{-/-}$ hearts. # $p < 0.05$. (C, D) High-resolution SDS-PAGE analysis of α- and MYH7 proteins in wild-type and *Asxl2*$^{-/-}$ hearts at 2 months (C) and 4 months (D). MYH7 protein became detectable in *Asxl2*$^{-/-}$ heart extract at 4 months.

3.4.6 ASXL2 and the PcG protein EZH2 co-localize to *Myh7* promoter

We have previously shown that ASXL2 functions as an enhancer of PcG protein activity (Baskind et al., 2009). *Asxl2*$^{-/-}$ hearts exhibited a significant reduction in the bulk level of H3K27me3, which is the product of PcG histone methyltransferase activity and a mark of silenced chromatin. To better understand the functional mechanism of ASXL2, we generated a polyclonal antibody against ASXL2, KC17. We made FLAG-tagged ASXL2 using a rabbit reticulocyte *in vitro* transcription/translation kit and performed Western blot analysis using either KC17 or the M2 monoclonal antibody against FLAG (Fig. 6A). KC17 and M2 recognized the same band in the FLAG-ASXL2 translation mixture, but not in the control luciferase translation mixture, confirming that KC17 recognizes ASXL2. Next, we performed chromatin immunoprecipitation (Chiplunkar et al.) assay with KC17 to examine the association between ASXL2 and the *Myh7* promoter. It has been previously reported that there are 5 conserved regions, designated b1-b5, within 5 kb upstream of the *Myh7* transcription start site (Koga et al., 2002). The TrxG protein BRG1 is associated with 4 of these 5 regions.

We found that ASXL2 was enriched at all 5 regions (Fig. 6B). Furthermore, the PcG protein EZH2, which is the histone methyltransferase responsible for PcG's histone methylation activity, co-localized with ASXL2 at 4 out of 5 of these regions. These results suggest that ASXL2 plays a direct role in the repression of *Myh7*, and this repression may involve EZH2 histone methyltransferase activity.

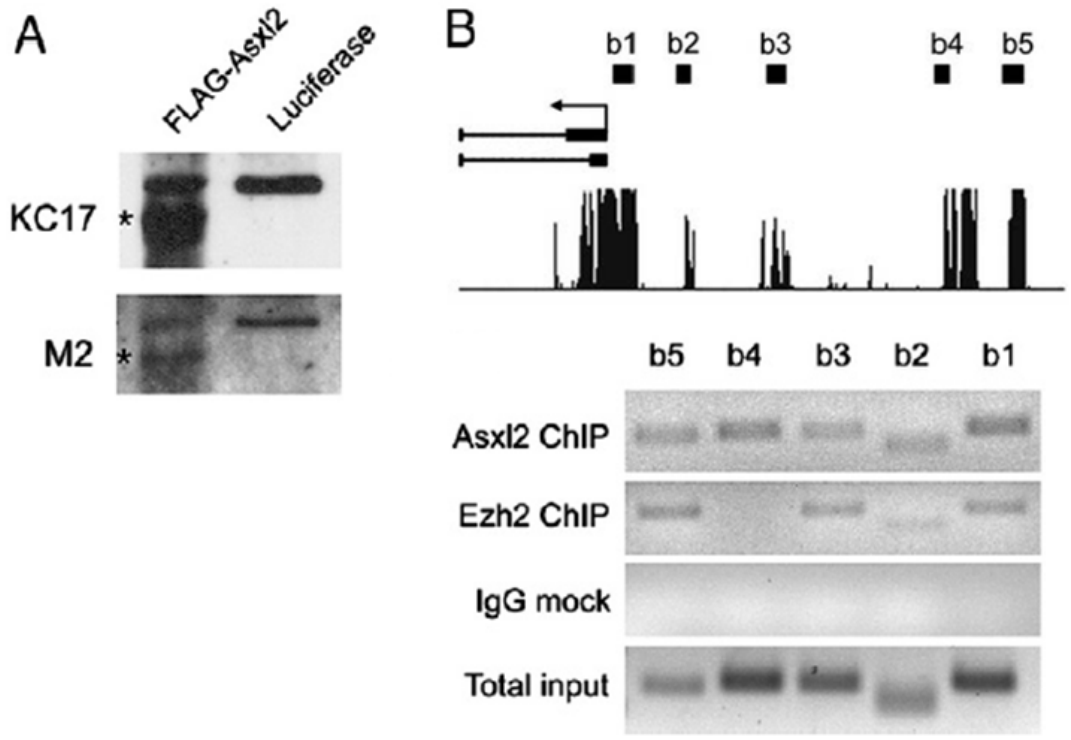

Figure 6: ASXL2 binds to *Myh7* promoter and co-localizes with EZH2 histone methyltransferase.

(A) The polyclonal antibody KC17 recognizes ASXL2 on Western blot. FLAG-tagged ASXL2 or luciferase was expressed using rabbit reticulocyte *in vitro* transcription/ translation system and probed with KC17 anti-ASXL2 antibody (top panel) or M2 anti-FLAG antibody (bottom panel). Both antibodies detected a band corresponding to the molecular weight of ASXL2 (asterisk) in the FLAG-ASXL2 translation mixture but not in the luciferase translation mixture. A non-specific band of higher molecular weight was detected in both mixtures and by both antibodies. (B) Alignment of mouse, rat and human genomic sequences, showing regions of sequence conservation in the *Myh7* promoter (top panel). Chromatin immunoprecipitation assays of ASXL2 and EZH2 enrichment at 5 conserved regions in the *Myh7* promoter. EZH2 and ASXL2 co-localize at 4 of the 5 regions analyzed (bottom panel).

3.4.7 *Asxl2*$^{-/-}$ hearts are not hypertrophic

De-repression of *Myh7* is often observed during hypertrophy, when the heart responds to stress by increasing the size of cardiomyocytes. De-repression of *Myh7* in *Asxl2*$^{-/-}$ heart could be the result of hypertrophic response — thus a secondary effect to ventricular dysfunction. Alternatively, it could be a primary effect of the loss of *Asxl2* and (at least part of) the cause of ventricular dysfunction. Indeed, transgenic expression of *Myh7* can cause ventricular dysfunction without inducing hypertrophy [32]. To distinguish the two possibilities, we examined the histology of *Asxl2*$^{-/-}$ hearts for signs of hypertrophy. *Asxl2*$^{-/-}$ hearts were not hypertrophic at any stage examined (Figs. 7A, B). WGA staining of wild-type and *Asxl2*$^{-/-}$ heart sections showed that cardiomyocyte diameter was comparable (Figs. 7C, D).

To further evaluate the size of cardiomyocytes, cardiomyocytes were isolated from 6-month-old wild-type and *Asxl2*$^{-/-}$ hearts. Consistent with the WGA staining result, wild-type and *Asxl2*$^{-/-}$ cardiomyocytes exhibited similar width (Fig. 7E). However, in comparison to wild-type, the average length of *Asxl2*$^{-/-}$ cardiomyocytes was 13% shorter, and the average area was 17% smaller (Figs. 7F, G). We conclude that *Asxl2*$^{-/-}$ hearts did not develop cellular hypertrophy. The gene *Nppa*, which encodes the atrial natriuretic factor (ANP), is normally highly expressed in the atria but not in the ventricles. Re-expression of *Nppa* in the ventricles is observed during hypertrophic response and considered a molecular hallmark of hypertrophy. We examined the expression of *Nppa* in *Asxl2*$^{-/-}$ LV by quantitative RT-PCR. Instead of observing *Nppa* de-repression, we observed ~67% down-regulation in *Asxl2*$^{-/-}$ LV at 1 month and ~52% down-regulation at 8 months (Fig. 7E). Therefore, *Asxl2*$^{-/-}$ heart did not develop hypertrophic response at the molecular level.

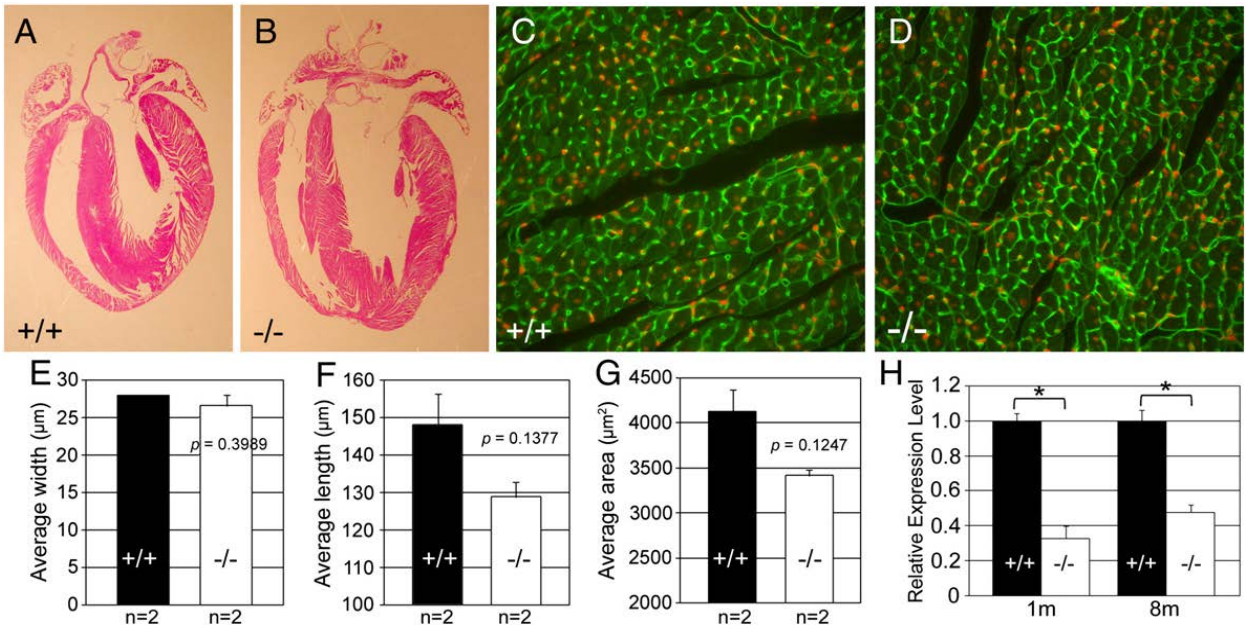

Figure 7: *Asxl2*$^{-/-}$ hearts did not develop hypertrophy.

(A, B) Wild-type and *Asxl2*$^{-/-}$ heart sections were stained with hematoxylin and eosin. (C, D) WGA stainings (green) of heart sections. Nuclei were stained with DAPI and pseudocolored in red. Both fluorescent images are shown at the same magnification. (E-G) Measurement of the average width (E), length (F) and area (G) of cardiomyocytes isolated from wild-type and *Asxl2*$^{-/-}$ hearts. (H) Quantitative RT-PCR analysis of *Nppa* transcription at 1 month and 8 months. * $p < 0.01$.

3.4.8 ASXL2 is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy

The CardioGenomics project has analyzed global gene expression pattern in the hearts of healthy individuals and patients with ischemic or idiopathic dilated cardiomyopathy by microarray. Expression in the LV was compared between 11 organ donors whose hearts could not be used in transplant, 15 patients with heart failure arising from idiopathic dilated cardiomyopathy and 11 patients with heart failure arising from ischemic cardiomyopathy.

We examined the expression of *Asxl* genes in the publically available microarray dataset (http://cardiogenomics.med.harvard.edu/public-data). There are three probe sets designated as *Asxl2* on the HgU133 Plus 2.0 array but only two of them, 218658_at and 1555266_a_at, map to the *Asxl2* locus. The third probe set, 226251_at, maps to an intergenic region downstream of *Asxl2* 3′UTR. Although 218659_at consistently showed higher signal intensity than 1555266_a_at, the two probe sets displayed the same trend showing that *Asxl2* expression is significantly down-regulated in both idiopathic and ischemic cardiomyopathy patients (Fig. 8). This suggests that the reduction of *Asxl2* expression may play a role in the etiology of some types of human cardiomyopathy. We were not able to determine with certainty whether the expression of *Asxl1* changes in human cardiomyopathy based on the microarray data because different probe sets (all of which map correctly to the *Asxl1* locus) gave different results (data not shown). *Asxl3* expression was not detected above noise level in the microarray dataset (data not shown).

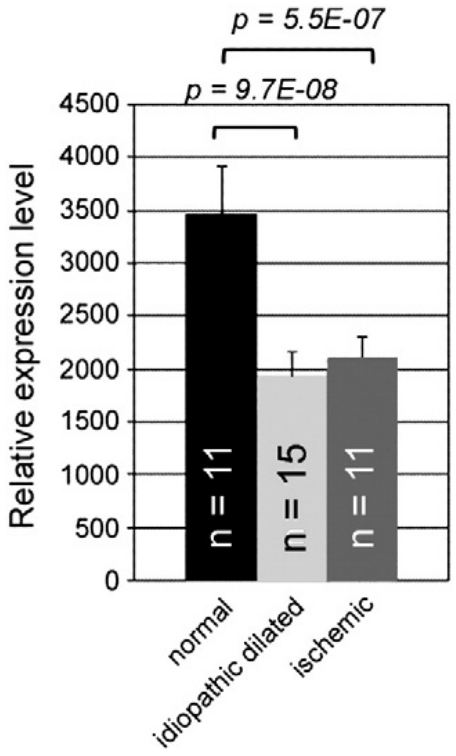

Figure 8: *Asxl2* expression in human cardiomyopathy patients.

Graphs were made using microarray data generated by the CardioGenomics project. Expression levels of *Asxl2* in the hearts of normal people (organ donors whose heart were healthy but could not be used in transplant), patients with idiopathic dilated cardiomyopathy, and patients with ischemic cardiomyopathy. Data shown in the graph represent probe set 218658_at, which gave the higher signal intensity. Probe set 1555266_a_at had lower signal intensity but similar trend.

3.5 Discussion

3.5.1 The molecular basis for ventricular dysfunction in *Asxl2*$^{-/-}$ heart

Contractility of *Asxl2*$^{-/-}$ hearts was comparable to that in wild-type at young age but deteriorated progressively over time. This suggests that ASXL2 has a specific role in the long-term maintenance of ventricular function. In parallel with the progressive decrease in contractility, we observed progressive de-repression of *Myh7* in *Asxl2*$^{-/-}$ hearts. The de-repression of *Myh7* was not a secondary result of stress induced hypertrophy, since *Asxl2*$^{-/-}$ hearts did not develop hypertrophy. Given that the TrxG protein BRG1 has been shown to be directly required for *Myh7* activation (Hang et al., 2010) , we reasoned that ASXL2, a PcG protein, may be directly required for *Myh7* repression.

ChIP assays showed that ASXL2 is enriched at the *Myh7* promoter, supporting a direct role for ASXL2 in *Myh7* repression. Thus, *Myh7* de-repression was likely a direct consequence of *Asxl2* deficiency. Interestingly, BRG1 functions not only as a *Myh7* activator but also as a *Myh6* repressor (Hang et al., 2010). The role of ASXL2 in *Myh6* expression remains to be addressed, but it is formally possible that ASXL2 normally stimulates *Myh6* expression, in which case the loss of *Asxl2* could lead to both direct and compensatory de-repression of *Myh6*. It has been previously shown that even a slight change in myosin composition – 12% of total myosin being MYH7 – can result in significant reduction in contractility (Tardiff et al., 2000). The protein level of MYH7 in *Asxl2*$^{-/-}$ hearts is ~6% of total myosin at 4 months. While this is a small change in myosin composition, it could have a detectable impact on contractility. The proportion of MYH7 in total myosin is likely much higher by 10 months, given that *Myh7* de-repression becomes more severe over age (Fig. 5A). Thus, we hypothesize that ventricular dysfunction in *Asxl2*$^{-/-}$ hearts could, at least partly, result from a failure to maintain *Myh7* in a silenced state.

Interestingly, *Asxl2*$^{-/-}$ hearts exhibited increased PKA phosphorylation of TNNI3 and PLB, both of which should increase the kinetics of Ca^{2+} transient and enhance contraction. Thus, contractility in *Asxl2*$^{-/-}$ hearts reflects the net result of a tug-of-war between abnormal myosin composition and enhanced PKA signaling. Additional, as-yet unidentified factors may also contribute to this tug-of-war. A proteomic approach would be well suited for the identification of such factors in the future.

3.5.2 The role of ASXL2 in cardiomyocyte hypertrophy

Systolic dysfunction usually induces cardiomyocyte hypertrophy as a compensatory mechanism. However, there is a disconnection between systolic function and hypertrophy in *Asxl2*$^{-/-}$ hearts. This is uncommon but not unprecedented. For example, *Myh7* transgenic mice have been reported to exhibit systolic dysfunction without developing hypertrophy (Tardiff et al., 2000). Furthermore, mutations in several genes – including the transcription factor Gata4 and the chromatin factor BRG1 – have been shown to significantly attenuate hypertrophic response induced by transverse aortic constriction (TAC), a powerful form of pressure overload that normally causes rapid development of hypertrophy (Hang et al., 2010; Oka et al., 2006). These reports suggest that while systolic function and cardiomyocyte growth are usually intricately connected, it is possible to uncouple them. An important task in future studies would be to characterize molecular pathways downstream of ASXL2, in particular pathways that regulate cellular growth. The ability to manipulate such pathways could facilitate the prevention of hypertrophy and its deleterious effects.

3.5.3 The role of chromatin factors in the long-term maintenance of cardiac gene expression and function

The adult mammalian heart has very limited regeneration capability. Cardiomyocytes need to maintain a cell type-appropriate gene expression pattern for a life time while constantly adjusting performance and metabolism in response to changes in physiological conditions, such as exercise, hormones, and pregnancy. Previous research has identified an array of transcription factors as important regulators of gene expression in the adult heart. Deficiency or mutation in these transcription factors leads to malfunction of cardiomyocytes and of the whole heart. For example, MEF2a, a typical transcription factor with both DNA binding and transactivating activities, is required for the maintenance of appropriate mitochondrial content and cyto-architectural integrity in the adult mouse heart (Naya et al., 2002).

In the past two decades, studies have revealed the importance of transcriptional regulation at the level of chromatin. Many chromatin factors have been reported. Among these, PcG proteins are particularly recognized for their role in creating and maintaining gene silencing. TrxG proteins, the antagonists of PcG proteins, can relieve PcG-mediated silencing. It has recently been shown that the TrxG protein BRG1 is required for MHC isoform switching during hypertrophic response in the adult mouse heart (Hang et al., 2010). BRG1 binds to 4 of the 5 conserved regions in the *Myh7* promoter and can activate *Myh7* reporters in an HDAC independent manner. If a TrxG protein is required for *Myh7* activation, do PcG proteins play a role in *Myh7* repression?

Our results suggest the answer is yes. ASXL2 is a chromatin factor and a regulator of histone methylation (Baskind et al., 2009) and histone ubiquitylation [H. Lai, submitted results]. *Asxl2*$^{-/-}$ hearts had decreased levels of bulk H3K27me3, a mark for silenced chromatin,

suggesting that ASXL2 normally promotes PcG-mediated gene repression. Here we report that *Myh7* is progressively de-repressed in *Asxl2*$^{-/-}$ hearts; furthermore, ASXL2 and the PcG histone methyltransferase EZH2 co-localize to 4 out of 5 conserved regions in the *Myh7* promoter. Thus, the expression of *Myh7* is likely controlled by opposing actions of PcG and TrxG proteins. In this model, PcG proteins, with the help of ASXL2, functions keep the *Myh7* locus in a silenced chromatin configuration. In the absence of *Asxl2*, PcG activity is reduced, resulting in progressive deterioration of the silenced chromatin configuration and thereby progressive *Myh7* de-repression. It is conceivable that there are more genes that require ASXL2/PcG proteins for repression in the adult heart. The identification and study of these genes will increase our understanding of the role of the chromatin based mechanisms in the long-term maintenance of cardiac gene expression and function.

3.5.4 Implication of ASXL2 in human heart disease

Heart diseases have been associated with mutations in a variety of genes that encode transcription factors, cytoskeleton components and signaling molecules. However, the molecular basis for a large number of heart diseases remains elusive. While genetic factors are certainly critical, epigenetic factors may be just as important. Epidemiological studies have led to the theory that many adult disorders, including cardiac disorders, may originate from fetal or early postnatal programming through epigenetic mechanisms (Wadhwa et al., 2009). Epigenetic programming can be influenced by maternal and infantile diet, by environmental factors, by mutations in genes that encode epigenetic regulators, or by the combinatorial effect of the above. We showed that *Asxl2* is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy. Down-regulation of *Asxl2* may be a mere consequence of these heart conditions and may not play any active role; in this case, it may serve as a molecular marker in

diagnosis. Alternatively, it may actively contribute to the onset or development of these conditions. This would raise the question of whether restoring ASXL2 function, or boosting the activity of histone modification enzymes that are regulated by ASXL2, can prevent or hinder disease progression.

