Identification and Characterization of the Protein Interaction between ASXL2 and WTIP

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy in Biological Sciences
in the Graduate College of the
University of Illinois at Chicago, 2014

Chicago, Illinois

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This thesis is dedicated to my family.

Without their constant encouragement and support,

this journey would not have been possible.

ACKNOWLEDGEMENTS

I would like to thank my PhD advisor, Dr. Qun-Tian Wang, for giving me the opportunity to pursue research in her lab. I feel very fortunate to have had such an extraordinary person as my mentor. She has taught me how to think independently, question everything, strive for the best, and constantly push my limits. She always saw potential in me that I sometimes failed to see myself. I thank her sincerely for always believing in me and constantly helping me fulfill my goals.

I am also grateful to my thesis committee members, Dr. Okkema, Dr. Kay, Dr. Orenic, and Dr. Tyner, for their continuous support and scientific advice. Their refreshing insights and unique perspectives helped me advance my research in the right direction.

The Wang lab has been like my second home these last several years. I would like to thank the following current and former lab members: Heather, Rima, Rachel, Yanyang, Zana, Annie, Maithy, and especially Hsiao-Lei and Andrea for all their support throughout this journey.

Many other labs in the MCDB department have helped me in one way or another in my graduate studies. I thank the Liebman lab for their help with all my yeast experiments, Schmidt lab and Orenic lab for always sharing equipment, reagents, and advice, and Kay lab for helping with antibodies and troubleshooting. I also want to thank Arjun for helping me with experiments while working in our lab. In addition, Judith, Omar and Corinna have always made sure that everything is running smoothly in our department and I thank them for all their hard work.

Lastly, I want to thank my family for their unconditional love, unwavering support, and incredible patience. My parents, Nasir Khan and Mehtab Khatoon, have been my pillars of support my entire life, but especially during my graduate studies. I want to thank them for making so many sacrifices for me and I hope that I will always make them proud. My husband Sobaan, has patiently been by my side through the ups and downs of graduate school. I want to thank him for helping me stay sane amidst all the chaos. I want to thank my babies, Rayyan and Gibran, for always giving me something to look forward to each and every day. I want to thank my brothers Ayub (Bhaiya) and Asif and my sister Farah (Baji) for helping me become the person that I am today. Without the love and support of my entire family, my dream would never have become a reality.

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LIST OF ABBREVIATIONS

AD	activation domain
Asx	Additional sex combs
ASXH	Asx-homology
ASXL	Additional sex combs-like
ATRA	*all trans*-retinoic acid
β-gal	beta-galactosidase
°C	degree Celsius
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle's Medium
E	Embryonic day
EMT	epithelial-to-mesenchymal transition
FBS	fetal bovine serum
GST	Glutathione S-transferase
H3K27me3	trimethylated histone H3 lysine 27
HARE-HTH	HARE helix-turn-helix
mm	millimeter
ng	nanogram
NR	nuclear receptor
OD	optical density
PBS	Phosphate-buffered saline
PcG	Polycomb group

LIST OF ABBREVIATIONS (continued)

PHD	plant homeodomain
Pho-RC	Pho-repressive complex
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PR-DUB	Polycomb repressive deubiquitinase
QDO	quadruple dropout media
RA	retinoic acid
RAR	retinoic acid receptor
RARE	Retinoic acid response element
rpm	rotations per minute
RT-PCR	reverse transcription polymerase chain reaction
SD/-	yeast synthetic minimal media lacking
TE-LiCl	Tris-EDTA lithium chloride
TF	transcription factor
TrxG	Trithorax group
ug	microgram
uH2A	mono-ubiquitinated histone H2A
ul	microliter
WT1	Wilms tumor 1
WTIP	Wilms tumor 1-interacting protein
YPDA	yeast peptone dextrose adenine

SUMMARY

Epigenetics refers to processes that result in heritable changes to gene expression patterns that do not require changes to the DNA sequence itself. These processes are critical during the development of an organism and have a major impact on disease development. One form of epigenetic regulation involves histone modifications. Polycomb Group (PcG) and Trithorax Group (TrxG) proteins are epigenetic regulators that regulate chromatin architecture through histone modifications. PcG and TrxG proteins form multi-protein complexes and have opposite effects on chromatin. PcG-mediated histone modifications result in a condensed or transcriptionally inactive chromatin. On the other hand, TrxG-mediated histone modifications result in an open or transcriptionally active state.

ASXL2 is an epigenetic regulator belonging to the Enhancers of Trithorax and Polycomb (ETP) group. ETP proteins play dual roles in transcriptional activation and repression. Our lab has developed a mutant mouse model for the study of ASXL2. Our research shows that ASXL2 is highly expressed in the heart, is required for normal cardiovascular development, and required for proper heart function. In order to understand the molecular mechanisms underlying ASXL2's functions in the heart, I have undertaken a study to identify ASXL2 protein partners and the functional implications of those protein interactions.

Herein, I present the identification and functional characterization of the protein interaction between ASXL2 and the ZYXIN family protein WTIP. ASXL2 and WTIP show direct interaction and are involved in the regulation of RA signaling. We propose that this regulation may play a role in the epicardium during heart development.

1. GENERAL INTRODUCTION

1.1 Epigenetics in development and disease

Epigenetic regulation in general refers to stable and heritable changes to gene expression patterns that do not involve changes to the DNA sequence itself (Goldberg et al. 2007). These changes are mediated mainly by three important mechanisms: DNA methylation, histone modifications, and non-coding RNA-based mechanisms (Handy et al. 2011). The most well-studied epigenetic modification is DNA methylation. In mammalian cells, DNA methylation occurs mainly at the cytosine residues of CpG islands, areas of the genome with high numbers of CpG dinucleotides, via DNA methyltransferases (Jaenisch and Bird 2003). Methylation of these islands is linked to the maintenance of long-term transcriptional repression (Goll and Bestor 2005).