IV. ADDITIONAL SEX COMBS-LIKE 2 IS REQUIRED FOR POLYCOMB REPRESSIVE COMPLEX 2 BINDING AT SELECT TARGETS

4.1 Abstract

Polycomb Group (PcG) proteins are epigenetic repressors of gene expression. The Drosophila *Additional sex combs (Asx)* gene and its mammalian homologs exhibit PcG protein function in genetic assays; however, the mechanism by which Asx family proteins mediate gene repression is not well understood. ASXL2, one of three mammalian homologs for Asx, is highly expressed in the mammalian heart and is required for the maintenance of cardiac function. We have previously shown that *Asxl2* deficiency results in a reduction in the bulk level of histone H3 lysine 27 trimethylation (H3K27me3), a repressive mark generated by the Polycomb Repressive Complex 2 (PRC2).

Here we identify several ASXL2 target genes in the heart and investigate the mechanism by which ASXL2 facilitates their repression. We show that the *Asxl2*-deficient heart is defective in converting H3K27me2 to H3K27me3 and in removing ubiquitin from mono-ubiquitinated histone H2A (uH2A). ASXL2 and PRC2 interact in the adult heart and co-localize to target promoters. Additionally, ASXL2 is required for the binding of PRC2 and for the enrichment of H3K27me3 at target promoters. These results add a new perspective to our understanding of the mechanisms that regulate PcG protein activity and gene repression.

4.2 Introduction

During the development and life of multicellular organisms, there is a need to both set up and maintain distinct identities in different types of cells and tissues. Epigenetic mechanisms play critical roles in the establishment and maintenance of cellular identity. Polycomb Group (PcG) proteins were originally identified in Drosophila as repressors of homeotic genes (*Hox* genes) [for a review of the early genetic studies that identified PcG proteins, see (Kennison, 1995)]. The balanced action of PcG proteins and their antagonists, the Trithorax Group (TrxG) protein epigenetic activators, is crucial for the maintenance of *Hox* expression domains along the anterior-posterior axis (Gould, 1997; Kennison, 1995). It has been discovered that PcG and TrxG proteins play essential roles in mammalian development, regulating the differentiation of a wide array of cell lineages (Ng and Gurdon, 2008; Schuettengruber and Cavalli, 2009; Surface et al., 2010).

PcG proteins form multi-subunit complexes and function at the level of chromatin. One of the best characterized PcG complexes is the Polycomb Repressive Complex 2 (PRC2). PRC2 is responsible for generation of histone H3 lysine 27 trimethylation (H3K27me3), a mark that is associated with a silent chromatin state (Cao et al., 2002; Kuzmichev et al., 2002). The core components of PRC2 are EZH2, SUZ12 and EED, and are necessary and sufficient for PRC2's histone methyltransferase (HMTase) activity (Cao et al., 2002; Cao and Zhang, 2004; Kuzmichev et al., 2002; Montgomery et al., 2005). The SET-domain protein EZH2 is the catalytic subunit (Cao et al., 2002; Kuzmichev et al., 2002). SUZ12 is required for the integrity of the PRC2 complex and for preventing proteolytic degradation of EZH2 (Cao and Zhang, 2004; Pasini et al., 2004). EED binds to H3 tails carrying trimethylated K27 and stimulates the

HMTase activity of EZH2, thereby facilitating the spread of the H3K27me3 mark to neighboring nucleosomes (Margueron et al., 2009).

The Drosophila *Additional sex combs (Asx)* gene was initially identified based on PcG-like mutant phenotypes and genetic interaction with other PcG genes (Simon et al., 1992). Recently, Asx was shown to associate with the histone deubiquitinase Calypso to form the Polycomb Repressive Deubiquitinase (PR-DUB) complex (Scheuermann et al., 2010). Asx plays at least two roles in the PR-DUB complex: to stabilize the Calypso protein and to stimulate its deubiquitinase activity, which is specific for mono-ubiquitinated histone H2A (uH2A). The deubiquitinase activity of PR-DUB is required for repression of *Ubx* in Drosophila wing disc. These results provided important insight into the biological function of Asx and PR-DUB. However, it remains unclear how H2A deubiquitination contributes to the repression of PcG protein target genes.

There are three Asx homologs in human and mouse genomes, *Asx-like 1* (*Asxl1*), *Asxl2* and *Asxl3* (Fisher et al., 2010a; Katoh, 2003, 2004). We have previously presented evidence suggesting functional conservation between *Asxl2* and *Asx* (Baskind et al., 2009). *Asxl2* is highly expressed in the heart. Interestingly, *Asxl2*$^{-/-}$ hearts exhibit significant reduction in the level of bulk H3K27me3, suggesting that ASXL2 regulates PRC2 activity (Baskind et al., 2009). Here we explore the molecular basis for this reduction and present evidence that ASXL2 is required for PRC2 binding at select target genes.

4.3 Materials and Methods

4.3.1 Chromatin immunoprecipitation

The experimental procedure was followed as previously described in 2.9. The antibodies used in this study were: rabbit anti- ASXL2 (8 µl per 300µl chromatin); mouse anti-EZH2 (BD

transduction laboratories; 8 µl per 300µl chromatin); rabbit IgG (Invitrogen; 2 µg per 300µl chromatin); mouse IgG (Invitrogen; 2 µg per 300µl chromatin); rabbit anti-H3K27me3 (cell signaling, 6 µl per 300µl chromatin); rabbit anti-SUZ12 (8µl per 300 µl chromatin, active motif); and rabbit anti-AcH3 (7 µl per 300 µl chromatin, Active motif). ChIPed DNAs were subjected to PCR analysis. The PCR program used for EZH2 and SUZ12 ChIP assay was as follows; (1) 95° 30 sec. (2) 95° 30 sec. (3) 57-60° 30 sec. (4) 72° 1 min. (5) repeat steps 2-4 for 31 times, (6) 72° 5 min. The qPCR program used for ASXL2, EZH2, H3K27me3, and AcH3 was as follows; (1) 95° 30 sec. (2) 95° 30 sec. (3) 57-60° 30 sec. (4) 72° 1 min. (5) repeat step 2-4 for 39 times (7) melting curve analysis

4.3.2 Immunoprecipitation

The experimental procedure was followed as previously described in 2.13. Antibodies used in this study were as follows: anti-pre-absorbed ASXL2* (16 µl per 600µl nuclei extract); anti-EZH2 (Millipore; 12 µl per 600 µl nuclei extract); rabbit IgG (Invitrogen; 2 µg 600µl nuclei extract); mouse IgG (Invitrogen; 2 µg per 600µl nuclei extract); and rabbit anti-SUZ12 (Active motif, 8µl per 600µl nuclei extract).

*100 µl Anti-ASXL2 was diluted in 100 µl of PBS and then was pre-absorbed in methanol fixed HEK293 cells for 1 hour at 4 ºC (pre-ASXL).

4.3.3 Western blot

The experimental procedure was followed as previously described in 2.14. The antibodies used in this study were: anti- ASXL2 (1:500; ~170KDa; anti rabbit HRP 1:10,000); anti- H3 (Active motif; 1:5000; ~15KDa; anti rabbit HRP 1:50,000); anti-GAPDH (Millipore; 1:1000; ~40KDa; anti mouse HRP 1:10,000); anti-EED (Millipore; 1:3000; ~36-72KDa; anti rabbit HRP 1:10,000); anti-SUZ12 (Active motif; 1:2000; ~85KDa; anti rabbit HRP 1:10,000); anti-EZH2

(BD transduction laboratories, 1:2000; ~90KDa; anti mouse HRP 1:10,000); anti-BAP1 (Millipore, 1:2000; ~100 KDa; anti mouse HRP 1:10,000); anti-ubiquityl-H2B (1:2000; ~25KDa; anti mouse HRP 1:10,000); and anti-ubiquityl-H2A (Millipore, 1:2000; ~27KDa; anti mouse IgM HRP 1:10,000).

4.4 Results

4.4.1 ASXL2 is associated with chromatin

Drosophila Asx is a chromatin-associated protein. Immunostaining of polytene chromosomes identified 90 Asx binding sites, ~70% of which overlapped with binding sites of other PcG proteins (Scheuermann et al., 2010). A recent ChIP-on-chip study identified 879 PR-DUB binding sites with high confidence in the Drosophila genome (Scheuermann et al., 2010). To confirm that murine ASXL2 is also associated with chromatin, we expressed FLAG-tagged ASXL2 in HEK293 cells and used biochemical fractionation (Mendez and Stillman, 2000) to separate chromatin-associated proteins from soluble nuclear proteins. Probing the fractions with either the anti-ASXL2 antibody KC17 or with anti-FLAG antibody M2 (Sigma) detected ASXL2 predominantly in the chromatin fraction (Fig. 9A). Similar results were obtained with endogenous ASXL2 in murine heart tissue (Fig. 9B).

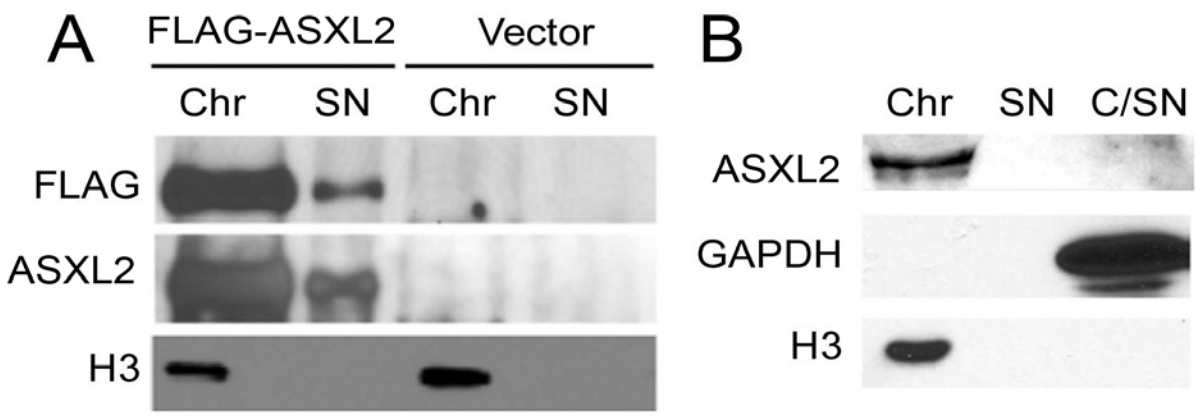

Figure 9: ASXL2 is associated with chromatin.

Biochemical fractions of cellular proteins were probed with antibodies indicated. (A) FLAG-ASXL2 is associated with chromatin in HEK293 cells. Fractions were prepared from HEK293 cells transfected with FLAG-ASXL2 and probed with M2 anti-FLAG antibody and KC17 anti-ASXL2 antibody, respectively. An anti-histone H3 antibody (Active Motif) was used to confirm the quality of fractionation. (B) Endogenous ASXL2 is associated with chromatin. Fractions were prepared from heart tissue and probed with KC17 antibody. Anti-GAPDH (Millipore) and anti-histone H3 antibodies were used to confirm the quality of fractionation. Chr: chromatin fraction. SN: soluble nuclear fraction. C/SN: cytosol fraction with trace soluble nuclear proteins.

4.4.2 ASXL2 is required for the normal expression of multiple cardiac genes

We have recently shown that ASXL2 is required for the long-term maintenance of cardiac function in adult mice (Lai et al., 2012). The loss of cardiac function in *Asxl2*$^{-/-}$ hearts is correlated with de-repression of *myosin, heavy chain 7, cardiac muscle, beta* (*Myh7*), and the fetal form of myosin heavy chain (MHC) that has lower ATPase activity than the adult alpha form (Lai et al., 2012). We showed that ASXL2 and the PRC2 core component EZH2 co-localized to multiple conserved regions within the *Myh7* promoter. This, along with our previous observation that the level of bulk H3K27me3 is significantly reduced in *Asxl2*$^{-/-}$ hearts, led us to hypothesize that ASXL2 and PRC2 may act together to regulate the expression of *Myh7* and other target genes.

To investigate this hypothesis, we first sought to identify additional targets of ASXL2 in the murine heart. We performed a microarray analysis on 1-month-old wild-type and *Asxl2*$^{-/-}$ hearts and identified 753 genes that are either induced or repressed more than 2 fold in *Asxl2*$^{-/-}$ hearts (Appendices L and M). The mis-expression of these genes is unlikely a secondary effect due to cardiac stress, because ventricular function is largely normal in *Asxl2*$^{-/-}$ hearts at this early stage (Lai et al., 2012). We chose to examine three genes, in addition to *Myh7*, in more detail: *Secreted frizzled-related protein 2* (*Sfrp2*); *Actin, alpha 1, skeletal muscle* (*Acta1*); and *G protein-coupled receptor kinase 5* (*Grk5*). First, query of the Broad Institute ChIP-seq database revealed that the promoters of these genes are enriched for PRC2 components and H3K27me3 in embryonic stem (ES) cells (Fig. 10). This suggests that these loci contain regulatory elements needed to recruit PcG protein activity. Therefore, they are good candidates as PcG protein target genes in not only ES cells but also in differentiated cells/tissues, including the heart. In fact, *Sfrp2* has been shown to be a PcG target in human embryonic fibroblasts (Bracken et al., 2006).

Second, all three genes have been implicated in congenital or acquired heart diseases/conditions in human and/or mouse (Chen et al., 2001; Feng and Marston, 2009; Huang et al., 2011; Kobayashi et al., 2009), suggesting that an understanding of their regulation could be clinically important. Using quantitative RT-PCR, we confirmed that *Sfrp2*, *Acta1* and *Grk5* are de-repressed in *Asxl2*$^{-/-}$ hearts by 4.6, 5.8, and 5.9 folds, respectively (Fig. 11).

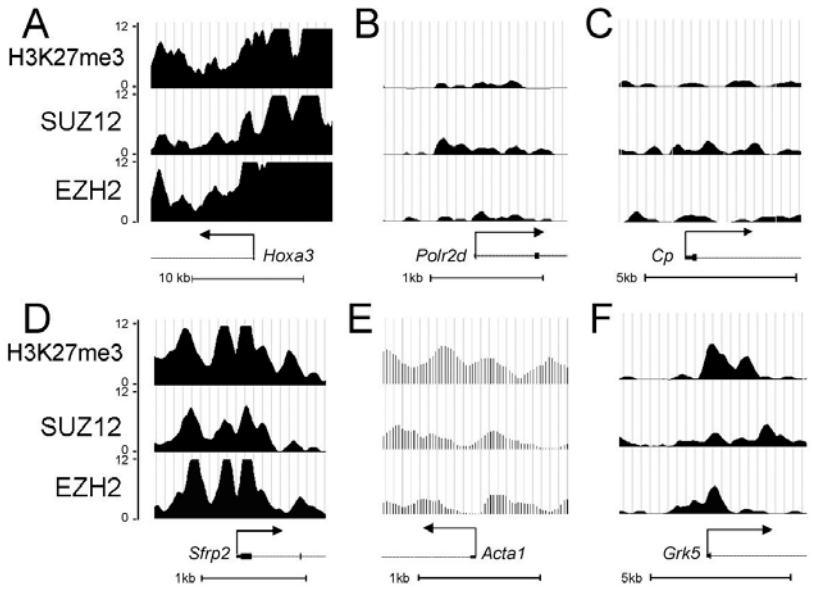

Figure 10: Epigenetic profiles at *Sfrp2*, *Acta1* and *Grk5* loci in ES cells.

The Broad Institute ChIP-seq database (http://www.broadinstitute.org/scientific-community/science/programs/epigenomics/chip-seqdata) was queried for the enrichment of H3K27me3, SUZ12, and EZH2 at the loci of interest. For each gene, only the genomic region around the transcriptional start site (TSS) is shown. Arrow points to the direction of transcription. The y axis is the relative level of enrichment. The scale bar for each panel is shown at the bottom of the panel. (A-C) Representative epigenetic profiles for three types of genes in ES cells: those that are repressed by PcG protein activity, those that are constitutively expressed and not regulated by PcG protein activity, and those that are repressed via PcG-independent mechanism. (A) The chromatin region near the TSS of *Hoxa3*, a classical PcG protein target gene, displays high levels of enrichment of H3K27me3, SUZ12 and EZH2. (B) The profile for *Polr2d*, a housekeeping gene that encodes an RNA polymerase II subunit, shows no enrichment of H3K27me3, SUZ12 or EZH2. (C) H3K27me3 and PRC2 components are not enriched near the TSS of *Cp*, a gene that is repressed in ES cells. (D-F) The epigenetic profiles around the TSS of *Sfrp2*, *Acta1* and *Grk5* resemble that for *Hoxa3*.

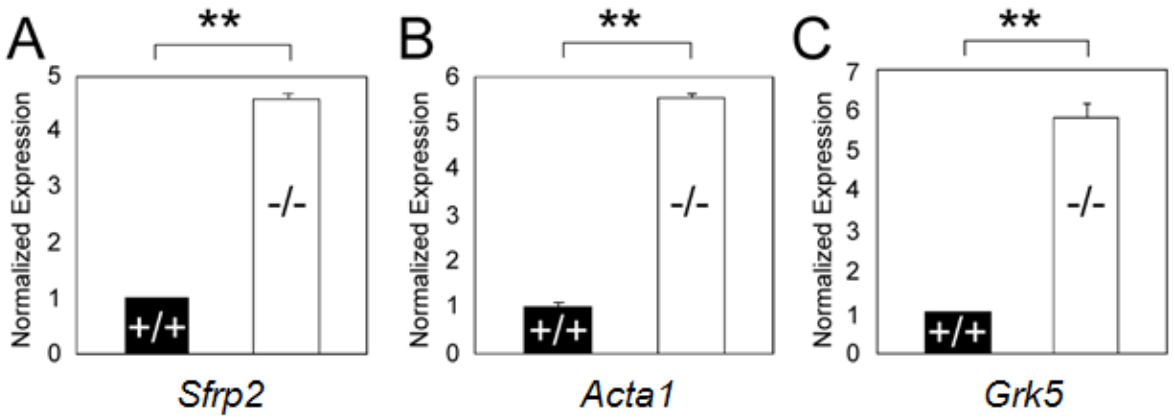

Figure 11: ASXL2 is required for the repression of select cardiac genes.

The mRNA levels of *Sfrp2* (A), *Acta1* (B), and *Grk5* (C) in 1mo-old wild-type and *Asxl2*$^{-/-}$ hearts were analyzed by quantitative RT-PCR. The expression level of each gene analyzed was normalized against that of ACTIN in the same sample. Each column shown is the mean value of data generated from three independent samples. ** $p < 0.01$; Error bar: standard deviation.

4.4.3 ASXL2 and PRC2 components co-localize at select target loci

Genome-wide studies have consistently found PRC2 components to be enriched at chromatin regions near the transcription start sites (TSSs) of target genes (Bernstein et al., 2006; Ku et al., 2008; Meissner et al., 2008; Mikkelsen et al., 2008; Mikkelsen et al., 2007; Pan et al., 2007; Schuettengruber et al., 2009; Zhao et al., 2007). To determine whether *Sfrp2*, *Acta1* and *Grk5* are directly repressed by ASXL2 and PRC2, we examined enrichment of ASXL2 and PRC2 components at these loci by ChIP-qPCR assays, focusing on regions between -2 kb and +2 kb of the TSS. For each locus, we selected 2-3 genomic sites that are conserved between mouse, rat and human (Fig. 12A-C). ASXL2 was enriched at most of these sites (Fig. 12D-F). Most of the ASXL2-enriched sites also exhibited enrichment of PRC2 core components EZH2 and SUZ12 (Fig. 12G-I).

To investigate the distribution of ASXL2 along target loci, we selected a series of conserved sites within the gene bodies of *Sfrp2* and *Grk5* and examined the level of ASXL2 enrichment by ChIP-qPCR assays. For both genes, ASXL2 was most highly enriched at the promoter, and the level of enrichment decreases from the 5' to 3' end of the gene body (Fig. 13A-B).

To confirm that we are detecting site-specific binding of ASXL2 instead of promiscuous binding to chromatin, ChIP assays were also performed for the *S100a10* locus, which was active in both wild-type and *Asxl2*$^{-/-}$ hearts. ASXL2 enrichment was not detected at any of the six sites that we analyzed for the *S100a10* locus (Fig. 14).