Histone proteins are also key players in epigenetics. DNA associates with core histone proteins in order to be packaged into chromatin. Histones are subjected to several types of covalent modifications including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Kouzarides 2007). These modifications are important in regulating chromatin structure dynamics, which in turn affects transcriptional activation and repression. More recently, RNA-based mechanisms have also been shown to be involved in epigenetic silencing (Liu and Pan 2014). Non-coding RNAs have been shown to regulate gene silencing by recruiting chromatin remodeling complexes such as PcG to target regions (Rinn et al. 2007), and also through DNA methylation (Morris 2009).

Epigenetic processes are essential during development and differentiation. They enable cells in an organism, despite carrying identical genetic information, to differentiate into multiple

different cell types and maintain those differentiated states. During development, epigenetic processes play important roles in various cellular events such as X-chromosome inactivation in female mammals, genomic imprinting, patterning by *Hox* genes, and neuronal development (Kiefer 2007). In recent years, numerous studies have also highlighted the importance of epigenetic regulation during normal cardiovascular development. Several chromatin remodeling proteins and histone-modifying factors have been shown to control cardiac gene expression in a time- and tissue-specific manner to regulate heart muscle formation, chamber development, vascular development, and angiogenesis (Chang and Bruneau 2012).

Epigenetic events also have a major impact on disease development. Disruption or alteration of epigenetic events can result in abnormal gene activation or repression, which can lead to cancer, neurological disorders, and autoimmune diseases (Portela and Esteller 2010). In cardiovascular disease, dysregulation of DNA methylation has been implicated in atherosclerosis and vascular inflammation (Webster et al. 2013). Certain histone modifications have also been implicated in angiogenesis and endogenous recovery after myocardial infarction (Webster et al. 2013). The study of epigenetics is crucial to our understanding of diseases and may be an important contributor to future therapies and diagnostic tests.

1.2 Epigenetic regulation by Polycomb Group and Trithorax Group proteins

Polycomb Group (PcG) and Trithorax Group (TrxG) proteins are important epigenetic factors that were originally discovered in *Drosophila* as regulators of *Hox* gene expression. *Hox* genes encode a group of transcription factors that specify the anterior/posterior body axis and segment identity during early embryonic development (Pearson et al. 2005). Phenotypically, it was observed that PcG and TrxG mutations have opposite effects on axial patterning. PcG

mutations cause posterior transformations, whereas TrxG mutations cause anterior transformations (Kennison 1995). PcG and TrxG proteins function antagonistically to each other and act upon chromatin to maintain a transcriptionally repressed or activated state, respectively (Papp and Müller 2006). The molecular mechanisms of action of these proteins involve the formation and recruitment of multi-protein histone-modifying complexes to DNA regulatory elements termed PcG and TrxG response elements (PREs and TREs) (Schuettengruber et al. 2007).

There are four known PcG complexes: PhoRC, Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2), and Polycomb repressive deubiquitinase (PR-DUB). The PhoRC complex contains the DNA binding protein Pho, which is known to target the complex to PREs (Fujioka et al. 2008). It also contains the protein dSfmbt, which binds histone H3 and H4 tails that have mono or dimethylated lysine residues (Papp and Müller 2006). In *Drosophila*, it was observed that E(z), the catalytic subunit of PRC2, can bind Pho and trimethylate K27 of histone H3 (H3K27) (Kuzmichev et al. 2002). This suggests that the PhoRC complex helps recruit the PRC2 complex to target chromatin. The Pc component of the PRC1 complex binds trimethylated H3K27 (H3K27me3) and has been shown to inhibit nucleosome remodeling (Schuettengruber et al. 2007). The most-recently discovered PcG complex is PR-DUB. PR-DUB removes ubiquitin from mono-ubiquitinated histone H2A lysine 119 (H2AK119ub1). The deubiquitinase activity of PR-DUB was shown to be required for proper *Hox* gene repression in *Drosophila* (Scheuermann et al. 2010).

The mechanisms that drive TrxG complexes to target chromatin are less clearly understood. The Set1-like complex of TrxG trimethylates lysine 4 of histone H3 to maintain transcriptional activation (Yokoyama et al. 2004). Another TrxG complex, known as the

SWI/SNF or BRM complex, contains ATP-dependent chromatin remodeling factors, such as BRM and SNF2L, and mediates nucleosome sliding (Papoulas et al. 1998). The MLL complex of TrxG is involved in both deposition of active histone marks and chromatin remodeling activity (Nakamura et al. 2002).

1.3 Enhancers of Trithorax and Polycomb group proteins

It was later discovered that mutations in a set of genes originally thought to be PcG members showed TrxG-related phenotypes (Gildea et al. 2000). These genes genetically interacted with both PcG and TrxG members: double mutations with PcG showed enhancement of posterior transformations and double mutations with TrxG showed enhancement of anterior transformations (Sinclair et al. 1992). Therefore, these genes are now termed Enhancers of Trithorax and Polycomb (ETP). Corto and DSP1 are two examples of ETP proteins. They have been shown to associate with PcG complexes and localize to the nucleus (Salvaing et al. 2006, 2003; Lopez et al. 2001). DSP1, in particular, was also observed to recruit PcG proteins to target chromatin (Salvaing et al. 2006). Overall, the functions of ETP proteins may involve recruitment of PcG/TrxG proteins to target chromatin, helping in formation of PcG/TrxG complexes, or aiding in the stabilization of those complexes.

1.4 The *Drosophila Additional sex combs*

The *Drosophila Additional sex combs (Asx)* is an ETP gene and displays genetic characteristics of this group. *Asx* was first discovered in *Drosophila* as a member of the PcG family of chromatin regulators (Jürgens 1985). *Asx* mutants displayed homeotic and segmentation defects at embryonic and adult stages (Sinclair et al. 1992). Interestingly, a *P*-element-induced *Asx* allele, Asx^{P1}, showed an unusual phenotype of both anterior and posterior

homeotic transformations within the same individual (Sinclair et al. 1992). It was concluded that *Asx* genetically interacts with both PcG and TrxG genes since most *Asx* mutations enhance homeotic transformations of PcG mutants as well as homeotic transformations of TrxG mutants (Campbell et al. 1995; Milne et al. 1999). In the nucleus, Asx binds to distinct loci on chromatin, with 70% of these loci overlapping with binding sites of other PcG proteins (Sinclair et al. 1998).