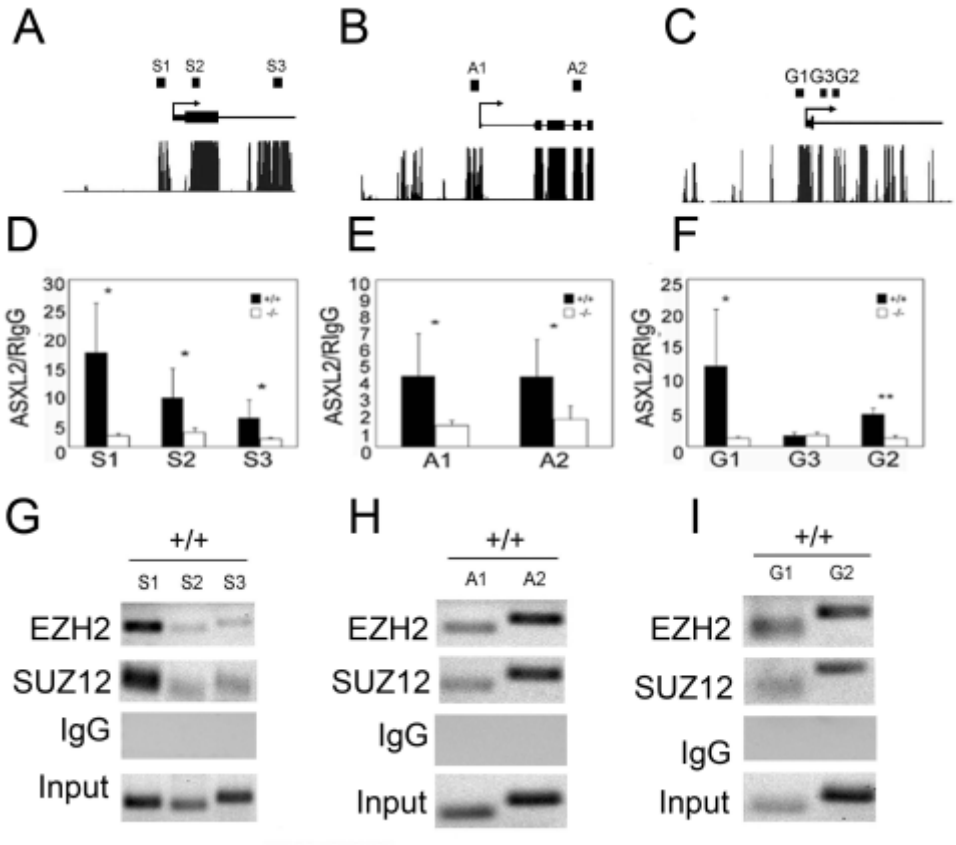

Figure 12: ASXL2 and PRC2 core components co-localize at select target loci.

(A-C) Alignment of mouse, rat and human genomic sequences from -2kb to +2kb of *Sfrp2* (A), *Acta1* (B), and *Grk5* (C). The peaks correspond to regions of sequence conservation. For each gene, 2-3 highly conserved regions (black bars on top of the graphs, designated S1-3, A1-2 and G1-3, respectively) were selected for ChIP-qPCR analysis. (D-F) ChIP-PCR assays of ASXL2 enrichment near *Sfrp2* (D), *Acta1* (E) and *Grk5* (F) TSSs in 1-month-old wild-type and *Asxl2*$^{-/-}$ hearts. Mock ChIPs were performed with rabbit IgG. Input: qPCR assays of 1:100 diluted total input. (G-I) ChIP-PCR assays of EZH2 and SUZ12 enrichment near *Sfrp2* (G), *Acta1* (H) and *Grk5* (I) TSSs in 1-month-old wild-type mouse hearts. * $p < 0.05$; ** $p < 0.01$; Error bar: standard deviation.

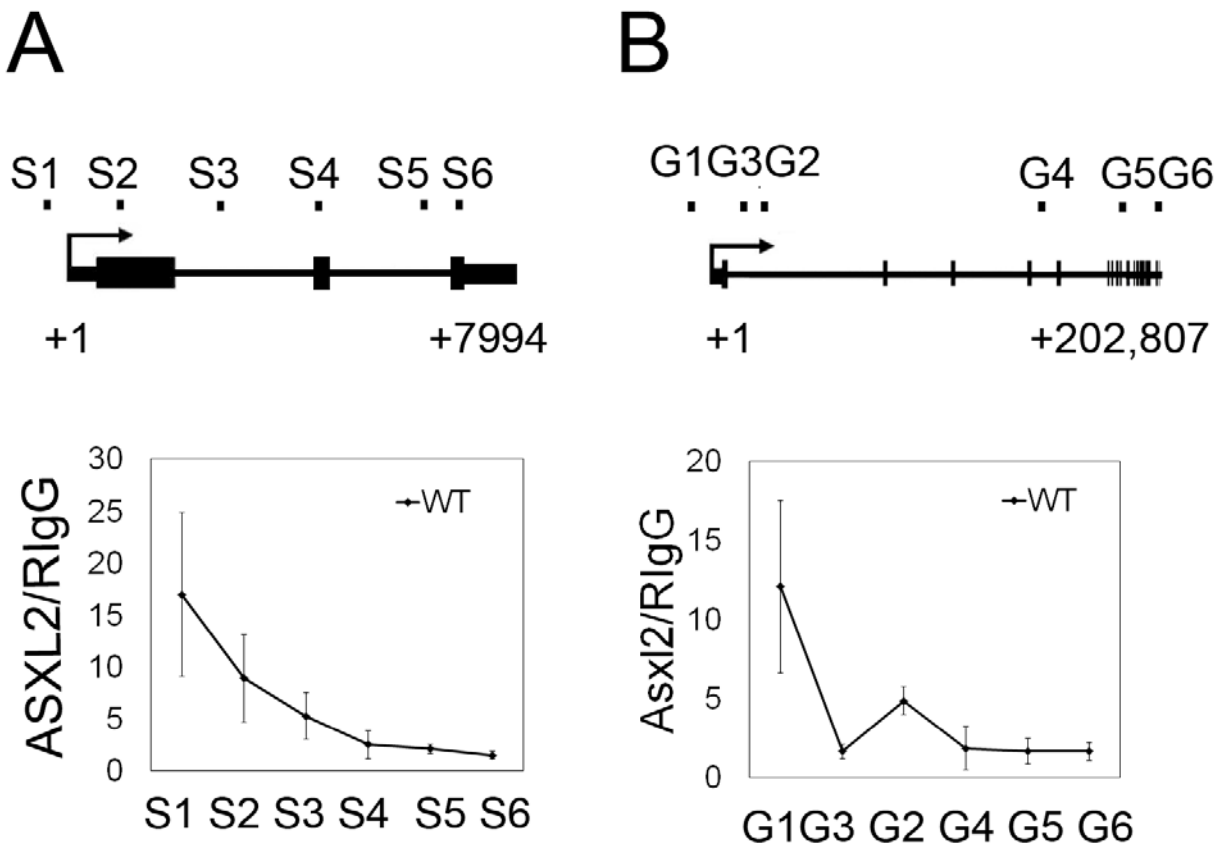

Figure 13: ASXL2 is not enriched at the coding regions of *Sfrp2* and *Grk5*.

ChIP-qPCR analysis of ASXL2 enrichment at *Sfrp2* (A) and *Grk5* (B) in 1-month-old wild-type hearts. Mock ChIPs were performed with rabbit IgG. Each column represents the mean value of data from three independent samples; Error bar: standard deviation. The x axis indicates the regions that were analyzed, as shown in the schematic representations of the *Sfrp2* and *Grk5* promoters and coding regions.

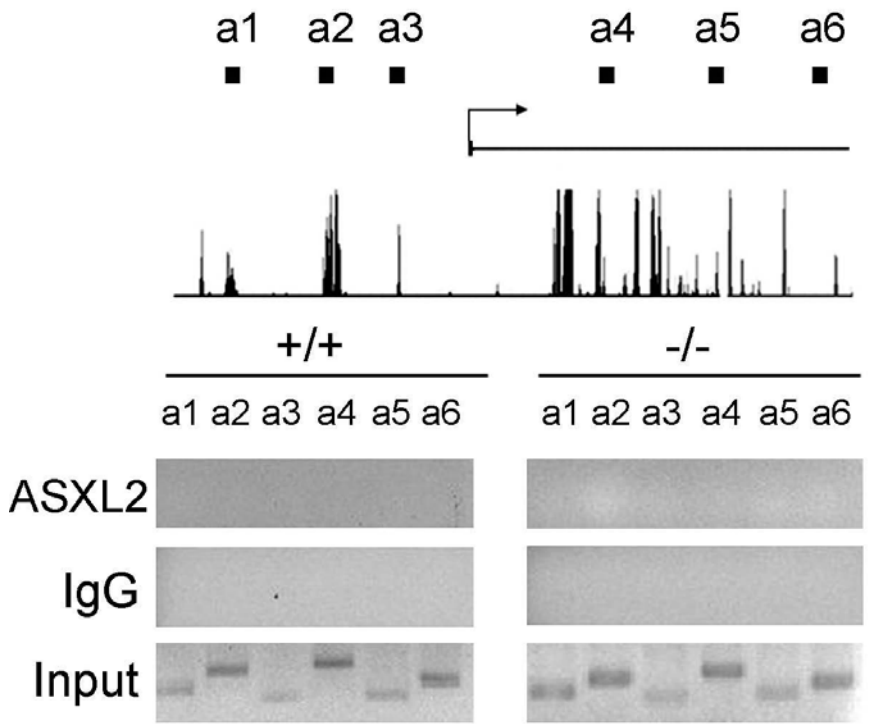

Figure 14: ASXL2 is not enriched at the *S100a10* locus. *S100a10* encodes a calcium binding protein and is highly expressed in both wild-type and *Asxl2*$^{-/-}$ hearts. Shown is anti-ASXL2 ChIP-PCR results for six chromatin sites (a1-a6) within -5kb to +5kb of *S100a10* TSS. Mock ChIP was performed with normal rabbit IgG. Input: PCR assay of 1:100 diluted total input chromatin.

4.4.4 H3K27me3 is significantly reduced at de-repressed Asxl2 target loci

We have previously shown that the bulk level of H3K27me3 is decreased in *Asxl2*$^{-/-}$ hearts (Baskind et al., 2009). This is consistent with genetic evidence in both Drosophila and mouse suggesting that *Asx* and *Asx-like* genes promote PcG protein activity (Baskind et al., 2009; Fisher et al., 2010a; Milne et al., 1999). We hypothesized that de-repression of *Myh7*, *Sfrp2*, *Acta1* and *Grk5* in the *Asxl2*$^{-/-}$ heart is due to a deficiency of H3K27me3 at these loci. ChIP-qPCR assay showed that in comparison to wild-type hearts, *Asxl2*$^{-/-}$ hearts exhibited significant reductions in the level of H3K27me3 enrichment at *Myh7, Sfrp2, Acta1* and *Grk5* promoters (Fig. 15A-D), confirming our hypothesis. In contrast, the level of H3K27me3 enrichment at the *Hoxb5* locus did not change in *Asxl2*$^{-/-}$ hearts (Fig. 16). Additionally, qRT-PCR detected extremely low, if any, *Hoxb5* transcription in both wild-type and *Asxl2*$^{-/-}$ hearts (Figure 17), suggesting that it does not require ASXL2 for repression. These results suggest that ASXL2 is specifically involved in the regulation of a subset of PcG protein targets.

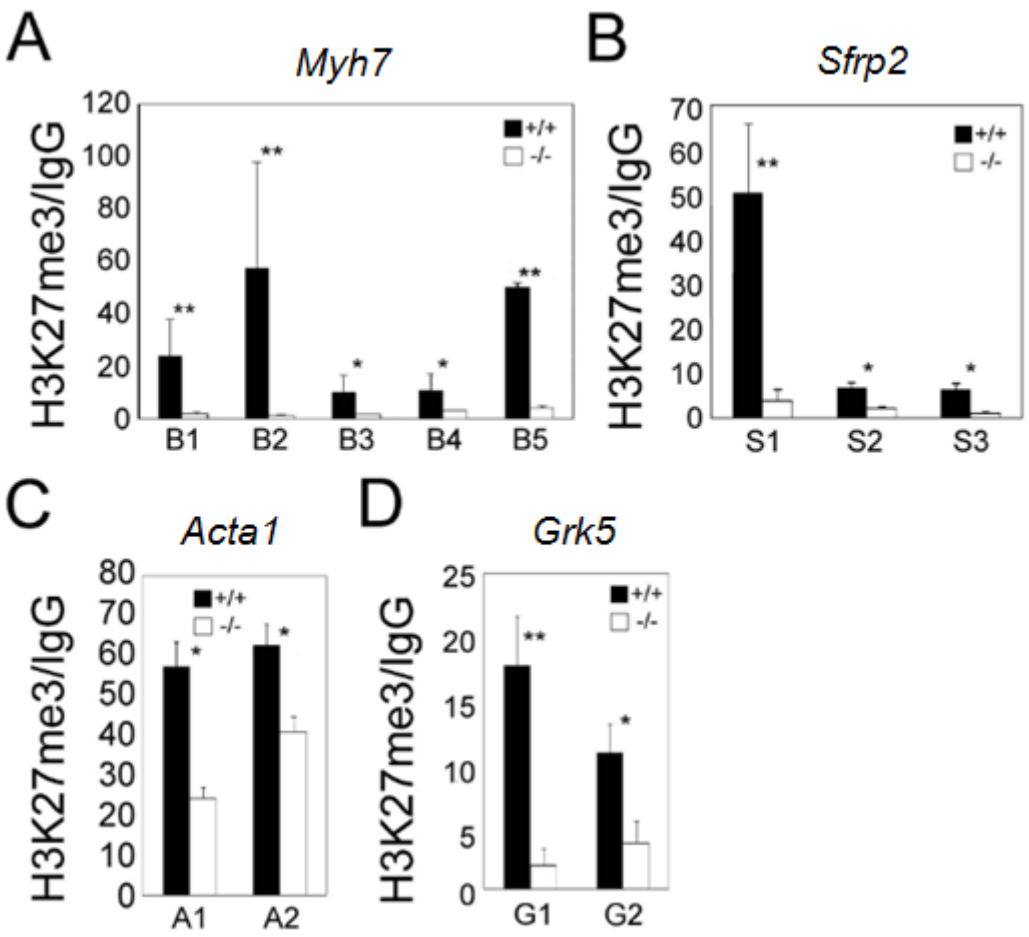

Figure 15: De-repression of ASXL2 target genes is accompanied by reduced levels of H3K27me3.

ChIP-qPCR assays were used to compare the levels of H3K27me3 at conserved regions surrounding *Myh7* (A), *Sfrp2* (B), *Acta1* (C), and *Grk5* (D) TSS in wild-type and *Asxl2*$^{-/-}$ hearts. Data from H3K27me3 ChIP were normalized against those from IgG mock ChIP. Each column represents the mean value of data from three independent samples. The five conserved regions in the *Myh7* promoter, B1-5, are as previously described (79). * $p < 0.05$; ** $p < 0.01$; Error bar: standard deviation.

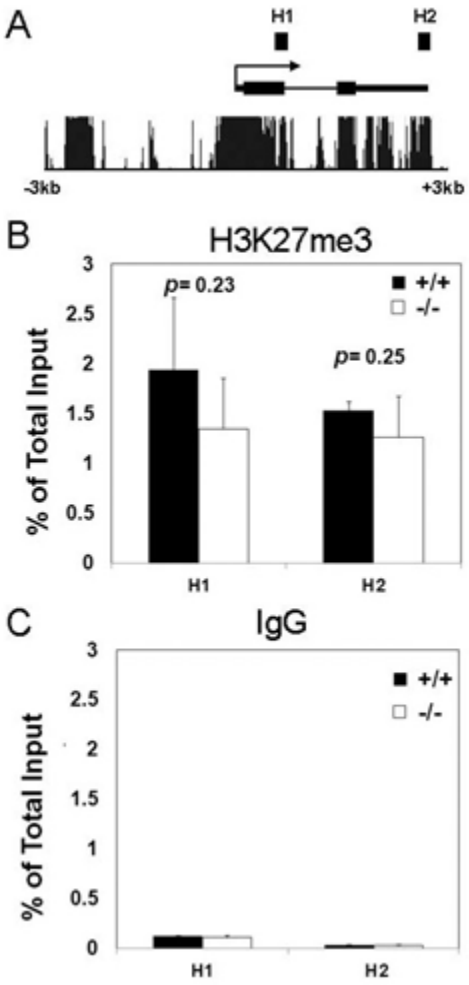

Figure 16: ChIP-qPCR analysis of H3K27me3 enrichment at the *Hoxb5* locus.

(A) Alignment of mouse, rat and human genomic sequences from -3kb to +3kb of *Hoxb5*. H1 and H2 are two highly conserved regions that were selected for ChIP-qPCR analysis. (B) H3K27me3 ChIP. (C) Mock IgG ChIP. Each column represents the mean value of data from three independent samples. Error bar: standard deviation.

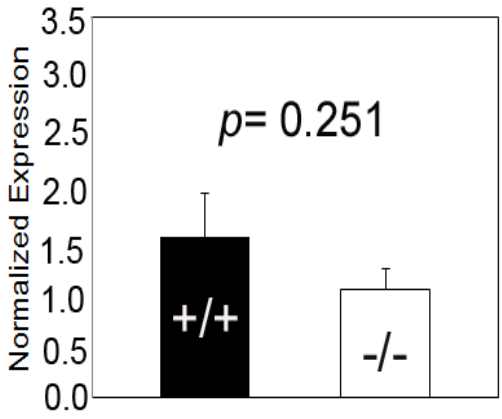

Figure 17: *Hoxb5* transcription levels were detected very low in both wild-type and *Asxl2*$^{-/-}$ hearts.

The mRNA levels of *Hoxb5* in 1mo-old wild-type and *Asxl2*$^{-/-}$ hearts were analyzed by quantitative RT-PCR. Each column shown is the mean value of data generated from two independent samples.

4.4.5 Acetylation of histone H3 (AcH3) is significantly increased at de-repressed *Asxl2* target loci

To test the possibility that the loss of *Asxl2* may result in depletion of nucleosomes or indiscriminate reduction of all histone modifications at target loci, we examined the enrichment of AcH3, an active histone mark (Delgado-Olguin et al., 2012). In the absence of *Asxl2*, the level of AcH3 enrichment increased significantly at *Myh7, Sfrp2, Acta1 and Grk5* – loci that are dependent on ASXL2 for repression (Fig. 18A-D). No increase of AcH3 was observed at the *Hoxb5* locus, which does not require ASXL2 for repression (Fig. 18E). The bulk level of AcH3 is comparable in wild-type and *Asxl2*$^{-/-}$ hearts (Fig. 18F). Taken together, *Asxl2* deficiency specifically affects H3K27 methylation.

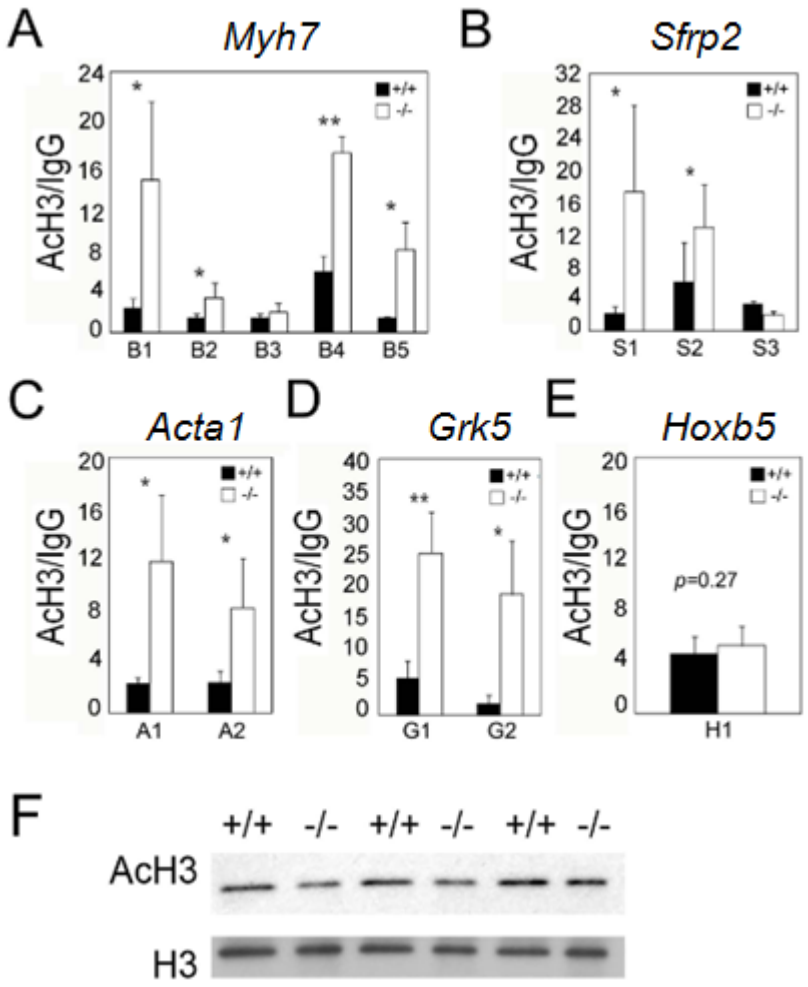

Figure 18: De-repression of ASXL2 target genes is accompanied by increased levels of AcH3.