In one study, it was observed that Asx truncation of the C-terminal 425 amino acids results in various wing defect phenotypes (Bischoff et al. 2009). These defects were linked to a dramatic upregulation of *Ultrabithorax (Ubx)*, a haltere fate marker, in the wing blade (Bischoff et al. 2009). These results indicated that Asx suppresses *Ubx* expression in the wing imaginal disc and demonstrated that the C-terminal region of Asx is essential for this function.

1.5 ASXL family proteins

1.5.1 Mammalian ASXL1, ASXL2 and ASXL3

The mouse and human genomes each has three *Asx* homologs, *Asx-like (ASXL) 1*, *2*, and *3*. These homologs contain four conserved regions. Progressing from the N- to C- terminus, they are: HARE helix-turn-helix (HARE-HTH) predicted DNA-binding domain, Asx-homology (ASXH) domain, a nuclear receptor (NR) binding motif, and the plant homeodomain (PHD) (Aravind and Iyer 2012; Fisher 2003; Cho et al. 2006) (Fig. 1). There is high sequence conservation between *Drosophila Asx* and its vertebrate homologs in two regions. The first region is the N-terminal ASXH domain containing a conserved LxxLL nuclear receptor binding motif. These motifs are found in several transcription factors (TFs) and thought to mediate protein interactions (Plevin et al. 2005). The second conserved region is the C-terminal PHD

domain. Various chromatin proteins, including PcG and TrxG proteins, contain PHD domains. This domain has known roles in protein-protein interactions, acetylation of chromatin proteins, and nucleosomal interactions (Baker et al. 2008; Papait et al. 2008). PHD fingers can function as histone-binding modules and can recognize modified/unmodified histone tails (Ramón-Maiques et al. 2007). Mutations in PHD domains relate to many human diseases (Baker et al. 2008).

There are two regions that are conserved among the ASXL homologs but not the *Drosophila* Asx. The first is the N-terminal HARE-HTH domain. This domain adopts the winged helix-turn-helix structural feature (wHTH) that is found in several chromatin proteins, restriction enzymes, and bacterial proteins and is predicted to bind DNA (Aravind and Iyer 2012). In order to investigate whether ASXL2 indeed binds DNA, we have collaborated with the Min Lab in the Chemistry Department of the University of Illinois at Chicago. We are actively conducting experiments to answer this question. The second feature conserved among ASXL homologs is a nuclear receptor-binding motif LVxxLL towards the middle of the protein. In ASXL1, this motif has been shown to interact with several nuclear receptors including RARα, RARβ, RXRα, and RXRβ (Cho et al. 2006).

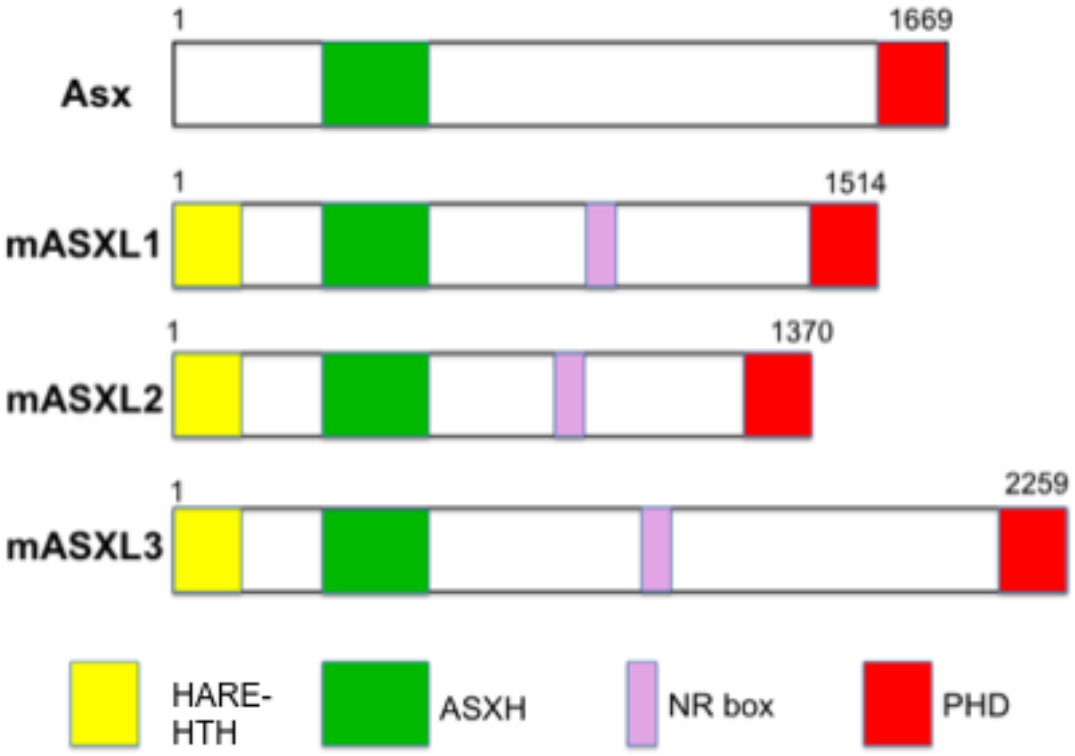


Figure 1. *Drosophila* Asx and mouse ASXL proteins. Schematic of protein sizes and domain organization. The ASXH and PHD domains are highly conserved between *Drosophila* Asx and its mammalian homologs. Mammalian ASXLs also share two additional regions of conservation, the HARE-HTH motif and the NR box region.

HARE-HTH: HARE helix-turn-helix, ASXH: Asx-homology, NR: nuclear receptor, PHD: plant homeodomain.