ChIP-PCR assays were used to compare the levels of AcH3 (Active Motif) at conserved regions surrounding *Myh7* (A), *Sfrp2* (B), *Acta1* (C), *Grk5* (D) and *Hoxb5* (E) TSS in wild-type and *Asxl2*$^{-/-}$ hearts. Data from AcH3 ChIP were normalized against those from IgG mock ChIP. Each column represents the mean value of data from three independent samples. The five conserved regions in the *Myh7* promoter, B1-5, are as previously described (Lai et al., 2012). Position of H1 within the *Hoxb5* locus is shown in Figure 8. * $p < 0.05$; ** $p < 0.01$; Error bar: standard deviation. (F) Western blot analysis of bulk AcH3.

4.4.6 PRC2 core subunits are expressed and form complexes in *Asxl2*$^{-/-}$ hearts

To understand the mechanism by which ASXL2 regulates H3K27me3 levels at target chromatin loci, we first asked whether ASXL2 is required for the stability of PRC2 core subunits. Nuclear protein extracts from wild-type and *Asxl2*$^{-/-}$ hearts were separated on SDS-PAGE and probed with antibodies against EZH2, SUZ12, and EED (Fig. 19A). The level of EZH2 protein in wild-type is by approximately of 3/8-fold in *Asxl2*$^{-/-}$ hearts (Fig. 20). The levels of SUZ12 and EED also increased but to lesser degrees. Quantitative RT-PCR showed that transcription of EZH2 is increased by 1.4-fold in *Asxl2*$^{-/-}$ hearts (Fig. 19B). Thus, the higher level of EZH2 protein in *Asxl2*$^{-/-}$ hearts is regulated at both the transcript and the protein levels. Taken together, these results suggest that *Asxl2* is not required for the expression of EZH2, SUZ12 or EED. Instead, the loss of *Asxl2* and the subsequent reduction in bulk H3K27me3 may have triggered a compensation pathway to express more PRC2 components.

Next, we asked whether deficiency in *Asxl2* affects the association between PRC2 core components. We immunoprecipitated SUZ12 and proteins associated with it from wild-type and *Asxl2*$^{-/-}$ heart extracts. Western blot analysis showed that EZH2 and EED co-IPed with SUZ12 in both wild-type and *Asxl2*$^{-/-}$ hearts (Fig. 19C). In addition, immunoprecipitation of EZH2 pulled down SUZ12 (Fig. 19D). These results suggest that *Asxl2* is dispensable for the formation of the PRC2 core complex.

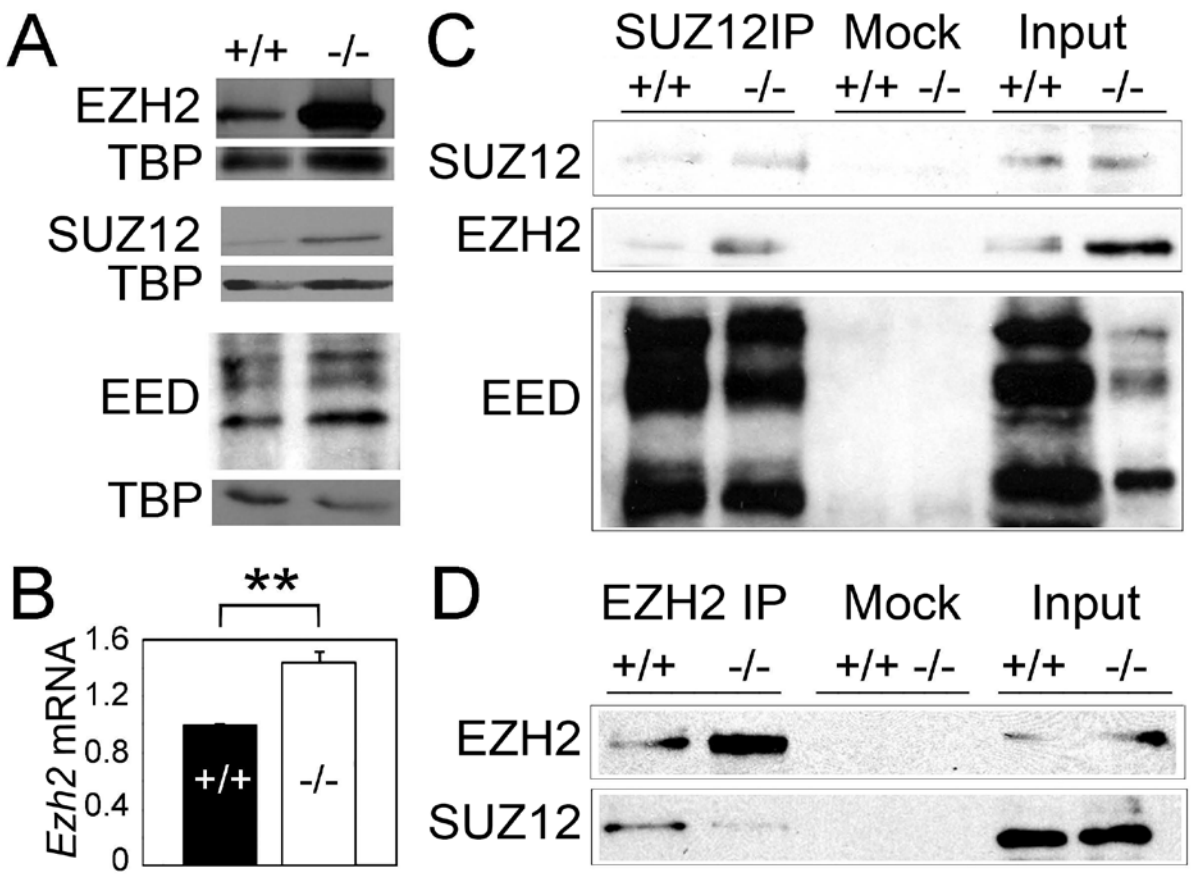

Figure 19: ASXL2 is not required for the protein stability of PRC2 core components or the integrity of PRC2 complex.

(A) Western blot analysis of protein levels of EZH2, SUZ12, and EED in wild-type and *Asxl2*$^{-/-}$ hearts. Western blot of TATA-binding protein (TBP) was used as a loading control. Three pairs of hearts were analyzed and a representative result was shown for each protein. (B) Quantitative RT-PCR analysis of EZH2 transcription in wild-type and *Asxl2*$^{-/-}$ hearts. (C, D) Co-IP analysis of PRC2 components. Wild-type and *Asxl2*$^{-/-}$ heart extracts were IPed using either an anti-SUZ12 antibody (C) or an anti-EZH2 antibody (D). Mock IP was performed with pre-immune serum (IgG). IPed samples were analyzed by Western blot using the indicated antibodies. ** $p < 0.01$; Error bar: standard deviation.

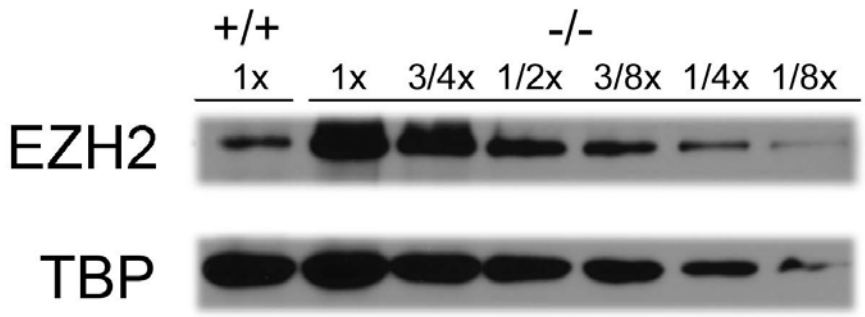

Figure 20: Comparison of EZH2 level in wild-type and *Asxl2*$^{-/-}$ hearts.

Serial dilutions of heart extracts were analyzed by SDS-PAGE and then probed with anti-EZH2 antibody. Western blot of TBP was used as a loading control.

4.4.7 ASXL2 is required for PRC2 binding at target loci

Next, we asked whether ASXL2 plays a role in the localization of PRC2 to target chromatin. We compared the level of EZH2 enrichment at *Myh7*, *Sfrp2*, *Acta1* and *Grk5* loci in wild-type and *Asxl2*$^{-/-}$ hearts by ChIP-qPCR. For all the sites that exhibited EZH2 enrichment above background in wild-type hearts, there is a significant reduction in chromatin-bound EZH2 in *Asxl2*$^{-/-}$ hearts (Fig. 21A-D). Therefore, although *Asxl2*$^{-/-}$ hearts expressed a much higher level of EZH2 protein (Fig. 19A), it failed to bind to ASXL2 target loci, which may account for reduced H3K27me3 levels at these loci and thereby de-repression.

4.4.8 ASXL2 interacts with PRC2 core components in the adult heart

Given that ASXL2 co-localizes with PRC2 at target loci and is required for PRC2 binding, we tested whether ASXL2 interacts with PRC2 *in vivo*. We immunoprecipitated ASXL2 from heart extracts and examined the presence of EZH2 and SUZ12. As shown in Fig. 21E, both PRC2 core components co-IPed with ASXL2. This suggests that ASXL2 associates with PRC2 in the heart and may regulate chromatin binding of PRC2 directly.

4.4.9 ASXL2 is specifically required for the addition of the third methyl group to H3K27

PRC2 mediates the mono-, di- and tri- methylation of H3K27. It has been proposed that a stable association of PRC2 with chromatin is specifically required for the conversion of H3K27me2 to H3K27me3 (Sarma et al., 2008). Since the loss of *Asxl2* resulted in a significant decrease in the bulk level of H3K27me3 (Baskind et al., 2009) and, at the same time, a decrease in PRC2 association with target loci (Fig. 21A-D), we asked whether ASXL2 is specifically required for the addition of the third methyl group. Western blot analysis showed a striking increase in the level of bulk H3K27me2 in *Asxl2*$^{-/-}$ hearts (Fig. 21F). This further confirms that PRC2 complex is intact and enzymatically active but fails to stably associate with chromatin in the absence of *Asxl2*.

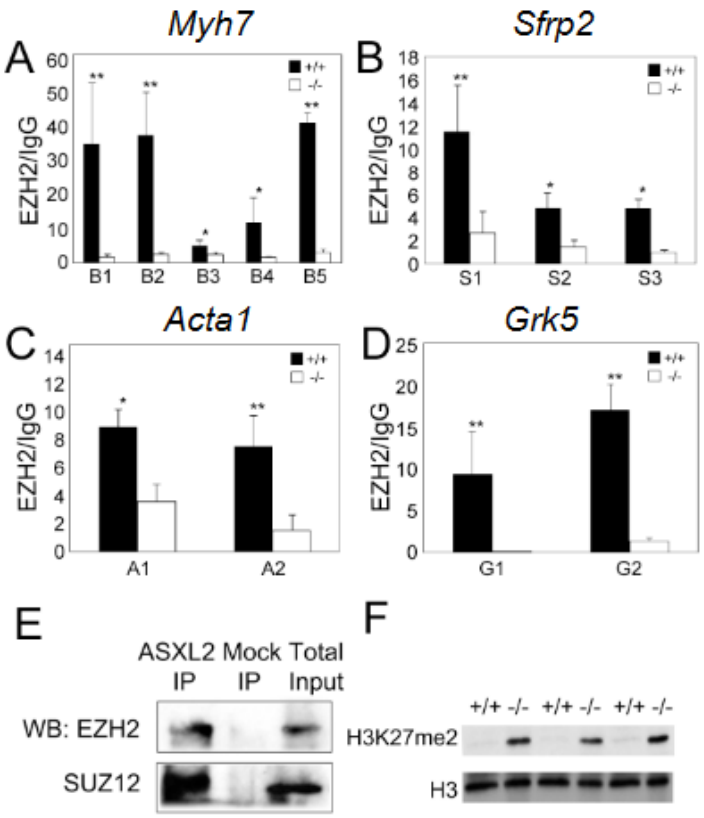

Figure 21: ASXL2 interacts with PRC2 and is required for recruitment of PRC2 to select target genes in the mouse heart.

The level of EZH2 enrichment at *Myh7* (A), *Sfrp2* (B), *Acta1* (C) and *Grk5* (D) in wild-type and *Asxl2*$^{-/-}$ hearts was compared by ChIP-qPCR. Data from EZH2 ChIP were normalized against those from IgG mock ChIP. Each column represents the mean value of data from three independent samples. (E) Co-IP analysis of the interaction between ASXL2 and PRC2 components. Wild-type heart extract was IPed using anti-ASXL2 antibody. Mock IP was performed with pre-immune serum (IgG). IPed samples were analyzed by Western blot using the indicated antibodies. *$p < 0.05$; **$p < 0.01$; Error bar: standard deviation. (F) Western blot analysis of bulk H3K27me2 in three pairs of wild-type and *Asxl2*$^{-/-}$ hearts. To control for comparable protein loading, the blot was stripped and re-blotted for histone H3.

4.4.10 ASXL2 interacts with BAP1 and is required for efficient uH2A deubiquitination *in vivo*

Drosophila Asx is a component of the PR-DUB complex and is required for efficient deubiquitination of uH2A (Scheuermann et al., 2010). To determine whether this function is conserved in ASXL2, we examined the interaction between ASXL2 and BAP1, the mammalian homolog of Calypso, and the effect of *Asxl2* deficiency on bulk uH2A level. We found that BAP1 co-IPed with ASXL2 from wild-type heart extract (Fig. 22A). In addition, the level of bulk uH2A was significantly increased in *Asxl2*$^{-/-}$ hearts (Fig. 22B). The level of bulk uH2B did not change, consistent with previous report that PR-DUB specifically deubiquitinates uH2A but not uH2B (Scheuermann et al., 2010). These results suggest that ASXL2 is a critical component of mammalian PR-DUB in the heart.

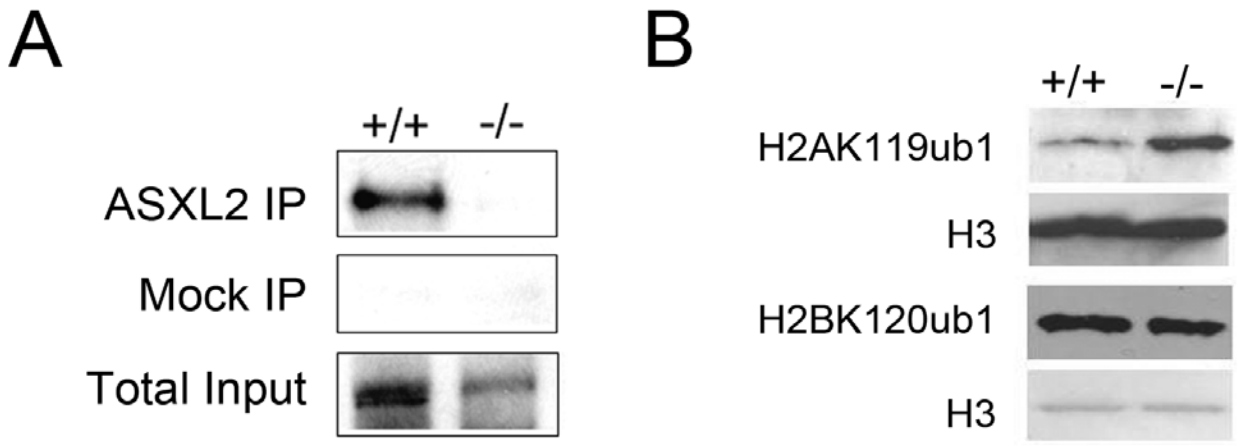

Figure 22: ASXL2 interacts with BAP1 *in vivo* and is required for efficient deubiquitination of uH2A.

(A) Co-IP analysis of interaction between ASXL2 and BAP1. Wild-type and *Asxl2*$^{-/-}$ heart extracts were IPed using anti-ASXL2 antibody. Mock IP was performed with pre-immune rabbit serum (IgG). IPed samples were analyzed by Western blot using anti-BAP1 (Millipore; 1:1500; ~100KDa). (B) Western blot analysis of protein levels of uH2A and uH2B in wild-type and *Asxl2*$^{-/-}$ hearts. Western blot of H3 was used as a loading control. Three pairs of hearts were analyzed and a representative result was shown for each protein.

4.4.11 The loss of *Asxl2* is not compensated by *Asxl1* or *Asxl3*

The *Asxl* family has three genes. To determine whether there is functional redundancy in the *Asxl2*$^{-/-}$ adult heart, we examined the mRNA levels of *Asxl1*, *2*, and *3* in 1 month-old wild-type and mutant hearts. *Asxl2* is the only *Asxl* gene that highly expressed in adult hearts. Also, the expression levels of *Asxl1* and *Asxl3* are not up-regulated in loss of *Asxl2*, suggesting *Asxl1* and *3* do not compensate upon loss of *Asxl2* in adult heart (Fig. 23).

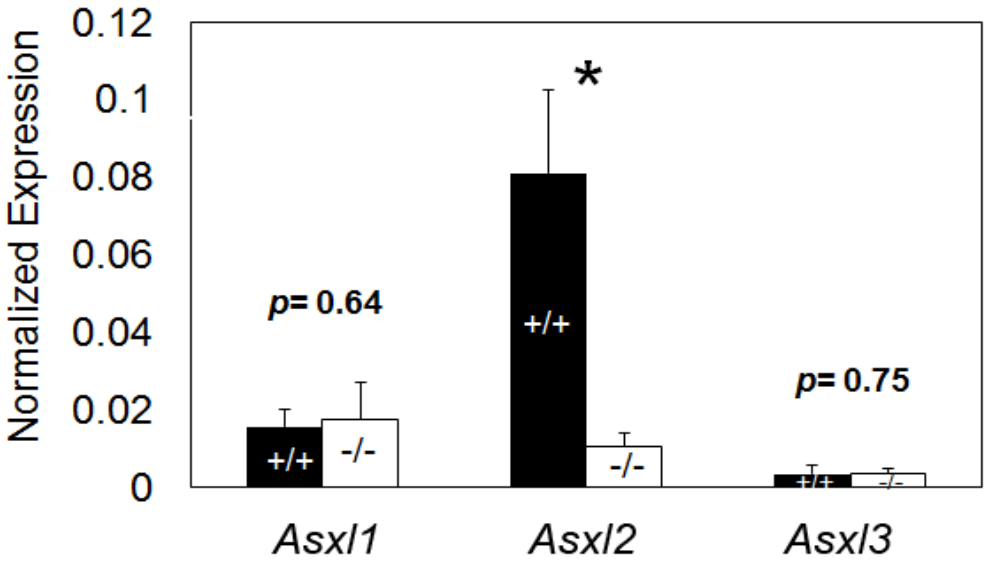

Figure 23: Expression of *Asxl* genes in the adult mouse heart.

The mRNA levels of *Asxl1*, *Asxl2*, and *Asxl3* in wild-type and *Asxl2*$^{-/-}$ hearts were analyzed by quantitative RT-PCR. Each column shown is the mean value of data generated from three independent samples. *$p < 0.05$; Error bar: standard deviation.

4.5 Discussion

4.5.1 ASXL2 regulates PRC2-chromatin association

We have previously observed that the level of bulk H3K27me3 was reduced in *Asxl2*$^{-/-}$ hearts, suggesting an important role for ASXL2 in the homeostasis of the H3K27me3 mark (Baskind et al., 2009). H3K27 methylation is catalyzed by the PRC2 complex. PRC2 alone is sufficient for the mono- and di- methylation of H3K27 both *in vitro* and *in vivo* (Nekrasov et al., 2007), and it has been proposed that H3K27 di-methylation may be accomplished prior to histone deposition (Sarma et al., 2008). On the other hand, efficient conversion of H3K27me2 to H3K27me3 is thought to require stable association of PRC2 with target chromatin (Nekrasov et al., 2007; Sarma et al., 2008).

Here we show that ASXL2 co-IPs with PRC2 and co-localizes with PRC2 at selected target loci. The loss of *Asxl2* results in loss of H3K27me3 enrichment at target promoters and gene de-repression. Further investigation showed that *Asxl2* deficiency did not reduce the expression of PRC2 components or prevent the formation of PRC2 complex, but specifically affected the association of PRC2 complex to target chromatin. Consistent with a requirement for *Asxl2* in PRC2 binding to chromatin, *Asxl2*$^{-/-}$ hearts exhibit a significant increase in the level of bulk H3K27me2. Taken together, these results strongly suggest that ASXL2 is a regulator of PRC2-chromatin association and specifically promotes the addition of the third methyl group to H3K27.

A recent paper has shown that ASXL1 is required for PRC2 binding at target loci in human hematopoietic cells (Abdel-Wahab et al., 2012), suggesting that it is a conserved function of ASXL proteins. Like ASXL2 in the heart, ASXL1 is required in hematopoietic cells for maintaining the normal level of bulk H3K27me3. It will be interesting to determine

whether there is an increase in H3K27me2 in *ASXL1* deficient blood cells. While the functional mechanism of ASXL1 and 2 may be similar, the two proteins are expressed in different tissues and have different target genes. *Asxl2* is the only *Asxl* gene that is highly expressed in the heart, and *Asxl2*$^{-/-}$ hearts did not exhibit up-regulation of either *Asxl1* or *Asxl3* (Fig. 22). ASXL1 is required for the enrichment of PRC2 and H3K27me3 at the *HOXA* gene cluster in the hematopoietic lineage (Abdel-Wahab et al., 2012). In the absence of *ASXL1*, *HOXA* genes are de-repressed. In contrast, ASXL2 appears dispensable for *Hox* gene repression in the heart (Appendix Table I); the loss of *Asxl2* did not disrupt PRC2 and H3K27me3 enrichment at the *Hoxb5* locus (Fig. 16, Fig. 17E). What could account for this difference? We propose that ASXL proteins are general facilitators of PRC2 recruitment and through their interaction with additional partners, such as transcription factors, target specificity in a given tissue can be achieved.

4.5.2 ASXL2 and PHF1 use different mechanisms to promote H3K27 trimethylation

The function of ASXL2 in promoting H3K27 trimethylation is reminiscent of PHF1 (also known as PCL1), which interacts with EZH2 (Cao et al., 2008; O'Connell et al., 2001; Sarma et al., 2008) and is essential for converting H3K27me2 to H3K27me3 at target loci (Nekrasov et al., 2007; Sarma et al., 2008). However, there are three important distinctions. First, PHF1 can be an integral component of PRC2 and co-purifies with the core components (Cao et al., 2008; Nekrasov et al., 2007; Sarma et al., 2008). Although ASXL2 co-IPs with PRC2 from heart extract, neither Asx nor any ASXL proteins have been found to be part of PRC2. The interaction between ASXL2 and PRC2 may be indirect.

Secondly, PHF1 deficiency does not affect the level of bulk H3K27me2 or H3K27me3

(Nekrasov et al., 2007). Thus, ASXL2 appears to play a broader role than PHF1 in the regulation of PRC2. One possible scenario is that different genes require different proteins for the promotion of H3K27 trimethylation. The effect of *Asxl2* deficiency on bulk H3K27me2/3 levels suggests that in the adult heart, most PRC2 targets require ASXL2. In contrast, PHF1 may be required for the regulation of just a small number of targets.

Finally, although a GAL4-PHF1 fusion protein is able to recruit PRC2 to transgenic UAS sites, EZH2 enrichment at target chromatin is independent of PHF1 (Sarma et al., 2008). In comparison, ASXL2 is more critically required for PRC2-chromatin association at its target loci. This suggests that the two proteins use different mechanisms for promoting H3K27 trimethylation. For example, for PRC2 to efficiently convert H3K27me2 to H3K27me3 on chromatin substrate, there might be two prerequisites: stable chromatin association, followed by stimulation of enzymatic activity by a co-factor that can be independently recruited to target chromatin. We propose that ASXL2 regulates the first step, while PHF1 acts as a PRC2 co-factor.

4.5.3 A potential link between histone H2A deubiquitination and H3K27 trimethylation?

Asx and ASXL proteins are core components of the PR-DUB complex, which specifically removes ubiquitin from histone H2A that is mono-ubiquitinated at lysine 119 (Scheuermann et al., 2010). The discovery that ASXL is required for PRC2 binding at target genes raises the question of whether PR-DUB deubiquitinase activity is involved in the regulation of PRC2 binding. In the mouse heart, ASXL2 is required for the homeostasis of both H3K27me3 and uH2A: the loss of *Asxl2* resulted in a decrease in the level of bulk H3K27me3 (Baskind et al., 2009) as well as an increase in the level of bulk uH2A (Fig.

21B). It remains to be answered whether there is any causative link between the changes in these two histone marks. On the other hand, in the hematopoietic cell lines studied by Abdel-Wahab *et al.*, the loss of *ASXL1* disrupted PRC2 and H3K27me3 enrichment at the *HOXA* gene cluster without disrupting the level of uH2A (Abdel-Wahab et al., 2012).

Furthermore, knocking down *Bap1* in the hematopoietic cell lines inactivated PR-DUB but did not reproduce the de-repression of *HOXA* genes as observed in *ASXL1*-deficient cells (Abdel-Wahab et al., 2012). This seems to suggest that PR-DUB and PRC2 act independently of each other at the *HOXA* cluster, and that the loss of PRC2 recruitment in *ASXL1*- deficient cells did not result from inactivation of PR-DUB. A comprehensive study of more gene loci is needed to answer whether there is a functional relationship between histone H2A deubiquitination and cells.

4.5.4 Potential PR-DUB-independent mechanisms to regulate PRC2 binding

ASXL1 and 2 are large proteins that interact with multiple proteins other than BAP1 (Cho et al., 2006; Lee et al., 2010; Yu et al., 2010). Interaction with histone and DNA has also been proposed (Aravind and Iyer, 2012). These interactions could translate into PR-DUB-independent regulation of PRC2 binding. In mammalian cells, ASXL1 and ASXL2 co-purify with the YY1 protein in a >1 MD, multi-subunit complex (Yu et al., 2010). The Drosophila homolog of YY1, Pleiohomeotic (Pho), is a sequence- specific DNA-binding protein that mediates the recruitment of other PcG proteins, including PRC2, to a subset of target chromatin sites (Brown et al., 2003; Brown et al., 1998; Mohd-Sarip et al., 2002). When expressed in Drosophila, YY1 can rescue the homeotic phenotypes in homozygous *Pho* mutants, suggesting a high degree of functional conservation (Atchison et al., 2003; Kim et al., 2006). In mouse embryos, YY1 was found to co-localize with other PcG proteins and with

H3K27me3 to upstream regulatory regions of *Hoxc8* and *Hoxa5* (Kim et al., 2006). Through its interaction with YY1, ASXL2 could potentially regulate YY1's ability to bind regulatory elements or other PcG proteins, thereby regulating PRC2 binding.

Asx and all ASXL proteins contain a highly conserved plant homeo domain (PHD) at the C- terminus (Atchison et al., 2003). The PHD finger is not involved in interaction with Calypso/Bap1 (Scheuermann et al., 2010), yet is required for repression of *Ubx* in the wing primordial (Bienz, 2006; Fisher et al., 2006). PHD fingers are found in many chromatin proteins and can mediate interactions with histones or non-histone protein partners [reviewed in (Bienz, 2006). For example, the PHD finger of Pcl is involved in binding to E(z) (O'Connell et al., 2001), and that of BPTF binds H3K4me3 (Li et al., 2006; Wysocka et al., 2006). If the PHD finger of ASXL2 interacts with PRC2 component(s) and/or with the nucleosome, it could directly contribute to PRC2 binding and/or to stabilizing PRC2 association with target chromatin.

A recent computational modeling study of ASXL proteins identified an N-terminal winged helix- turn-helix (wHTH) domain that is predicted to bind DNA (Aravind and Iyer, 2012). This domain is also found in a number of other eukaryotic and prokaryotic proteins that are known to bind DNA, including certain restriction endonucleases, DNA glycosylases, and the RNA polymerase delta subunit of Gram-positive bacteria. A wHTH-DNA interaction may increase the affinity of ASXL2/PRC2 to chromatin.

4.5.5 Functional divergence between Asx and ASXL

The level of bulk H3K27me3 was dependent on ASXL1/2 in mammalian cells but was unaffected in Drosophila embryos carrying a homozygous null mutation of *Asx* (Scheuermann et al., 2010). Moreover, RNAi knock-down of *Trx* severely disrupted binding

of Asx, but not of PRC2, to polytene chromosomes (Petruk et al., 2008), suggesting that PRC2 does not require Asx for chromatin association. What could account for this apparent discrepancy between the functional requirements for Drosophila Asx and for mouse ASXL1/2?

While the mechanism that regulates PRC2 binding is far from well understood, differences between mammals and Drosophila have been observed (Schuettengruber and Cavalli, 2009). ASXL proteins may have evolved new functions, not possessed by Asx, to meet the specific needs of PRC2 regulation in mammals. Two lines of evidence are consistent with the scenario of functional divergence. First, although Asx family proteins range in size from 1370 to 2204-aa, homology between Asx and ASXL is largely restricted to the 32-aa PHD domain and the 120-aa ASXH domain (Fisher et al., 2006). Secondly, while PRC2 and ASXL1/2 co-IP in human cells (Abdel-Wahab et al., 2012) and mouse tissue (this study), Asx did not co-purify with Drosophila PRC2 in cultured cells (Scheuermann et al., 2010).

Alternatively, the role of Asx/ASXL in PRC2 binding to chromatin may be dependent on the chromatin loci and/or on the cell type. For example, we showed that not all PcG protein targets require *Asxl2* for H3K27 trimethylation in the heart (Fig. 16, Fig 17E). The ratio of Asx/ASXL- dependent targets versus independent targets in a given tissue at a given developmental time may determine whether there is a detectable change in the level of bulk H3K27me3 in the mutant.

V. GENERAL DISCUSSION

5.1 ETP proteins modulate gene expression via interaction with different partners

ETP proteins genetically regulate both PcG and TrxG protein activities, and mutations in *ETP* genes result in both anterior and posterior transformation phenotypes. The functional mechanisms of a number of ETP proteins have been studied (Table II). Many ETP proteins are known to interact with PcG and/or TrxG proteins. Additionally, some ETP proteins have been shown to partially co-localize with PcG/TrxG proteins at multiple chromatin sites. For example, Asx binds ~ 90 loci on polytene chromosome, but only shares 63 binding sites with PRC1 subunits Pc and Ph (Milne et al., 1999). ETP proteins also interact with various non-PcG/TrxG partners, and some of these interactions have been shown to be functionally important.

TABLE II: ETP GENES AND THEIR KNOWN MOLECULAR MECHANISMS

ETP protein	Species	Molecular mechanism
Asx	Drosophila	- Forms the PR-DUB complex with Calypso; stabilizing Calypso protein and promoting its deubiquitinase activity (Abdel-Wahab et al., 2012)
ASXL1	Human	- Acts as co-activator of RAR and RXR through association with SRC-1, a c-Src tyrosine kinase (Cho et al., 2006) - Acts as co-repressor through recruitment of LSD1 and HP-1 to target genes in a cell-type specific manner (Lee et al., 2010) - Component of the PR-DUB complex (Scheuermann et al., 2010) - Required for EZH2 enrichment at the promoters of *HOX A* gene cluster (Abdel-Wahab et al., 2012)
ASXL2	Human	- Acts as co-activator for PPARγ together with the TrxG protein MLL1 (Park et al., 2011)
ASXL2	Mouse	- Required for PRC2 enrichment at target promoters and promoting the conversion of H3K27me2 to H3K27me3 (Lai submitted manuscript).

Corto	Drosophila	- An activator when interacting with Dsp1 on *Scr* maintenance element (ME) (Salvaing et al., 2006) - A repressor when localizing alone on *Scr* ME (Salvaing et al., 2006)
Dsp1	Drosophila	- Binds to PREs and facilitates Pho binding to PREs. Once bound, Pho recruits PRC2 to targets (Dejardin et al., 2005)
EPL1	Saccharomyces cerevisiae	- A component of the NuA4 histone acetyltransferase complex (Boudreault et al., 2003)
EPC	Human	- A component of the NuA4 histone acetyltransferase complex (Doyon et al., 2004) - Interacts with RET finger protein, a transcriptional repressor (Shimono et al., 2000)
Rm62	Drosophila	- Interacts with Dsp1 (Lamiable et al., 2010)
Psc	Drosophila	- Recruits histone deacetylases (HDACs) to targets (Breiling et al., 2001). - A component of PRC1 (Francis et al., 2001)
Scm	Drosophila	- A component of PRC1 (Peterson et al., 2004)

My study has primarily focused on the functional mechanism of ASXL2, in particular the mechanism of its repressor function. I have shown that ASXL2 interacts with both PRC2 and BAP1. ASXL2 is required for PRC2 enrichment at target promoters. The *Asxl2*$^{-/-}$ heart is defective in conversion of H3K27me2 to H3K27me3 and in deubiquitination of uH2A. Studies by others have shown that ASXL2 can also interact with the TrxG protein MLL1 and function as an activator (Park et al., 2011).

ASXL2's protein interactions also include O-linked N acetylglucosamine transferase (OGT), HCF-1, and LSD1. LSD1 is a H3K4 demethylase and required for *Hox* gene expression in Drosophila (Di Stefano et al., 2007). OGT modification of transcription factors is an important mechanism that modulates transcription activity (Ozcan et al., 2010). HCF-1 is a transcription factor and is involved in control of the cell cycle (Li et al., 2008).

While ASXL2 interacts with BAP1 and is required for BAP1 activity in the adult heart, it is unclear what the functional importance of this interaction is. Since *Bap1* mutant mice do not survive embryogenesis, a conditional knockout *Bap1* in adult heart is required for further investigation of their roles in heart. The interaction of Drosophila Asx and Calypso (Drosophila homolog of *Bap1*) has repressive role in the regulation of *Ubx*. I hypothesize that the ASXL2-BAP1 interaction in the heart is functionally important for the repression of at least a subset of, if not all, ASXL2 target genes. To test this hypothesis, we first need to identify genes that are directly regulated by both ASXL2 and BAP1. Such genes should meet two criteria:1) their expression should be mis-regulated in *Bap1*$^{-/-}$ and *Asxl2*$^{-/-}$ hearts, and 2) BAP1 and ASXL2 should co-localize at their promoter regions. *Asxl2*$^{-/-}$ hearts exhibit an increased level of bulk uH2A, suggesting ASXL2 is required for BAP1 activity. Once we have identified common target genes of ASXL2 and BAP1, we will test whether the loss of ASXL2/BAP1 has the same effect

on uH2A distribution/levels at the target genes by ChIP assay. If there is a positive correlation between distribution/levels of uH2A with gene expression states, it will support our hypothesis that ASXL2 regulates gene expression by associating with BAP1. On the other hand, it is also possible that (1) there will be no common targets identified or (2) they have common target genes but there will be no or different effects on uH2A distribution/levels upon loss of *Asxl2*/*Bap1*. These alternative scenarios would suggest that ASXL2 regulates cardiac gene expression independently of BAP1 activity.

5.2 Sequence and functional divergence within ASXL members

Although ASXL1, 2, and 3 share a few conserved domains and some conserved protein interactions, their sequence similarity is only 14.38 % (Fisher et al., 2010a). Some protein interactions appear to be specific to certain member(s) of the family. For example, an HP1 binding motif resides in ASXL1 and ASXL3, but is absent from the corresponding region in ASXL2 (Lee et al., 2010). Consistently, HP1 only interacts with ASXL1 but not with ASXL2 (Lee et al., 2010). Overexpression of *Asxl1* inhibits peroxisome proliferator-activated receptor's (PPARγ) target gene expression by associating with HP1 (Abdel-Wahab et al., 2012). In contrast, overexpression of *Asxl2* activates PPARγ target gene expression by associating with TrxG protein MLL (Abdel-Wahab et al., 2012). Deletion of the HP1 binding site from ASXL1 results in a mutant protein that enchances PPARγ target gene expression, suggesting the HP1 binding site is required for the repressive role of ASXL1 in PPARγ targets (Park et al., 2011). Thus, ASXL1 and ASXL2, two members of the same family, have opposite roles in the transcription of PPARγ target genes due to the divergence in their protein sequences.

Why is there sequence and functional divergence between different ASXL proteins? I propose that divergence at the protein sequences in the ASXL family is important for ASXL proteins to interact with different protein partners that are expressed in different tissues. This would facilitate differential regulation of distinct sets of target genes; as well allow regulation by different pathways that are present in different tissues.

First, ASXL proteins are expressed in different tissues. Each gene may have evolved differently to meet the transcriptional needs in the tissues that express it. Northern blot analysis suggested that *Asxl1* is highly expressed in brain, kidney, and lung and expressed very low in heart and skeletal muscle (Fisher et al., 2006). The expression of the *Asxl2* gene trap was detected in the heart, the retina, the ovary and the testis, but not in the liver, the brain, the spleen, the pancreas, or the lung by X-gal staining (Baskind et al., 2009). We have specifically confirmed that *Asxl2* is highly expressed in mouse embryonic and adult hearts by qRT-PCR assay (A. McGinley unpublished data; Lai submitted). The mRNA levels of *Asxl1* and *Asxl3* are barely detected in the heart, and *Asxl1* and *Asxl3* are not up-regulated in *Asxl2*$^{-/-}$ hearts. Second, in different tissues, there are different target genes that need to be expressed or repressed, and there are different transcription factors to activate or repress them. For example, repression of *Ubx* in the wing blade is important for wing development in Drosophila, and de-repression of *Ubx* in Drosophila wing results in the wing defects. In contrast, de-repression of *Ubx* in the central nerve system (CNS) has no significant effect (Bischoff et al., 2009; Milne et al., 1999). Accumulated evidence suggests that ASXLs regulate transcriptional repression and activation of homeotic and non-homeotic genes in a cell context-dependent manner (Abdel-Wahab et al., 2012; Cho et al., 2006; Fisher et al., 2010b; Lai et al., 2012; Lamiable et al., 2010; Milne et al., 1999).

In summary, the ability of ASXL family to interact with diverse partners is consistent with the roles of other ETP proteins in transcriptional regulation. These partners allow flexibility in adapting regulation needs to target-, cell-, and situation- specific contexts.

5.3 Whether ASXL2 has a role in cellular memory?

Transcription factors are responsible for the establishment of cell identities during development. "Cellular memory" mechanisms enable cells to remember their fate long after the initiating factors are gone (Hemberger et al., 2009). PcG proteins are known to maintain the repressed state of developmental genes in succeeding cell cycles (Dejardin and Cavalli, 2005; Ringrose and Paro, 2004; Struhl and Akam, 1985). For example, Hox genes are essential developmental genes that control the body plan of the embryo along the anterior-posterior axis (Hughes and Kaufman, 2002). Mutations in Hox genes lead to transformation of body segments from one into the identity of another. PcG proteins are required to maintain Hox gene repression outside of their expression domains throughout developmental stages (Lewis, 1982). Mutations in PcG genes result in homeotic transformation phenotypes (Lewis, 1978). PcG proteins also have been implicated in responsible for mediating the silencing of one of the X chromosomes (Plath et al., 2003). X-inactivation is a classic epigenetic phenomenon by which one of the X chromosomes present in female is inactivated, resulting in only a copy of the X chromosome gene product is generated in female (Penny et al., 1996). PRC2 subunits, EED and EZH2 were found to be required for long-term X inactivation by establishment of the H3K27me3 marks on one of the X chromosomes. Thus, PcG proteins might be the molecular basis of the cellular memory.

PcG proteins must be able to perform three functions to have a role in maintaining cellular memory (1) PcG proteins need to find the target gene; (2) PcG proteins must deposit the histone mark that can implement the repressive state. (3) PcG proteins will assure the histone marks are renewed correctly in every cell cycle.

Deposition of H3K27 methylation marks to target chromatin is tightly associated with PcG- mediated gene repression. Several mechanisms have been proposed to explain how PcG proteins guarantee the histone marks regenerated correctly at each cell division. Old histones are disrupted but the core H3/H4 tetramer is randomly redistributed to the two daughter DNA molecules during DNA replication. New histones must be deposited to newly replicated DNA to fill the gaps. Also, the histone marks that are on the old chromatin would be diluted to two-fold. PRC2 complex can associate with the replication fork and recognize the H3K27me3 mark through PRC2 subunit EED. This allows its own self- renewal by recruiting more PRC2 complexes to newly replicated DNA (Han et al., 2007). Loss of ESC, Drosophila EED homolog, results in de-repression of Hox genes, and dramatic depletion of both H3K27me3 and H3K27me2 at target chromatin. Furthermore, it is essential to restore full H3K27me3 to maintain the PRC2 activity. Recombinant PRC2 complex generate mono, di, and tri methylation of H3K27. However, the presence of H3K27me3 peptides in the reaction can greatly stimulate the catalytic activity.