1.5.2 Role of ASXL2 in heart development and function

In an attempt to understand the role of Asx-like proteins in the mammalian system and elucidate their mechanisms of action, our lab has generated a mutant mouse model for *Asxl2* using a gene-trapped ES cell line. In this cell line, the gene trap cassette, containing a β-geo reporter, is integrated in the first intron of *Asxl2*, resulting in a truncated mRNA product which leads to a protein product that is missing all of ASXL's conserved domains (Baskind et al. 2009). The truncated protein product is expected to be functionally null. The β-geo reporter is under the control of the endogenous *Asxl2* promoter, allowing us to study endogenous *Asxl2* expression.

We initially noticed that the *Asxl2*$^{-/-}$ animals had disproportionately enlarged hearts and using X-gal staining, we also observed that the *Asxl2* promoter was highly active in the heart, both at embryonic and adult stages (Baskind et al. 2009). This led us to hypothesize that Asxl2 may play a role in heart development and function. Indeed, recent work from our lab has shown that *Asxl2* is required for normal cardiovascular development. In the inbred C57BL/6J background, using *in situ* hybridization, we observed that *Asxl2* is highly expressed in the heart at embryonic stages E9.5-10.5. *Asxl2*$^{-/-}$ mice died shortly after birth and displayed congenital heart defects including thickened left ventricle walls, interventricular septal defects and atrioventricular valve defects (McGinley et al. 2014).

Additionally, we have discovered that *Asxl2* is required for the maintenance of proper cardiac function in the adult. In the C57BL/6J;129Sv F1genetic background, *Asxl2*$^{-/-}$ mice displayed ventricular dysfunction over time (Lai et al. 2012). This may be attributed to *Asxl2* being directly involved in the repression of β-MHC, an important cardiac gene whose expression levels correlate with the contractile function of the heart (Lai et al. 2012).

1.6 Functional mechanisms of ASXL1 and ASXL2

Since ASXL family members do not have a direct DNA-binding domain or enzymatic activity, it is speculated that their functions are carried out through protein-protein interactions. Through these interactions, they help regulate chromatin dynamics, which in turn regulates transcription by either activating or repressing target genes in a context-dependent manner. Several studies have highlighted the role of ASXL1 and ASXL2 in PcG-mediated and nuclear receptor-mediated functions. The results of these studies are detailed below and summarized in Table 1.

1.6.1 PRC2-mediated functions of ASXL1 and ASXL2

ASXL1 mutations commonly occur in several human myeloid malignancies (Boultwood et al. 2010; Gelsi-Boyer et al. 2009). It was observed in hematopoietic cells that loss of *ASXL1* results in upregulated expression of genes in the *HOXA* gene cluster, which is known to contribute to myeloid transformations, and global loss of the histone mark H3K27me3 (Abdel-Wahab et al. 2012). Further analysis revealed that loss of *ASXL1* resulted in a loss of EZH2 enrichment at the *HOXA* locus. Furthermore, co-immunoprecipitation (co-IP) experiments revealed that ASXL1 physically interacts with EZH2 and SUZ12. These results indicate that ASXL1 is directly involved in the recruitment of PRC2 to the *HOXA* locus and thereby, is required for PRC-mediated gene repression.

Work from our lab has shown that loss of *Asxl2* results in reduced levels of H3K27me3 in the mouse heart (Baskind et al. 2009). We later discovered that Asxl2 is required for repressing select cardiac genes: *β-MHC, Sfrp2, Acta1* and *Grk5* (Lai and Wang 2013). ASXl2 co-localizes with EZH2 and SUZ12 at these cardiac genes and is required for PRC2 binding at their

promoters (Lai and Wang 2013). Furthermore, there was a significant reduction in H3K27me3 levels at the promoter regions of all the target genes analyzed (Lai and Wang 2013). Together, these results showed that ASXL2 interacts with PRC, regulates its enzymatic activity, and is required for PRC2-mediated gene repression of select cardiac genes in the adult heart.

1.6.2 PR-DUB-mediated functions of ASXL1 and ASXL2

One study has shown that the N-terminal 365 amino acids of human ASXL1 directly interact with the deubiquitinating enzyme BAP1, forming a stable complex that is now called PR-DUB (Scheuermann et al. 2010). It was observed that PR-DUB bound to the promoters of a large number of PcG target genes such as the HOX genes, along with members of the PhoRC complex (Scheuermann et al. 2010). Furthermore, this group discovered that the deubiquitinase activity of PR-DUB is required for proper HOX gene repression in *Drosophila*. This research identified ASXL1 as a core component of the PR-DUB complex. Our lab has recently shown that ASXL2 also interacts with BAP1 in the mouse heart (Lai and Wang 2013). In addition, *Asxl2*$^{-/-}$ hearts showed increased bulk level of uH2A, suggesting that *Asxl2* is required for efficient deubiquitination of uH2A in the heart.

1.6.3 RARα-related functions of ASXL1

There is evidence supporting the role of ASXL family in retinoic acid (RA) signaling. Cell-based molecular studies have shown that ASXL1 physically interacts with and co-localizes with the nuclear hormone receptor RARα in a ligand-dependent manner (Cho et al. 2006). Functionally, ASXL1 enhanced RARα-mediated transcriptional activity in a dose-dependent manner. While investigating the mechanism behind the transcriptional activation associated with ASXL1, the authors learned that ASXL1 cooperates with other transcriptional co-activators, such

as SRC-1, to synergistically increase RA-dependent activity of RARα (Cho et al. 2006). They also observed that ASXL1 itself was enriched at the promoter region of RARα target gene (RARβ2) and further enhanced ligand-dependent RARα recruitment as well. These results identified ASXL1 as a novel regulator of RAR activity and implicated PcG involvement in RA signaling.

There is also evidence showing that ASXL1 acts as a ligand-dependent co-repressor for RA signaling. It was observed that the N-terminal region of ASXL1 (aa1-655) inhibits RA-induced transcriptional activity of RARα in a dose-dependent manner (Lee et al. 2010). Furthermore, it was observed that ASXL1 forms a ternary complex with the proteins HP1 and LSD1 to carry out its repressive activity (Lee et al. 2010). HP1 is a heterochromatin protein implicated in gene silencing through formation of condensed chromatin structure (Eissenberg and Elgin 2000). LSD1 is a lysine-specific demethylase that has dual roles in transcriptional regulation. It carries out its repressive role by removing methyl groups from mono and dimethylated lysine 4 of histone H3. Conversely, it carries out its activating role by removing mono- and di-methylated lysine 9 of histone H3 (Forneris et al. 2008). While investigating the mechanism through which ASXL1 represses RA signaling activity, the authors discovered that ASXL1 recruits HP1 to the promoter of RA-responsive gene (RARβ2) and overexpression of ASXL1 results in more H3K9 di- and tri-methylation and less H3K4 di- and tri-methylation (Lee et al. 2010).

1.6.4 PPARγ-related functions of ASXL1 and ASXL2

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent TFs belonging to the nuclear receptor superfamily. PPARγ functions mainly in adipocyte (fat cell)

differentiation (Semple et al. 2006). It is induced during adipogenesis and is necessary and sufficient for proper differentiation. Without ligand, PPARγ is associated with co-repressors. In the presence of ligand, it undergoes conformational changes that release co-repressors and recruit co-activators such as histone lysine acetyl transferases to allow transcription (Park et al. 2011).

ASXL1 and ASXL2 both showed direct interaction with ligand-bound PPARγ in HEK293 cells and ASXL1 repressed PPARγ transcriptional activity while ASXL2 enhanced it. (Park et al. 2011). ASXL1 overexpression resulted in an increase in repressive histone mark (H3K9me3) and a decrease in activating histone marks (H3K9ac, H3K4me3) and the methyltransferase MLL1. Conversely, ASXL2 overexpression resulted in an increase in activating histone marks and MLL1 (Park et al. 2011). Using these mechanisms, ASXL1 inhibits lipid accumulation while ASXL2 enhances it, showing the opposite roles of these proteins in adipocyte differentiation.

1.6.5 LXRα-related functions of ASXL1 and ASXL2

More recently, ASXL1 and ASXL2 have also been implicated in transcriptional regulation during lipogenesis. Liver X receptors (LXRs) are ligand-dependent TFs also belonging to the nuclear receptor superfamily. They are expressed in the liver, spleen, kidneys, and intestine and regulate target gene expression to maintain homeostasis of lipid and cholesterol metabolism (Baranowski 2008). It was observed in rat hepatoma cells (H4IIE) that ASXL1/2 interact with LXRα in a ligand-dependent manner (Park et al. 2014). ASXL1 was shown to repress LXRα transcriptional activity while ASXL2 enhanced it. ASXL1/2 were recruited to promoter regions of LXRα target genes along with LXRα, in a ligand-dependent manner and

regulate transcription. Furthermore, it was observed in H4IIE cells that ASXL1 plays a repressive role in lipid synthesis while ASXL2 plays an activating role (Park et al. 2014).

Table I: Functional mechanisms of ASXL1 and ASXL2.

Functional mechanisms of ASXL1 and ASXL2		
	ASXL1	**ASXL2**
PcG-mediated	PRC2-mediated gene repression in hematopoietic cells (Abdel-Wahab et al. 2012)	PRC2-mediated gene repression in adult mouse heart (Lai and Wang 2013)
	PR-DUB-mediated gene repression in *Drosophila* (Scheuermann et al. 2010)	PR-DUB enzymatic activity in adult mouse heart (Lai and Wang 2013)
Nuclear receptor-mediated	Transcriptional co-activator for RARα activity (HeLa cells) (Cho et al. 2006)	
	Transcriptional co-repressor for RARα activity (HEK293 cells) (Lee et al. 2010)	
	Transcriptional co-repressor for PPARγ activity (3T3-L1 preadipocyte cells) (Park et al. 2011)	Transcriptional co-activator for PPARγ activity (3T3-L1 preadipocyte cells) (Park et al. 2011)
	Transcriptional co-repressor for LXRα activity (HEK293 cells) (Park et al. 2014)	Transcriptional co-activator for LXRα activity (HEK293 cells) (Park et al. 2014)

1.7 The LIM domain proteins

LIM domains are 50-60 amino acid long cysteine- and histidine-rich sequences that form two highly conserved zinc-finger motifs. It was initially hypothesized that LIM domains are involved in DNA-binding due to these zinc-finger motifs. However, there has been no evidence showing that they bind DNA (Zheng and Zhao 2007). There is, however, a plethora of evidence supporting the idea that LIM domains function in mediating protein-protein interactions and act as adaptors or scaffolds to support the assembly of multi-protein complexes (Bach 2000). LIM domain proteins contain one or more of these domains and are broadly categorized into four groups based on the arrangement of the LIM domains. They are further separated into families based on their cellular localization and function.

LIM domain proteins have diverse biological functions, both in the cytoplasm and in the nucleus. In the cytoplasm, they have been shown to regulate actin cytoskeletal organization and interact with cell/extra-cellular matrix focal adhesion components to maintain cell shape, motility and signal transduction events (Kadrmas and Beckerle 2004). In the nucleus, they are involved in transcriptional regulation though interactions with TFs and other nuclear proteins. Many of them have been shown to shuttle between the cytoplasmic and nuclear compartments, connecting extracellular and cytoplasmic events to gene expression (Kadrmas and Beckerle 2004).

1.7.1 ZYXIN family of LIM proteins

There are seven members of the ZYXIN family of LIM proteins: Ajuba, LIMD1, LPP, TRIP6, Migfillin, WTIP, and ZYXIN (Fig. 2A). Members in this group contain a divergent proline-rich N-terminal region and a highly conserved C-terminal region containing three LIM domains (Fig. 2B) (Zheng and Zhao 2007). Although a majority of the ZYXIN family members

have been shown to localize to focal-adhesion points at the cell-plasma membrane interface, more and more research is uncovering their roles in transcriptional regulation via nuclear shuttling (Kadrmas and Beckerle 2004).