How does PcG protein recognize target chromatin? Several molecules have been implicated in PcG targeting to target chromatin (See 1.5.2). Here, we have shown that ETP protein ASXL2 interacts with PRC2 in adult heart. ASXL2 is also required for PRC2 binding and the enrichment of H3K27me3 at target chromatin. Loss of Asxl2 results in a significant decrease in the bulk level of H3K27me3 and a concomitant increase in H3K27me2y, suggesting

that ASXL2 is specifically required for the addition of the third methyl group to H3K27. It has been proposed that H3K27 di-methylation may be accomplished prior to histone deposition. Also, the efficient conversion of H3K27me2 to H3K27me3 is thought to be required for stable association of PRC2 with target chromatin. Thus, we hypothesize that ASXL2 is required for PRC2 complexes stabilization at target chromatin. PRC2 will then further be stimulated to catalyze H3K27me3 formation to maintain repressive state of target genes. In order to test the hypothesis, we have to first determine whether loss of ASXL2 would affect the H3K27me2 level at target chromatin. If we see the increase level of H3K27me2 at target chromatin upon loss of ASXL2, it would strengthen our hypothesis that ASXL2 is required for PRC2 stability at target chromatin. A tetracycline-inducible Gal4-tagged EZH2 in the 293 Trex TK Luc cell line gave rise to H3K27me2 but not H3K27me3 at the TK promoter (Sarma et al., 2008). We can test whether overexpressed GAL-ASXL2 fusion protein in above system can stabilize EZH2 at TK promoter, which should result in generation of full strength of H3K27methylaton. On the other hand, if H3K27me2 level is decreased at target chromatin, ASXL2 may play a role in PRC2 recruitment to target chromatin.

The hallmark of PcG activity is to establish and maintain long-term developmental decisions. Histone modifications, PcG proteins and their interacting proteins seem to play an essential role in this process.

VI. APPENDICES

APPENDIX A: PRIMERS USED IN CHIP ASSAY

TABLE III: PRIMERS USED IN CHIP ASSAY

ChIP primers	Sequence (5'-3')	Tm
Grk5-1-F (G1F)	AGACAGTTGCAGGGACGAGT	60
Grk5-1-R (G1R)	ATTGGCACGTTTTCTTGCTT	60
Grk5-2-F (G2F)	AACACGGTCTTGCTGAAAGC	60
Grk5-2-R (G2R)	CACTCGCACCAACTCACTGT	60
Grk5-3-F (G3F)	GAGGAGAATGGAGTGACAGA	60
Grk5-3-R (G3R)	CTTCTTCCTCCCTCTGTGT	60
Grk5-4-F (G4F)	GCTTCCATTGCCCTACTTCA	58
Grk5-4-R (G4R)	TCAGACAGCACTTTCCATGC	58
Grk5-5-F (G5F)	TGCTATGCCATTGTCCTTCA	58
Grk5-5-R (G5R)	CAAGGGACCTGCATCATCTT	58
Grk5-6-F (G6F)	CCTGGCCTATGCCTATGAAA	58
Grk5-6-R (G6R)	TAAGGCTCGCTCTTCCTCAA	58
Acta1-1-F (A1F)	CCCTTGCACAGGTTTTTA	60
Acta1-1-R (A1R)	AAATATGGCTTGGAAGG	60
Acta1-2-F (A2F)	CCACGCTCAGTGAGGATTTT	60
Acta1-2-R (A2R)	TGCCCATCTATGAGGGCTAT	60
Sfrp2-1-F (S1F)	CAGCCCGACTTCTCCTACAA	60
Sfrp2-1-R (S1R)	AGCCGCATGTTCTGGTACTC	60

APPENDIX A (continued)

ChIP primers	Sequence (5'-3')	Tm
Sfrp2-2-F (S2F)	CCAGCCCCAGAAAGTAGT	60
sfrp2-2-R (S2R)	AATCTGGAGGTGGAGGAG	60
Sfrp2-4-F (S4F)	ACGACAACGACATCATGGAA	58
Sfrp2-4-R (S4R)	GACTTTAGGGACCGGGAGAG	58
Sfrp2-5-F (S5F)	TCCAATAGGGGAGTCGTTTG	58
Sfrp2-5-R (S5R)	TTCCAACCAGCTATCGTTCC	58
Sfrp2-6-F (S6F)	GAGAGTTCAAGCGCATCTCC	58
Sfrp2-6-R (S6R)	GGAGCGGAAGTGGTCTACAG	58
β-MHC-F1 (B1F)	GGAACCAGCGGAGTACAAAA	58
β-MHC-R1 (B1R)	TGTATCCCCTGACCTTGGAG	58
β-MHC-F2 (B2F)	CTGCCCCTTTGTCTTGTCTC	58
β-MHC-R2 (B2R)	CCCAGGCTTCAGAGTACAGC	58
β-MHC-F3 (B3F)	TGCAACTGCATTCTGAGGAC	58
β-MHC-R3 (B3R)	ACCAAAGCAGGGGTAAGGAT	58
β-MHC-F4 (B4F)	CAACTTCCTATCTGCTGAGG	58
β-MHC-R4 (B4R)	GATCCATTTAAGTGCTTTGC	58
β-MHC-F5 (B5F)	GGGGAGGATACTGGAAATAG	58
β-MHC-R5 (B5R)	GAGAGTAAGCTGACCACGAC	58
S100a10-1-F (a1F)	GGGGTTTCTGAGGGTAAAGG	60
S100a10-1-R (a1R)	AGAGCACATACGTGGCACTG	60
S100a10-2-F (a2F)	GCCTAGCTGGTTGCTGATTC	60

APPENDIX A (continued)

ChIP primers	Sequence (5'-3')	Tm
S100a10-2-R (a2R)	GGCAGCTCAGACAAGAAAC	60
S100a10-3-F (a3F)	AGCTCCTGAAGCTGACAAGC	60
S100a10-3-R (a3R)	TGTTGAGTGCAGAACCAAGG	60
S100a10-4-F (a4F)	CCTGAGATTTCCTCCACAGC	60
S100a10-4-R (a4R)	AATTCTCTATGCGCCACCAC	60
S100a10-5-F (a5F)	TGATGTTGTTGGTTGGGTTG	60
S100a10-5-R (a5R)	AAAGGAAGCTCCAGATGCAA	60
S100a10-6-F (a6F)	CACCCCATCTGGAGTGAAGT	60
S100a10-6-R (a6R)	GTCCTAACCAATCCCCCATT	60
Hoxb5-1-F (H1F)	TGAGGAAGCTTCACATCAGCCACG	58
Hoxb5-1-R (H1R)	CCAAGCTTTGCTCGCCCCCAC	58
Hoxb5-2-F (H2F)	TGCCAGGCCTGTCTCAGTGATT	58
Hoxb5-2-R (H2R)	ACAAGTAGAGGGCACTGGAGTGG	58

APPENDIX B: PRIMERS USED IN QUANTITATIVE RT-PCR ASSAY

TABLE IV: PRIMERS USED IN QUANTITATIV RT-PCR ASSAY

RT-PCR primers	Sequence (5'-3')	Tm
Acta1-F	ACCGCTCTTGTGTGTGACAA	60
Acta1-R	GGAGTCCTTCTGACCCATACC	60
β-Actin-F	TCACCCACACTGTGCCCATCTACGA	60
β-Actin-R	TGGTGAAGCTGTAGCCACGCT	60
ANP-F	AGGAGAAGATGCCGGTAGAAGA	60
ANP-R	GCTTCCTCAGTCTGCTCACTCA	60
Asxl1-F	TAAAGAGGAGCCCAAAGTCCCG	58
Asxl1-R	GGCAGGAGGACTCCGTGATG	58
Asxl2-F	CTCCTGAAATGCAGGTGAGA	58
Asxl2-R	TTGCTTTGGGATCACTTGAG	58
Asxl3-F	CTTCAAAATCCCTGGAAAGTCG	58
Asxl3-R	ATCTCAGCGCCATCCAGGTC	58
Ezh2-F	ATCTGAGAAGGGACCGGTTT	60
Ezh2-R	TGTGCACAGGCTGTATCCTC	60
Grk5-F	GGAAGGGGGTGGAGGAAAG	60
Grk5-R	AGAACTGTCGAAAAAGCAGTCTC	60
αMHC-F	GTCACCAACAACCCATACGACTAC	60
αMHC -R	CAGCACATCAAAGGCACTATCAGT	60
βMHC-F	TCTCCTGCTGTTTCCTTACTTGCT	60
βMHC -R	CAGGCCTGTAGGAGAGCTGTACTC	60
Sfrp2-F	CGTGGGCTCTTCCTCTTCG	60
Sfrp2-R	ATGTTCTGGTACTCGATGCCG	60

APPENDIX B (continued)

RT-PCR primers	Sequence (5’-3’)	Tm
rBap1-1F	AGCCAGCATGGATATGAAGG	58
rBap1-R	TCTCAAGGAGGTGGAGA	58
rAsxl1-F	TTCCAGCAGCAACTCCTCTT	58
rAsxl1-R	GCGTGGGTGAAAAACTCATT	58
rAsxl2-F	GGAGAAAAGACCACGGATCA	58
rAsxl2-R	ATCGGGGAGATTCTGGAGAC	58
rAsxl3-F	GGGTTCCCCCTCTCAAGATA	58
Aurkb-F	AGGTCTGCAGGGAGAACTGA	58
Aurkb-R	TCATCTCTGGGGGCAGATAG	58
Ccnb1-F	GCGTGTGCCTGTGACAGTTA	58
Ccnb1-R	CCTAGCGTTTTTGCTTCCCTT	58
Ccnd1-F	CCCAACAACTTCCTCTCCTG	58
Ccnd1-R	TCCAGAAGGGCTTCAATCTG	58
Cdk1-F	TGCCAGAGCGTTTGGAATAC	58
Cdk1-R	GATGTCAACCGGAGTGGAGT	58
Cdk4-F	CTGGTACCGAGCTCCTGAAG	58
Cdk4-R	GTCGGCTTCAGAGTTTCCAC	58
Rb1-F-1	TTCACCCTTACGGATTCCTG	58
Rb1-R-1	TGTCCCAAATGATTCACCAA	58
P130-F	TGAGAGCAGAAGCCATCAGA	58
P130-R	CCGTGAGTCGAGTTGGTGTA	58

APPENDIX C: ANTIBODIES USED IN CHIP ASSAY

TABLE V: ANTIBODIES USED IN CHIP ASSAY

ChIP Ab	Catalog number	Carrier	Concentration of the stock antibody	Amount used (µl) per 300µl chromatin
ASXL2 (KC17)		Biomatik	5 mg/ml	8
AcH3	39139	Active motif	n/d	7
EZH2	612667	BD Transduction laboratory	n/d	8
H3K27me3	C36B11	Cell signaling	n/d	6
SUZ12	39357	Active motif	n/d	8
Rabbit IgG	FL1021	Invitrogen	2.5 mg/ml	1.6 (4µg)
Mouse IgG	02-6502	Invitrogen	2.5 mg/ml	1.6 (4µg)

n/d: not determined

APPENDIX D: ANTIBODIES USED IN WESTERN BLOTTING

TABLE VI: ANTIBODIES USED IN WESTERN BLOTTING

WB Ab	Dilution	Size (KDa)	2°Ab dilution (α-mouse HRP)	2°Ab dilution (α-rabbit HRP)	Carrier (Catalog number)
AcH3	1:2000	~15		1:10,000	Active motif (39139)
ASXL2	1:500	~170		1:10,000	Biomatik
ACTIN	1:3000	~40			Solaro Lab
BAP1	1:1500	~100	1:10,000		Millipore (05-671)
EZH1	1:2000	~100		1:10,000	Abcam (ab64850)
EZH2	1:2000	~90	1:10.000		Millipore (17-662)
EED	1:3000	36-76		1:10,000	Millipore (09-774)
GAPDH	1:2000	~35	1:10,000		Millipore (MAB374)
H3	1:5000	~15		1:50,000	Active motif (39163)

APPENDIX D (continued)

WB Ab	Dilution	Size (KDa)	2ºAb dilution (α-mouse HRP)	2ºAb dilution (α-rabbit HRP)	Carrier
uH2A	1:2000	~25	1:10,000 (α-mouse IgM HRP)		Millipore (05-678)
uH2B	1:2000	~20	1:20,000		Millipore (05-1312)
PLB	1:5000	~20	1:20,000		Solaro Lab
PLB^{ser16}	1:2000	~21	1:10,000		Solaro Lab
RNAPoII	1:3000	~170	1:10,000		Sigma (ab5408)
P130	1:2000	~130		1:10.000	Santa Cruz (C-20)
RB	1:2000	~130		1:10,000	Santa Cruz (C-15)
FLAG	1:1000			1:10,000	Sigma (F7425)
TNNI3	1:5000	~25	1:50,000		Solaro's Lab

APPENDIX D (continued)

WB Ab	Dilution	Size (KDa)	2ºAb dilution (α-mouse HRP)	2ºAb dilution (α-rabbit HRP)	Carrier
PKA-TNNI3^{ser23}	1:1000	~26		1:40,000	Solaro's Lab
TBP	1:3000	~40	1:10.000		Sigma (58C9)
SUZ12	1:2000	~85		1:10,000	Active motif (39357)

APPENDIX E: ANTIBODIES USED FOR IMMUNOPRECIPITATION (IP) ASSAY

TABLE VII: ANTIBODIES USED FOR IMMUNOPRECIPITATION (IP) ASSAY

IP Ab	Catalog number	Carrier	Amount used (µl) per 600µl Nucleus extract
Pre-ASXL2 (KC17)		Biomatik	16
EZH2	Ac22	BD Transduction laboratory	12
SUZ12	39357	Active motif	8
Rabbit IgG	FL1021	Invitrogen	2 (5µg)
Mouse IgG	02-6502	Invitrogen	2 (5µg)

APPENDIX F: ANTIBODIES USED FOR IMMUNOFLUORESCENCE (IF)

TABLE VIII: ANTIBODIES USED FOR IMMUNOFLUORESCENCE (IF)

IF Ab	Dilution	2°Ab dilution		Carrier
		(α-mouse Alexa 488)	(α-rabbit Texas Red)	
Pre-ASXL2 (KC17)	1:25		1:2000	Biomatik
BMI-1	1:50	1:2000		Helin Lab
EED	1:50	1:2000		Millipore (09-774)

APPENDIX G: PLASMIDS

TABLE VIIII: PLASMIDS

Plasmid	Note
pCAG-ASXL2 (1-390)	Destination vector
pCAG-Asxl2(72-1370)	Destination vector
pCAG-Asxl2(1-660)	Destination vector
pCAG-Asxl2(FL)	Destination vector
pCAG-Asxl2(Δ240-350)	Destination vector
pCAG-Asxl1(Δ PHD)	Destination vector
pCAG-Asxl2(1-720)	Destination vector
pCR8-Asxl2(1-720)	Entry clone
pCR8-Asxl1(Δ PHD)	Entry clone
pCR8-Asxl2(72-1370)	Entry clone
pCR8-Asxl2(1-660)	Entry clone
pCR8-Asxl2(1-390)	Entry clone
pCR8-Asxl2(FL)	Entry clone
pCR8-Asxl2(Δ240-350)	Entry clone
pTREX-DEST30-BAP1 (C91S)	Dominant negative mutant of BAP1

APPENDIX H: SHRNA PLASMIDS

TABLE X: SHRNA PLASMIDS

Plasmid	shRNA Sequence (5'-3')
Bap1-Rat, shRNA in retroviral GFP vector	
pGFP-V-RS (GI723441)	ACCAGTTCTGCCATCAGATACAAGCGGAA
pGFP-V-RS (GI723442)	AACCTGGTGGAGCAGAACATCTCAGTGCG
pGFP-V-RS (GI723443)	ACGGACACAGCCTCTGAGATTGGCAGTGC
pGFP-V-RS (GI723444)	AGTCACAACTGCCTGAGGAGAGCAAGCCA
ASXL2-Rat, shRNA in retroviral GFP vector	
pGFP-V-RS (GI726225)	AACCAGTTGCCTCTGCTGAACAAGAATCT
pGFP-V-RS (GI726226)	ACAGGTTGGTCCAGACGGCTTGATGAAGT
pGFP-V-RS (GI726227)	TGCCTCCAGCACCTGTTAGCGACCACATC
pGFP-V-RS (GI726228)	AGCAGCCTCTCCTTGCCACTCGCAAAGAAC
TR30013	Scrambled negative control non-effective shRNA

APPENDIX I: THE SUBCELLULAR LOCALIZATION OF ASXL2 IN HEK293 CELLS

I.1 Purpose and objective

PcG proteins form a unique discrete nuclear structure called PcG bodies (Pirrotta and Li, 2012). Drosophila Asx co-localizes at ~ 63 loci with Ph and Pc on polytene chromosome (Milne et al., 1999). Our study also shows that ASXL2 co-localizes with PRC2 at target promoter regions in adult heart. To determine whether ASXL2 is associated with PcG proteins in nucleus, immunofluorescence assay was used to detect the subcellular localization of ASXL2 and a PRC1 protein BMI-1 in HEK293 cells.

I.2 Materials and Methods

1 µg of pCDNA3.1 FLAG-Asxl2 plasmid was overexpressed in HEK293 cells for 24 hours using 3 µl polyethylenimine (PEI) transfection reagent. The cells were fixed with methanol for 15 minutes at RT and then blocked with 5% donkey and goat serum overnight at 4ºC. ASXL2 subcellular localization was determined by double staining with mouse anti-FLAG (1:500; Sigma) and anti-pre-absorbed ASXL2 (1:25; KC17) in HEK293 cells. After several wash steps with PBS buffer, the cells were double stained with anti-rabbit Texas Red (1:2000; Jackson) and anti-mouse Alexa Fluor 488 (1:2000; Jackson) for 2 hours at RT. U2OS cells were stained with anti- BMI-1 (1:25; Helin Lab) overnight at 4 ºC and then stained with anti-mouse Alexa Fluor 488 (1:2000; Jackson) for signal detection. DAPI stain was used to stain the nucleus in cells. All images were collected on a Zeiss Axiovert 200M equipped with a digital camera.

I.3 Result and conclusion

The FLAG and ASXL2 signals were overlapped in the nucleus of HEK293 cells, suggesting the detected signals are specific to FLAG-ASXL2 (Fig. 24A). Endogenous BMI-1 staining was detected in the nuclei of U2OS cells (Fig 24B). No cell was stained in the negative control experiments (data not shown). The punctate staining pattern of ASXL2 and BMI-1 were similar in two cell lines. However, we cannot be certain whether ASXL2 co-localizes with BMI-1 in the cell. Double staining of both proteins in one cell line is necessary to confirm the co-localization of ASXL2 and BMI1. If ASXL2 co-localizes with BMI-1 in cells, this would suggest that ASXL2 may be associated with PcG bodies.

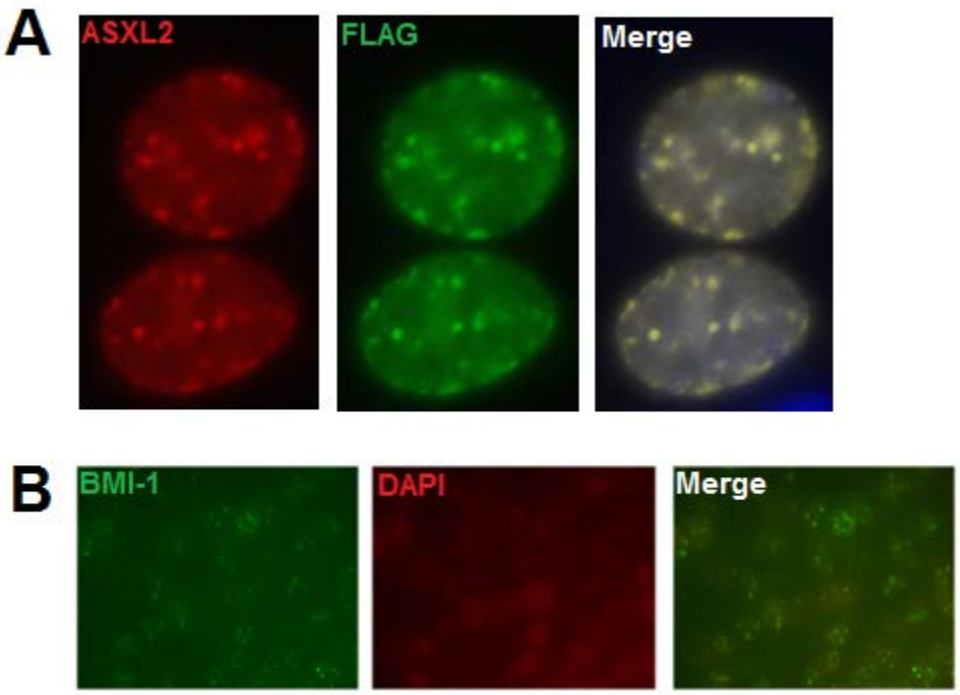

Figure 24: Nuclear localization of FLAG-ASXL2 and BMI-1 in HEK293 and U2OS cells, respectively.

HEK293 cells were transfected with an FLAG-ASXL2 expression construct and double stained with anti-FLAG and an anti-ASXL2 (A). The localization of endogenous BMI-1 was detected by staining with anti-BMI-1 (B). DAPI stain was used to label nuclei.