TRIP6 is a ZYXIN family member that has a conserved nuclear export sequence and multiple transactivation domains. It was shown to interact with TFs AP-1 and NF-κB and is recruited to the promoter regions of their target genes (Kassel et al. 2004). There, it acts as a molecular platform for enabling either activating or repressive signals (Kassel et al. 2004). Similarly, LPP also contains a nuclear export sequence and interacts with TFs, however, it was also observed to be an independent TF, with or without binding to other TFs (Grunewald et al. 2009). It has been characterized as a novel protooncogene with important roles in a number of human cancers including, leukemia, lung carcinoma, and soft tissue sarcoma (Grunewald et al. 2009). Ajuba has been shown to selectively interact with RARs in a ligand-dependent manner and negatively regulate retinoic acid signaling (Hou et al. 2010). Clearly, the ZYXIN family proteins have important roles in diverse biological functions and may be key in linking cellular/extracellular events to gene transcription.

A

ZYXIN Family	Shuttle in/out of nucleus	Role in transcriptional regulation
Ajuba	Yes	Yes
LIMD1	Yes	Yes
LPP	Yes	Yes
Migfillin	Yes	Yes
TRIP6	Yes	Yes
WTIP	Yes	Yes
ZYXIN	Yes	Yes

B

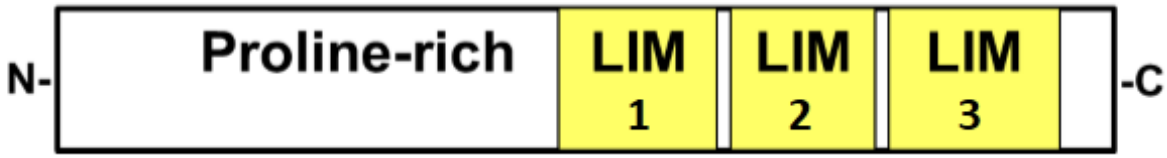


Figure 2. ZYXIN family proteins. A) Identified members of the ZYXIN family and their characteristics. B) General domain organization of ZYXIN family members includes a proline-rich N-terminal region followed by three LIM domains at the C-terminus.

1.7.2 Wilms tumor 1-interacting protein (WTIP)

Wilms tumor 1-interacting protein (WTIP) is a member of the ZYXIN family of LIM proteins. It was originally discovered by the Sedor group in a yeast two-hybrid screen using the Wilms tumor 1 protein (WT1) as bait (Srichai et al. 2004). WT1 is a transcription factor that is important in the development of several organs including kidneys, gonads, cardiac vasculature, and spleen (Hohenstein and Hastie 2006). Protein sequence analysis of WTIP identified a nuclear export sequence (NES) and two SH3 binding sites at the N-terminal region and three LIM domains and a PDZ binding site at the C-terminal region (Srichai et al. 2004) (Fig. 3). The domain structure of WTIP suggests that it is a molecular scaffold for multi-protein interactions.

In cell-based assays, full-length WTIP localized in the cytoplasm at cell edges and cell-cell contacts, while LIM domain-only region (lacking the NES) co-localized with WT1 in the nuclei (Srichai et al. 2004). Furthermore, luciferase assays in 3T3 and HeLa cells showed that WTIP represses WT1-dependent transcriptional activity (Srichai et al. 2004). In a separate study, it was observed that WTIP physically interacts with RARα in 293 cells and represses the transcriptional activity of a retinoic acid response element (RARE)-driven luciferase reporter in P19 cells (Hou et al. 2010). Another study found that WTIP represses canonical Wnt signaling activity in Cos-1 cells and in *Xenopus* embryos, it represses Wnt-induced secondary axis formation (van Wijk et al. 2009). Combined, this data suggests that WTIP interacts with TFs and nuclear receptors and functions as a transcriptional repressor in RA signaling and Wnt signaling pathways.

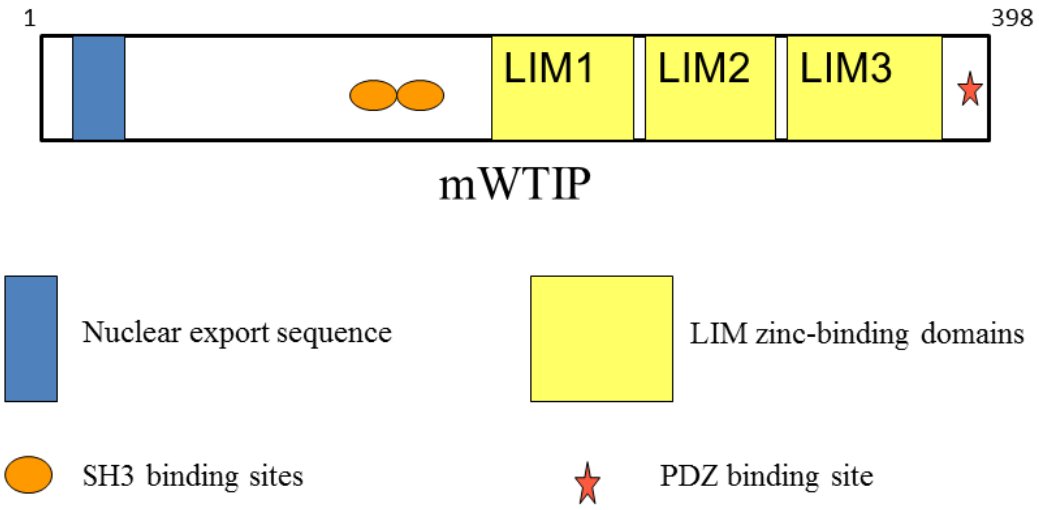


Figure 3. WTIP protein organization. Schematic of mouse WTIP protein and its key features.

1.8 Retinoic acid signaling in heart development and disease

Retinoic acid (RA) is the active metabolite of vitamin A (retinol). It plays important roles in the regulation of many biological processes including cell proliferation, differentiation, and apoptosis (Kumar and Duester 2011). During development, RA regulates gene expression to allow proper patterning and organogenesis. Numerous studies have shown that disruptions in RA signaling lead to several developmental disorders. These include skeletal patterning defects, central nervous system defects, craniofacial defects and cardiac defects (Niederreither and Dollé 2008; Duester 2008).

RA synthesis begins when vitamin A, which is carried in blood plasma via retinol-binding protein (RBP4), enters a cell expressing the retinol receptor (STRA6) (Fig. 4). Once in the cell, retinol either gets converted to retinyl ester for storage, or gets converted to retinaldehyde and then to retinoic acid through a series of oxidation reactions. Retinaldehyde dehydrogenases (RALDHs) are key enzymes in RA synthesis. RA is then released and taken up by surrounding cells. In a non-RA-responding cell, it gets degraded and excreted, while in RA-responding cells, it gets transported to the nucleus, where it binds to RXR-RAR heterodimers to regulate target gene expression (Niederreither and Dollé 2008).

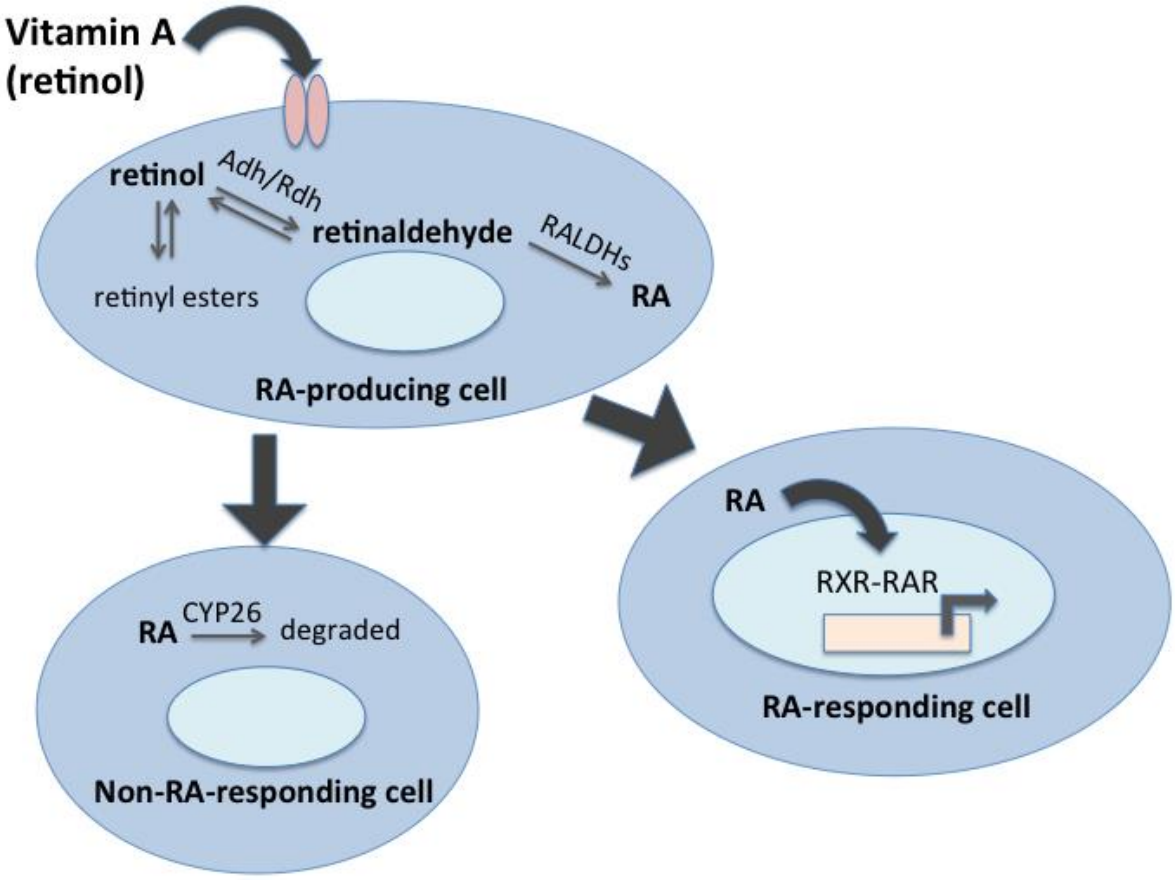


Figure 4. RA signaling. Vitamin A from blood serum is transported into cells via special receptors. Once in the cell, it is either converted to retinyl esters for storage or is oxidized to form the active metabolite retinoic acid (RA). In non-target cells, RA is broken down and excreted. In target cells, RA gets translocated to the nucleus, where it binds to retinoic acid receptors and activates gene transcription.

Studies using avian, rat, and mouse models have shown that proper RA signaling is essential for many aspects of cardiac development, including differentiation of embryonic ventricular cardiomyocytes, anterior/posterior patterning of the heart muscle, and proper looping of the heart tube (Kastner et al. 1997; Rosenthal and Xavier-Neto 2000; Niederreither et al. 2001). During the early stages of cardiac development, retinoids are required to prevent early differentiation, maintain cell proliferation, and regulate the morphology of ventricular cardiomyocytes (Kastner et al. 1997). Another study found that when RAR is overexpressed in the heart, it leads to dilated cardiomyopathy and reduces cardiac contractility, which eventually manifests into congestive heart failure (Colbert et al. 1997). A mutant mouse model for *Raldh2*, the enzyme essential for retinoic acid synthesis, showed that these mutants had a single dilated ventricle cavity, no left/right symmetry and no chamber formation (Niederreither et al. 2001). Evidence suggests that the Raldh2$^{-/-}$ phenotype is mainly due to abnormal development of the secondary heart field (SHF), a population of undifferentiated cardiac precursor cells (Ryckebusch et al. 2008).