APPENDIX J: ALLTHE TESTED ASXL2 DELETION/TRUNCATION FRAGMENTS INTERACT WITH CHROMATIN

J.1 Purpose and objective

Based on our preliminary data, we know that ASXL2 (1) is a chromatin associated nuclear protein; (2) regulates PRC2 and (3) PR-DUB activities. However, the structural basis for ASXL2 function is not well understood. ASXL2 contains four conserved domains: ASXL-BOX1, ASX-H, ASXL-BOX2, and PHD domains (Fisher et al., 2006). To gain a better understanding of the function of ASXL2 domains, I tested which domain(s) of ASXL2 is/are necessary for chromatin association.

J.2 Materials and Methods

Full-length pCAG-FLAG-ASXL2 and various ASXL2 deletion/truncations (pCAG-ASXL2(1-390); pCAG-ASXL2(1-660); pCAG-ASXL2(1-720); pCAG-ASXL2(82-1370); pCAG(Δ240-350)) were transiently expressed separately in HEK293 cells. The cells transfected with empty vector were a negative control. Chromatin fractionation was performed to separate each cell lysate into whole cell lysate (WL), chromatin (P3), soluble nuclear (SN) and cytosolic fractions (C). FLAG-ASXL2 fragments were detected by Western blot analysis using anti-rabbit FLAG (1:2000; Sigma).

J.3 Result and conclusion

Western blot analysis shows that full-length ASXL2 and all five ASXL2 deletion/truncation forms were present in the whole cell lysate, and chromatin fractions, suggesting they are associated with chromatin (Fig 24). Negative control lysates did not have detectable proteins, suggesting the protein band in the experimental group is specific. More

deletion/truncation constructs will need to be generated in the future to determine which domain is necessary for chromatin association of ASXL2.

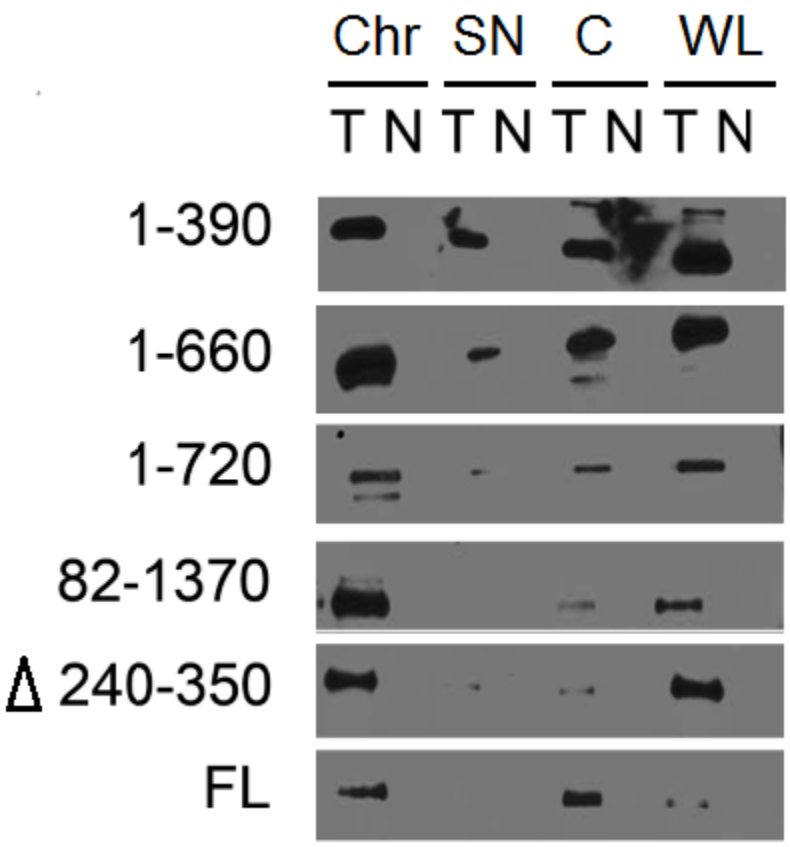

Figure 25: Full-length ASXL2 and various ASXL2 deletion/truncation constructs are predominantly associated with chromatin.

HEK293 cell proteins were separated into four fractions: chromatin (Chr); soluble nuclear proteins (SN), cytosol proteins (C), and whole cell lysate (WL). Western blot analysis of FLAG-ASXL2 protein fragments with anti-FLAG antibody in the four fractions.

APPENDIX K: PRIMERS USED IN UH2A CHIP ASSAY

TABLE IX: PRIMERS USED IN UH2A CHIP ASSAY

Primer	Sequence (5'-3')	Tested and worked
Sfrp2-(+750)F	ACTCCCAAGAAGCCTGAGGT	
Sfrp2-(+750)R	GCAAGGAGAGCTGCCATATC	
Sfrp2-(+418)F	ACGAGACCATGAAGGAGGTG	
Sfrp2-(+418)R	GAAGAGCGAGCACAGGAACT	
Sfrp2-(+1096)F	GCCTCTCTCTGCACGTTCTT	x
Sfrp2-(+1096)F	CACCTGGGGAGTTAAAACCA	x
Grk5-(+976)F	GTGAGTTGGTGCGAGTGGTA	x
Grk5-(+976)R	ACTCGTCCCTGCAACTGTCT	x
Grk5-(+831)F	AATGGAGCTGGAAAACATCG	x
Grk5-(+831)R	CACTCGCACCAACTCACTGT	x
Grk5-(+248)F	GGTTTCGTTTTGGCTGAGTC	x
Grk5-(+248)R	CTCTATTGCACCGGGACTGT	x
Grk5-(+183)F	CTTGTGCTTTCCGCTTTCTC	x
Grk5-(+183)R	GGCAGGAGTCAAAAACGACT	x
bMHC(+494)F	GGGCTACCAGGAAATGATGA	x
bMHC(+494)R	GCAGAATGTCTGCCCTCTTC	x
bMHC(+730)F	CAATGTCACCTTGCTCCTGA	x
bMHC(+730)R	TGGCTCCATGGACAACACTA	x

APPENDIX K (continued)

Primer	Sequence (5’-3’)	Tested and worked
bMHC(+293)F	CCCAAGCCTTGTGGTTAGAG	
bMHC(+293)R	GTGCAAAAGGAGGACCTGAA	
bMHC(+1018)F	GGGGAGGTGGGAACTAAAGA	
bMHC(+1018)R	ACCACGTCCAGCTAAGCACT	
bMHC(+77)F	ATCAGGTGGAGGGTAGGTGA	
bMHC(+77)R	TCCTTCCAGCTCCAAACCTA	
Acta1(+709)F	TGCAGGGGATATCCTGAGAC	
Acta1(+709)R	GCAGCGTGCCTTAATACCTC	
Acta1(+113)F	CCCCCAGGCATATCCTAAAT	
Acta1(+113)R	TTGAGCCTTGGGCTTGTATT	
Acta1(+862)F	TCCGCTTAACCCATCTTCAC	
Acta1(+862)R	AGCACAGCCTTAAGCTGGAA	
Acta1(+1086)F	GCAGCCTGACCTGGTGAC	
Acta1(+1086)R	TTGTGTGTGACAACGGCTCT	

APPENDIX L: GENES THAT ARE DE-REPRESSED BY AT LEAST TWO-FOLD IN ASXL2 MUTANT HEARTS

TABLE IIX: GENES THAT ARE DE-REPRESSED BY AT LEAST TWO-FOLD IN ASXL2 MUTANT HEARTS AS DETERMINED BY MICROARRAY ANALYSIS

GeneID	gene
71911	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)
22628	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
11431	acid phosphatase 1, soluble
11459	actin, alpha 1, skeletal muscle
71985	Acyl-Coenzyme A dehydrogenase family, member 10
77794	ADAMTS-like 2
269959	ADAMTS-like 3
110532	adenosine deaminase, RNA-specific, B1
211673	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)
11569	AE binding protein 2
22589	alpha thalassemia/mental retardation syndrome X-linked homolog (human)
235633	ALS2 C-terminal like
11820	Amyloid beta (A4) precursor protein
70827	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3 (human)
74018	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)
57875	angiopoietin-like 4
68743	anillin, actin binding protein (scraps homolog, Drosophila)
17345	antigen identified by monoclonal antibody Ki 67
11622	aryl-hydrocarbon receptor
66929	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)
23808	ash2 (absent, small, or homeotic)-like (Drosophila)
104112	ATP citrate lyase
70472	ATPase family, AAA domain containing 2
235574	ATPase, Ca++-sequestering
320940	Atpase, class VI, type 11C
76184	ATP-binding cassette, sub-family A (ABC1), member 6
13876	avian erythroblastosis virus E-26 (v-ets) oncogene related
17940	baculoviral IAP repeat-containing 1a
17948	baculoviral IAP repeat-containing 1b
11798	baculoviral IAP repeat-containing 4
11799	baculoviral IAP repeat-containing 5

APPENDIX L (continued)

Gene ID	gene
94043	beta-amyloid binding protein precursor
12064	brain derived neurotrophic factor
12372	calsequestrin 1
12349	carbonic anhydrase 2
12319	carbonic anhydrase 8
55987	carboxypeptidase X 2 (M14 family)
12894	carnitine palmitoyltransferase 1a, liver
13030	cathepsin B
235505	CD109 antigen
12484	CD24a antigen
68916	CDK5 regulatory subunit associated protein 1-like 1
105278	cell cycle related kinase
12534	cell division cycle 2 homolog A (S. pombe)
107995	cell division cycle 20 homolog (S. cerevisiae)
52276	cell division cycle associated 8
216991	centaurin, alpha 2
12585	cerebellar degeneration-related 2
12870	ceruloplasmin
12772	chemokine (C-C motif) receptor 2
13051	chemokine (C-X3-C) receptor 1
57266	chemokine (C-X-C motif) ligand 14
57349	chemokine (C-X-C motif) ligand 7
224796	chloride intracellular channel 5
13004	chondroitin sulfate proteoglycan 3
12667	chordin
107932	chromodomain helicase DNA binding protein 4
67337	cleavage stimulation factor, 3' pre-RNA, subunit 1
66983	coiled-coil domain containing 16
235415	complexin 3
194231	connector enhancer of kinase suppressor of Ras 1
69274	CTD (carboxy-terminal domain, RNA polymerase II polypeptide A) small phosphatase-like
23845	c-type lectin domain family 5, member a
74322	CXXC finger 1 (PHD domain)
12428	cyclin A2
12576	cyclin-dependent kinase inhibitor 1B (P27)
50766	cysteine-rich motor neuron 1
230459	cytochrome P450, family 2, subfamily j, polypeptide 1

APPENDIX L (continued)

Gene ID	gene
74519	cytochrome P450, family 2, subfamily j, polypeptide 9
70101	cytochrome P450, family 4, subfamily f, polypeptide 16
12877	cytoplasmic polyadenylation element binding protein 1
80986	cytoskeleton associated protein 2
212880	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46
13388	delta-like 1 (Drosophila)
14357	deltex 1 homolog (Drosophila)
13356	diGeorge syndrome critical region gene 2
66369	dihydrouridine synthase 2-like (SMM1, S. cerevisiae)
13482	dipeptidylpeptidase 4
80915	dual specificity phosphatase 12
13429	dynamin 1
13591	early B-cell factor 1
93685	ectonucleoside triphosphate diphosphohydrolase 7
230316	EGF-like-domain, multiple 5
13639	ephrin A4
13642	ephrin B2
77781	EPM2A (laforin) interacting protein 1
71889	epsin 3
192193	ER degradation enhancer, mannosidase alpha-like 1
269587	erythrocyte protein band 4.1
170812	erythroid associated factor
26380	estrogen related receptor, beta
239528	eukaryotic translation initiation factor 2C, 2
116701	fibroblast growth factor receptor-like 1
20378	frizzled-related protein
56095	FtsJ homolog 3 (E. coli)
73068	fucosyltransferase 11
233079	G protein-coupled receptor 43
14773	G protein-coupled receptor kinase 5
118454	gap junction membrane channel protein alpha 12
233863	general transcription factor III C 1
23885	germ cell-less homolog (Drosophila)
384009	GLI pathogenesis-related 2
14585	glial cell line derived neurotrophic factor family receptor alpha 1
21847	kruppel-like factor 10
14809	glutamate receptor, ionotropic, kainate 5 (gamma 2)

APPENDIX L (continued)

Gene ID	gene
14809	glutamate receptor, ionotropic, kainate 5 (gamma 2)
76282	glutamic pyruvic transaminase 1, soluble
14594	glycoprotein galactosyltransferase alpha 1, 3
94221	golgi associated PDZ and coiled-coil motif containing
13197	growth arrest and DNA-damage-inducible 45 alpha
210710	growth factor receptor bound protein 2-associated protein 3
14544	guanine deaminase
14695	guanine nucleotide binding protein, beta 3
70676	GULP, engulfment adaptor PTB domain containing 1
56422	Hbs1-like (S. cerevisiae)
233490	HCF-binding transcription factor Zhangfei
15482	heat shock protein 1-like
23908	Heparan sulfate 2-O-sulfotransferase 1
171285	hepatitis A virus cellular receptor 2
50926	heterogeneous nuclear ribonucleoprotein D-like
433785 /// 433788	high mobility group box 2 /// similar to high mobility group protein B2
15466	histamine receptor H 2
15115	histidyl-tRNA synthetase
14950	histocompatibility 13
319165	histone 1, H2ad /// CDNA clone MGC:103288 IMAGE:5150365, complete cds
79221	histone deacetylase 9
27281	HRAS-like suppressor
330790	hyaluronan and proteoglycan link protein 4
15366	hyaluronan mediated motility receptor (RHAMM)
15586 /// 56441	hyaluronidase 1 /// N-acetyltransferase 6
15483	hydroxysteroid 11-beta dehydrogenase 1
16324	inhibin beta-B
16150	inhibitor of kappaB kinase beta
16319	inner centromere protein
233011	inositol 1,4,5-trisphosphate 3-kinase C
223272	integrin, beta-like 1
57444	interferon-stimulated protein
16477	Jun-B oncogene
19348	kinesin family member 20A
16570	kinesin family member 3C
67557	la ribonucleoprotein domain family, member 6
71835	lanC (bacterial lantibiotic synthetase component C)-like 2

APPENDIX L (continued)

Gene ID	gene
93730	leucine zipper transcription factor-like 1
329252	leucine-rich repeat-containing G protein-coupled receptor 6
74201	leucine-rich repeats and IQ motif containing 2
16840	leukocyte cell derived chemotaxin 1
16881	ligase I, DNA, ATP-dependent
16826	LIM domain binding 2
69605	limb and neural patterns
64899	lipin 3
100702	macrophage activation 2 like
54484	makorin, ring finger protein, 1
17389	matrix metalloproteinase 16
104362	meiosis expressed gene 1
17765	metal response element binding transcription factor 2
17768	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase
216760	microfibrillar-associated protein 3
171580	microtubule associated monoxygenase, calponin and LIM domain containing 1
225164	mindbomb homolog 1 (Drosophila)
17215	minichromosome maintenance deficient 3 (S. cerevisiae)
17218	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)
17219	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)
26400	mitogen activated protein kinase kinase 7
17344	Msx-interacting-zinc finger
76626	Musashi homolog 2 (Drosophila)
27418	Muskelin 1, intracellular mediator containing kelch motifs
17153	myelin and lymphocyte protein, T-cell differentiation protein
233199	myosin binding protein C, fast-type
74376	myosin XVIIIb
18104	NAD(P)H dehydrogenase, quinone 1
234258	nei like 3 (E. coli)
235627	neurobeachin-like 2
74513	neuropilin (NRP) and tolloid (TLL)-like 2
74455	NOL1/NOP2/Sun domain family 6
108907	nucleolar and spindle associated protein 1
67528	nudix (nucleoside diphosphate linked moiety X)-type motif 7
68767	open reading frame 19
231805	paired immunoglobin-like type 2 receptor alpha

APPENDIX L (continued)

Gene ID	gene
18933	paired related homeobox 1
19228	parathyroid hormone receptor 1
18771	Pbx/knotted 1 homeobox
56376	PDZ and LIM domain 5
170761	PDZ domain containing 2
237504	peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor
75725	PHD finger protein 14
74769	phosphatidylinositol 3-kinase, catalytic, beta polypeptide
18578	phosphodiesterase 4B, cAMP specific
107272	phosphoserine aminotransferase 1
18792	plasminogen activator, urokinase
71785	platelet-derived growth factor, D polypeptide
67448	plexin domain containing 2
20873	polo-like kinase 4 (Drosophila)
218832	polymerase (RNA) III (DNA directed) polypeptide A
19285	polymerase I and transcript release factor
16526	potassium channel, subfamily K, member 2
16513	potassium inwardly-rectifying channel, subfamily J, member 10
211480	potassium inwardly-rectifying channel, subfamily J, member 14
70673	PR domain containing 16
18514	Pre B-cell leukemia transcription factor 1
12828	procollagen, type IV, alpha 3
12837	procollagen, type VIII, alpha 1
12817	procollagen, type XIII, alpha 1
12818	procollagen, type XIV, alpha 1
67505	prolactin like protein O
19200	proline-serine-threonine phosphatase-interacting protein 1
19183	proteasome (prosome, macropain) 26S subunit, ATPase 3, interacting protein
80708	protein kinase C and casein kinase substrate in neurons 3
19106	protein kinase, interferon-inducible double stranded RNA dependent
69847	protein kinase, lysine deficient 4
19055	protein phosphatase 3, catalytic subunit, alpha isoform
233406	protein regulator of cytokinesis 1
19263	protein tyrosine phosphatase, receptor type, B
19268	protein tyrosine phosphatase, receptor type, F
53601	protocadherin 12
14083	PTK2 protein tyrosine kinase 2
18546	Purkinje cell protein 4

APPENDIX L (continued)

Gene ID	gene
26934	Rac GTPase-activating protein 1
19362	RAD51 associated protein 1
19361	RAD51 homolog (S. cerevisiae)
58185	radical S-adenosyl methionine domain containing 2
192786	Rap guanine nucleotide exchange factor (GEF) 6
51869	Rap1 interacting factor 1 homolog (yeast)
19883	RAR-related orphan receptor alpha
192656	receptor (TNFRSF)-interacting serine-threonine kinase 2
19724	regulatory factor X, 1 (influences HLA class II expression)
214742	REST corepressor 3
65079	reticulon 4 receptor
19889	retinitis pigmentosa 2 homolog (human)
328365	retinoic acid induced 17
105014	retinol dehydrogenase 14 (all-trans and 9-cis)
103142	retinol dehydrogenase 9
75415	Rho GTPase activating protein 12
19819	ribonuclease H1
382985	ribonucleotide reductase M2 B (TP53 inducible)
20088	ribosomal protein S24
110651	ribosomal protein S6 kinase polypeptide 3
30054	ring finger protein 17
19881	rod outer segment membrane protein 1
230257	ROD1 regulator of differentiation 1 (S. pombe)
229675	rosbin, round spermatid basic protein 1
20202	S100 calcium binding protein A9 (calgranulin B)
83997	sarcolemma associated protein
20319	secreted frizzled-related sequence protein 2
22287	secretoglobin, family 1A, member 1 (uteroglobin)
56747	seizure related 6 homolog (mouse)-like
233878	seizure related 6 homolog (mouse)-like 2
20347	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
20360	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C
214968	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D
18787	serine (or cysteine) proteinase inhibitor, clade E, member 1
20817	serine/arginine-rich protein specific kinase 2
20437 /// 20438	seven in absentia 1A /// seven in absentia 1B

APPENDIX L (continued)

Gene ID	gene
269016	SH3 domain containing ring finger 2
59009	SH3 multiple domains 2
20419	Shc SH2-domain binding protein 1
20612	sialoadhesin
272713	Similar to development- and differentiation-enhancing factor 2; PYK2 C terminus-associated protein
546041	similar to p47 protein isoform a
384254	similar to ribosomal protein L18a
20292	small chemokine (C-C motif) ligand 11
30927	snail homolog 3 (Drosophila)
399548	sodium channel, type IV, beta
65221	solute carrier family 15, member 3
105355	solute carrier family 17 (sodium phosphate), member 3
20515	solute carrier family 20, member 1
67712	solute carrier family 25, member 37
215085	solute carrier family 35, member F1
106957	solute carrier family 39 (metal ion transporter), member 6
20533	solute carrier family 4 (anion exchanger), member 1
20537	solute carrier family 5 (sodium/glucose cotransporter), member 1
20658	son cell proliferation protein
20662	son of sevenless homolog 1 (Drosophila)
20411	sorbin and SH3 domain containing 1
109552	sorcin
20660	sortilin-related receptor, LDLR class A repeats-containing
432572	spectrin domain with coiled-coils 1
66234	sterol-C4-methyl oxidase-like
67902	sulfatase modifying factor 2
20975	synaptojanin 2
54525	synaptotagmin 7
269397	synovial sarcoma translocation gene on chromosome 18-like 1
268996	synovial sarcoma translocation, Chromosome 18
20617	synuclein, alpha
230908	TAR DNA binding protein
21923	tenascin C
217449	tetratricopeptide repeat domain 15
73666	THO complex 3
58916	titin immunoglobulin domain protein (myotilin)
21899	toll-like receptor 6

APPENDIX L (continued)

Gene ID	gene
21973	topoisomerase (DNA) II alpha
21974	topoisomerase (DNA) II beta
21419	transcription factor AP-2 beta
81004	transducin (beta)-like 1X-linked receptor 1
67226	transmembrane protein 19
224090	transmembrane protein 44
80890	tripartite motif protein 2
217069	tripartite motif protein 25
94089	tripartite motif protein 7
22004	tropomyosin 2, beta
21953	troponin I, skeletal, fast 2
22035	tumor necrosis factor (ligand) superfamily, member 1
79202	tumor necrosis factor receptor superfamily, member 22
22222	ubiquitin protein ligase E3 component n-recognin 1
230484	ubiquitin specific protease 1
319651	ubiquitin specific protease 37
252870	ubiquitin specific protease 7
140499	ubiquitin-conjugating enzyme E2, J2 homolog (yeast)
68612	ubiquitin-conjugating enzyme E2C
56791	ubiquitin-conjugating enzyme E2L 6
18140	ubiquitin-like, containing PHD and RING finger domains, 1
320011	UDP-glucose ceramide glucosyltransferase-like 1
22229	uncoupling protein 3 (mitochondrial, proton carrier)
171530	urocortin 2
22310 /// 22311	vomeronasal 2, receptor, 4 /// vomeronasal 2, receptor, 5
73674	WD repeat domain 75
22376	Wiskott-Aldrich syndrome homolog (human)
229055	zinc finger and BTB domain containing 10
268294	zinc finger and BTB domain containing 24
16969	zinc finger and BTB domain containing 7a
106205	zinc finger CCCH type containing 7
22697	zinc finger proliferation 1
22666	zinc finger protein 161
193452	zinc finger protein 184 (Kruppel-like)
101095	zinc finger protein 282
68910	zinc finger protein 467
218820	zinc finger protein 503

APPENDIX L (continued)

Gene ID	gene
22775	zinc finger protein interacting with K protein 1
22764	zinc finger protein X-linked
224454	zinc finger, DHHC domain containing 14
75965	zinc finger, DHHC domain containing 20

APPENDIX M: GENES THAT ARE REPRESSED BY AT LEAST TWO-FOLD IN ASXL2 MUTANT HEARTS

TABLE IIIX: GENES THAT ARE REPRESSED BY AT LEAST TWO-FOLD IN ASXL2 MUTANT HEARTS AS DETERMINED BY MICROARRAY

GeneID	gene
246729	2'-5' oligoadenylate synthetase 1H
246728	2'-5' oligoadenylate synthetase 2
18640	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
13522	a disintegrin and metalloprotease domain 28
11475	actin, alpha 2, smooth muscle, aorta
11474	actinin alpha 3
216739	acyl-CoA synthetase long-chain family member 6
75302	additional sex combs like 2 (Drosophila)
11450	adiponectin, C1Q and collagen domain containing
11537	adipsin
218639	ADP-ribosylation factor related protein 2
11875	ADP-ribosyltransferase 5
14266	AF4/FMR2 family, member 2
93736	AF4/FMR2 family, member 4
211064	alkB, alkylation repair homolog (E. coli)
232345	alpha-2-macroglobulin
23923	aminoadipate aminotransferase
72823	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 19
11600	angiopoietin 1
70008	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2
70797	ankyrin repeat and IBR domain containing 1
208117	anterior pharynx defective 1b homolog (C. elegans)
110542	anti-Mullerian hormone type 2 receptor
11800	apoptosis inhibitor 5
11829	aquaporin 4
11831	aquaporin 6
70882	armadillo repeat containing 3
11899	astrotactin 1
72174	ataxin 7-like 4
11946	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1
50771	ATPas, class II, type 9B
70472	ATPase family, AAA domain containing 2
74772	ATPase type 13A2

APPENDIX M (continued)

GeneID	gene
109880	braf transforming gene
108100	brain-specific angiogenesis inhibitor 1-associated protein 2
76960	breast carcinoma amplified sequence 1
192197	breast carcinoma amplified sequence 3
76809	bri3 binding protein
22385	bromodomain adjacent to zinc finger domain, 1B
12300	calcium channel, voltage-dependent, gamma subunit 2
12288	calcium channel, voltage-dependent, L type, alpha 1C subunit
227541	calcium/calmodulin-dependent protein kinase ID
12374	calcium-sensing receptor
442829	calicin
231991	cAMP responsive element binding protein 5
12350	carbonic anhydrase 3
104158	carboxylesterase 3
17079	CD180 antigen
12478	CD19 antigen
171486	Cd99 antigen-like 2
229776	CDC14 cell division cycle 14 homolog A (S. cerevisiae)
14311	cell death-inducing DFFA-like effector c
212285	centaurin, delta 1
108000	centromere autoantigen F
12622	cerberus 1 homolog (Xenopus laevis)
12405	cerebellin 2 precursor protein
105513	CHMP family, member 7
75677	claudin 22
12739	claudin 3
66098	coiled-coil-helix-coiled-coil-helix domain containing 6
320924	collagen and calcium binding EGF domains 1
14219	connective tissue growth factor
21367	contactin 2
18488	contactin 3
12808	cordon-bleu
224912	crumbs homolog 3 (Drosophila)
12965 /// 12966	crystallin, gamma B /// crystallin, gamma C
51811	c-type lectin domain family 4, member f
74100	cyclic AMP-regulated phosphoprotein, 21

APPENDIX M (continued)

GeneID	gene
51813	cyclin C
94219	cyclin M2
13008	cysteine and glycine-rich protein 2
12583	cysteine dioxygenase 1, cytosolic
13075	cytochrome P450, family 19, subfamily a, polypeptide 1
13106	cytochrome P450, family 2, subfamily e, polypeptide 1
231162	cytokine like 1
229459	Dachsous 2 (Drosophila)
66573	DAZ interacting protein 1
74351	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23
66705	deoxyribonuclease 1-like 2
13367	diaphanous homolog 1 (Drosophila)
50781	dickkopf homolog 3 (Xenopus laevis)
12305	discoidin domain receptor family, member 1
330938	DIX domain containing 1
13434	DNA methyltransferase 2
73284	DNA-damage-inducible transcript 4-like
13447	double C2, beta
13175	double cortin and calcium/calmodulin-dependent protein kinase-like 1
216164	downstream of Stk11
18218	dual specificity phosphatase 8
13656	early growth response 4
67868	Elastase 3B, pancreatic
106389	ELL associated factor 2
269344	elongation factor RNA polymerase II-like 3
13796	empty spiracles homolog 1 (Drosophila)
13797	empty spiracles homolog 2 (Drosophila)
107522	endothelin converting enzyme 2
13839	Eph receptor A5
18612	Ets variant gene 4 (E1A enhancer binding protein, E1AF)
13664 /// 266459	eukaryotic translation initiation factor 1A /// similar to eukaryotic translation initiation factor 1A (eIF-1A) (eIF-4C)

APPENDIX M (continued)

GeneID	gene
230861	eukaryotic translation initiation factor 4 gamma, 3
13684	eukaryotic translation initiation factor 4E
14050	eyes absent 3 homolog (Drosophila)
14104	fatty acid synthase
314322	FBJ murine osteosarcoma viral oncogene homolog
57443	F-box only protein 3
14119	fibrillin 2
14167	fibroblast growth factor 12
14169	fibroblast growth factor 14
14264	fibromodulin
15227	forkhead box F1a
114142	forkhead box P2
14221	four jointed box 1 (Drosophila)
14266	fragile X mental retardation 2 homolog
14352	friend virus susceptibility 4
57265	frizzled homolog 2 (Drosophila)
67391	FUN14 domain containing 2
213054	GA repeat binding protein, beta 2
14403	Gamma-aminobutyric acid (GABA-A) receptor, subunit delta
14611	gap junction membrane channel protein alpha 3
14617	gap junction membrane channel protein alpha 9
66790	GH regulated TBC protein 1
14537	glucosaminyl (N-acetyl) transferase 1, core 2
14538	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme
14810	glutamate receptor, ionotropic, NMDA1 (zeta 1)
14583	glutamine fructose-6-phosphate transaminase 1
68312	glutathione S-transferase, mu 7
78926	growth arrest-specific 2 like 1
14559 /// 93898	growth differentiation factor 1 /// longevity assurance homolog 1 (S. cerevisiae)
227960	grancalcin

APPENDIX M (continued)

GeneID	gene
14702	guanine nucleotide binding protein (G protein), gamma 2 subunit
14709	guanine nucleotide binding protein (G protein), gamma 8 subunit
14955	H19 fetal liver mRNA
15439	haptoglobin
26386	heat shock transcription factor 4
59026	HECT, UBA and WWE domain containing 1
84506	hepcidin antimicrobial peptide 1
15388	heterogeneous nuclear ribonucleoprotein L
14950	histocompatibility 13
14968	histocompatibility 2, class II antigen E alpha
68024	histone 1, H2bc
15394	homeo box A1
15424	homeo box C5
75828	HORMA domain containing 2
330723	HtrA serine peptidase 4
53323	huntingtin interacting protein 2
77042	hyaluronoglucosaminidase 4
18518	immunoglobulin (CD79A) binding protein 1
16061	immunoglobulin heavy chain (J558 family)
16017	immunoglobulin heavy chain 4 (serum IgG1)
16071	immunoglobulin kappa chain, constant region
16142	immunoglobulin lambda chain, variable 1
209268	immunoglobulin superfamily, member 1
54725	immunoglobulin superfamily, member 4A
27993	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)
16438	Inositol 1,4,5-triphosphate receptor 1
75426	insulin-like growth factor binding protein-like 1
16398	integrin alpha 2
270110	interferon regulatory factor 2 binding protein 2
66845	mitochondrial ribosomal protein L33

APPENDIX M (continued)

GeneID	gene
16190	interleukin 4 receptor, alpha
75605	jumonji, AT rich interactive domain 1B (Rbp2 like)
57340	junctophilin 3
63830	KCNQ1 overlapping transcript 1
16673	keratin complex 1, acidic, gene 5
110308	keratin complex 2, basic, gene 5
16578	kinesin family member 9
84035	kringle containing transmembrane protein 1
237339	l(3)mbt-like 3 (Drosophila)
79235	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)
70361	lectin, mannose-binding, 1
76612	leucine rich repeat containing 27
216028	leucine rich repeat transmembrane neuronal 3
329252	leucine-rich repeat-containing G protein-coupled receptor 6
16871	LIM homeobox protein 3
16876	LIM homeobox protein 9
68311	ly6/Plaur domain containing 2
23934	lymphocyte antigen 6 complex, locus H
16842	lymphoid enhancer binding factor 1
15064	major histocompatibility complex, class I-related
320772	MAM domain containing 1
17174	mannan-binding lectin serine protease 1
17160	mannosidase 2, alpha B2
17184	matrin 3
17387	matrix metalloproteinase 14 (membrane-inserted)
17122	max dimerization protein 4
56524	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)
170813	membrane-spanning 4-domains, subfamily A, member 3
17752	metallothionein 4
17150	microfibrillar-associated protein 2
71306	microfibrillar-associated protein 3-like
328329	microtubule associated serine/threonine kinase family member 4
17318	midline 1
60441	mitochondrial ribosomal protein L38

APPENDIX M (continued)

Gene ID	gene
23939	mitogen activated protein kinase 7
60597	mitogen-activated protein kinase 8 interacting protein 2
83456	moloney leukemia virus 10-like 1
109731	monoamine oxidase B
67973	m-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
67014	myc induced nuclear antigen
17536	myeloid ecotropic viral integration site-related gene 1
17260	myocyte enhancer factor 2C
17918	myosin Va
17901	myosin, light polypeptide 1
17896	myosin, light polypeptide 4
17898	myosin, light polypeptide 7, regulatory
98932	myosin, light polypeptide 9, regulatory
217214	N-acetylglutamate synthase
230899	natriuretic peptide precursor type A
53885	nephronophthisis 1 (juvenile) homolog (human)
57764	netrin 4
80883	netrin G1
18011	neuralized-like homolog (Drosophila)
235627	neurobeachin-like 2
320840	neuronal growth regulator 1
18211	neurotrophic tyrosine kinase, receptor, type 1
18188	neurturin
66866	NHL repeat containing 2
18092	NK2 transcription factor related, locus 6 (Drosophila)
18124	nuclear receptor subfamily 4, group A, member 3
18226	nucleoporin 62
218121	o-acyltransferase (membrane bound) domain containing 1
18300	oncoprotein induced transcript 1
15379	one cut domain, family member 1
56374	open reading frame 18
80909	opposite strand transcription unit to Stag3
330962	organic solute transporter beta
70061	orphan short chain dehydrogenase/reductase
105689	pam, highwire, rpm 1
93742	Par-3 (partitioning defective 3) homolog (C. elegans)
53318	PDZ and LIM domain 3
18599	peptidyl arginine deiminase, type I
19132	peripherin 1
19679	phosphatidylinositol transfer protein, membrane-associated 2

APPENDIX M (continued)

Gene ID	gene
29863	phosphodiesterase 7B
227120	phospholipase C-like 1
110094	phosphorylase kinase alpha 2
56460	plakophilin 3
68797	platelet-derived growth factor receptor-like
215632	pleckstrin and Sec7 domain containing 4
231999	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8
211945	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1
378460	PML-RAR alpha-regulated adaptor molecule 1
319655	podocalyxin-like 2
16519	potassium inwardly-rectifying channel, subfamily J, member 3
16509	potassium voltage-gated channel, Isk-related subfamily, member 1
16508	potassium voltage-gated channel, Shal-related family, member 2
211468	potassium voltage-gated channel, subfamily H (eag-related), member 8
64243	Prader-Willi chromosome region 1 homolog (human)
237759	procollagen, type XXIII, alpha 1
74229	progestin and adipoQ receptor family member VIII
170952	proline rich membrane anchor 1
65116	proline-rich Gla (G-carboxyglutamic acid) polypeptide 2
19220	prostaglandin F receptor
67151	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
19088	protein kinase, cAMP dependent regulatory, type II beta
19091	protein kinase, cGMP-dependent, type I
320472	protein phosphatase 1E (PP2C domain containing)
68507	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4
211712	protocadherin 9
93886	protocadherin beta 15
230596	PRP38 pre-mRNA processing factor 38 (yeast) domain containing A
101631	PWWP domain containing 2
18770	pyruvate kinase liver and red blood cell
29809	RAB GTPase activating protein 1-like
98710	RAB interacting factor
227746	rab9 effector protein with kelch motifs
114714	rad51 homolog c (S. cerevisiae)

APPENDIX M (continued)

Gene ID	gene
78255	ral GEF with PH domain and SH3 binding motif 2
19765	ralA binding protein 1
320292	RasGEF domain family, member 1B
51801	receptor (calcitonin) activity modifying protein 1
19715	reduced expression 2
19716	reduced expression 3
51791	regulator of G-protein signaling 14
50779	regulator of G-protein signaling 6
243923	regulator of G-protein signalling 9 binding protein
328280	regulator of sex-limitation candidate 24
57262	resistin like alpha
104001	reticulon 1
19662	retinol binding protein 4, plasma
171207	Rho GTPase activating protein 4
19989	ribosomal protein L7
68925	RNA polymerase II associated protein 1
20191	ryanodine receptor 2, cardiac
66402	sarcolipin
140740	SEC63-like (S. cerevisiae)
20338	Sel1 (suppressor of lin-12) 1 homolog (C. elegans)
20350	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3 F
20700 /// 20701/3/4 /// 544889	serine (or cysteine) proteinase inhibitor, clade A, member 1a /// 1b /// 1d /// 1e /// similar to Serpina1a protein
12406	serine (or cysteine) proteinase inhibitor, clade H, member 1
56726	SH3-binding domain glutamic acid-rich protein like
20404	SH3-domain GRB2-like 2
237979	sidekick homolog 2 (chicken)
107513	signal sequence receptor, alpha
432700	similar to Ig heavy chain V-III region J606
546611	similar to kelch-like 9
435350	similar to NK13
546020	similar to secreted gel-forming mucin
20473	sine oculis-related homeobox 3 homolog (Drosophila)
75345	SLAM family member 7
20563	slit homolog 2 (Drosophila)
20365	small EDRK-rich factor 1
20269	sodium channel, voltage-gated, type III, alpha
20493	solute carrier family 10 (sodium/bile acid cotransporter family), member 1
56643	solute carrier family 15 (oligopeptide transporter), member 1

APPENDIX M (continued)

Gene ID	gene
20519	solute carrier family 22 (organic cation transporter), member 3
22232	solute carrier family 35 (UDP-galactose transporter), member 2
58246	solute carrier family 35, member B4
53945	solute carrier family 40 (iron-regulated transporter), member 1
246787	solute carrier family 5 (sodium/glucose cotransporter), member 2
230612	solute carrier family 5 (sodium/glucose cotransporter), member 9
20541	solute carrier family 8 (sodium/calcium exchanger), member 1
226999	solute carrier family 9 (sodium/hydrogen exchanger), member 2
77031	solute carrier family 9 (sodium/hydrogen exchanger), member 8
69024	sorting nexin 15
75469	spermatogenesis associated 19
57815	spermatogenesis associated 5
27401	s-phase kinase-associated protein 2 (p45)
56632	sphingosine kinase 2
278240	spindlin-like
114715	sprouty protein with EVH-1 domain 1, related sequence
20775	squalene epoxidase
20669	SRY-box containing gene 14
20680	SRY-box containing gene 7
20447	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3
56018	START domain containing 10
20249	stearoyl-Coenzyme A desaturase 1
74480	sterile alpha motif domain containing 4
78925	steroid 5 alpha-reductase 1
20655	superoxide dismutase 1, soluble
93762	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
214804	synapse defective 1, Rho GTPase, homolog 2 (C. elegans)
64176	synaptic vesicle glycoprotein 2 b
52440	Tax1 (human T-cell leukemia virus type I) binding protein 1
83993	T-box 19
57246	T-box 20
319939	tensin 3
60600	testis specific gene A8
223431	TGF-beta1-induced anti-apoptotic factor 2

APPENDIX M (continued)

Gene ID	gene
223431	TGF-beta1-induced anti-apoptotic factor 2
234723	thioredoxin-like 4B
21834	thyroid hormone receptor beta
76367	TP53 regulating kinase
69014	TRAF2 and NCK interacting kinase
21419	transcription factor AP-2 beta
252973	transcription factor CP2-like 3
57259	transducer of ERBB2, 2
21372	transducin (beta)-like 1 X-linked
22059	transformation related protein 53
21808	transforming growth factor, beta 2
331046	transglutaminase 4 (prostate)
67564	transmembrane protein 35
235135	transmembrane protein 45b
227331	trinucleotide repeat containing 15
229644	tripartite motif-containing 45
67525	tripartite motif-containing 62
50876	tropomodulin 2
319953	tubulin tyrosine ligase-like 1
67534	tubulin tyrosine ligase-like family, member 4
233276	tubulin, gamma complex associated protein 5
24099	tumor necrosis factor (ligand) superfamily, member 13b
21939	tumor necrosis factor receptor superfamily, member 5
22070	tumor protein, translationally-controlled 1
13345	twist homolog 2 (Drosophila)
234724	tyrosine aminotransferase
83813	tyrosine kinase, non-receptor, 1
93961	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
14425	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
22227	uncoupling protein 1 (mitochondrial, proton carrier)
22256	Uracil-DNA glycosylase
103149	ureidopropionase, beta
338362	Uronyl-2-sulfotransferase
22268	uroplakin 1B
100647	uroplakin 3B
22283	Usher syndrome 2A (autosomal recessive, mild) homolog (human)
195434	UTP14, U3 small nucleolar ribonucleoprotein, homolog B (yeast)
212190	UBX domain containing 3

APPENDIX M (continued)

Gene ID	gene
74199	vitrin
83767	WASP family 1
22408	wingless-related MMTV integration site 1
22416	Wingless-related MMTV integration site 3A
24117	Wnt inhibitory factor 1
22402	WNT1 inducible signaling pathway protein 1
74254	XPA binding protein 1
235320	zinc finger and BTB domain containing 16
75580	zinc finger and BTB domain containing 4
16969	zinc finger and BTB domain containing 7a
56869	zinc finger protein 109
57908	zinc finger protein 318
328977	zinc finger protein 532
241494	zinc finger protein 533
233887	zinc finger protein 553
69234	zinc finger protein 688
213436	zinc finger, CCHC domain containing 5
433204 /// 66980	zinc finger, DHHC domain containing 6 /// similar to Zinc finger DHHC domain containing protein 6 (H4 homolog)
53604	zona pellucida binding protein
80292	ZXD family zinc finger C

CITED LITERATURE

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PUBLICATIONS

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PRESENTATIONS

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