1.9 Wnt signaling in heart development

The Wnt signaling pathway has important roles during embryonic development. Wnt proteins are secreted molecules that regulate cell proliferation, differentiation, cell fate specification, migration, and survival (Kim et al. 2013). The canonical Wnt signaling pathway mediates many of these events. The Wnt signaling pathway is activated when Wnt protein ligand binds to the Frizzled (Fzd)/lipoprotein receptor-related protein (LRP) receptor complex and passes on the biological signal to activate the effector protein disheveled (Dvl). Dvl inhibits the β-catenin destruction complex, which contains the proteins Axin, adenomatous polyposis coli (APC), protein phosphatase 2A (PP2A), the kinases GSK-3 and CK1, and the E3-ubiquitin ligase

β-TrCPaxin. Binding of Wnt results in a series of events that disrupt the β-catenin destruction complex, which results in accumulation of β-catenin, its subsequent translocation to the nucleus and activation of Wnt target genes via binding to LEF/TCF DNA-binding proteins (Kim et al. 2013) (Fig. 5).

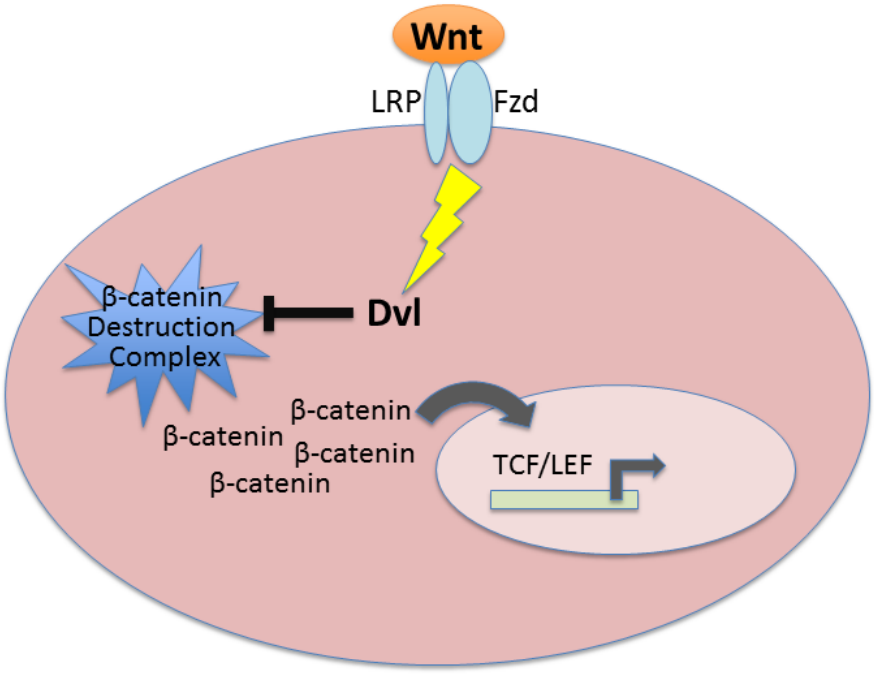


Figure 5. Wnt signaling. In the canonical Wnt signaling pathway, Wnt proteins bind to Fzd/LRP receptors and pass on the biological signal to activate Dvl effector protein. Dvl inhibits the activity of the β-catenin Destruction Complex to allow β-catenin accumulation and translocation to the nucleus. In the nucleus, β-catenin binds to the DNA binding proteins TCF/LEF to activate target gene transcription.

Numerous studies have established the significance of Wnt signaling in cardiovascular development, especially in cardiac progenitor cell self-renewal and differentiation and heart morphogenesis. It was observed that early in heart development, Wnt signaling promotes self-renewal of embryonic stem (ES) cells and is required for mesoderm formation (Dravid et al.; Lindsley et al. 2006). Later on, Wnt signaling is essential for specification and expansion for the $Isl1^{+}$ cardiac progenitor population (Qyang et al. 2007; Cohen et al. 2007). Furthermore, it was shown to promote proliferation of committed cardiomyocytes (Ai et al. 2007). Overall, the Wnt signaling pathway is an important regulator at multiple stages during heart development.

1.10 Purpose of this study

In an attempt to understand the role of Asx-like proteins in the mammalian system and elucidate their mechanism(s) of action, our lab has developed a mouse model for the study of ASXL2. We have discovered that ASXL2 is essential for normal cardiac development and proper cardiac function. Furthermore, our research shows that ASXL2 is a mammalian ETP protein and is involved in PcG/TrxG-mediated gene regulation. The purpose of this study was to identify novel interactions of ASXL2 in order to advance our understanding of the molecular mechanisms underlying the cardiac phenotype of *Asxl2*$^{-/-}$ mice. Here, I investigate the interaction between ASXL2 and the LIM protein WTIP and its functional significance. We find that ASXL2 and WTIP directly interact and regulate RA signaling. We provide evidence that this regulation may play a role in the epicardium during heart development. We also report a potential role for the ASXL2-WTIP interaction in Wnt signaling.

2. MATERIALS AND METHODS

2.1 Plasmids

For yeast two-hybrid experiments, the following ASXL2 cDNAs were cloned in frame into pGBKT7 vector (Clontech), and expressed as GAL4-DBD fusion proteins: full-length ASXL2, N-terminal region ($ASXL2_{1-599}$), C-terminal region ($ASXL2_{600-1370}$), C-terminal region missing the PHD domain ($ASXL2_{600-1295}$), and PHD region alone ($ASXL2_{1313-1370}$). The prey plasmid isolated from the initial screen, pACT2-$hWTIP_{245-430}$, was used in all confirmation matings.

For negative control yeast experiments, pACT2-Gal4-AD (Clontech) and pGBKT7-Gal4 DNA-BD empty vectors were used. For positive control experiments, pGBKT7-53 (Clontech) in AH109 and pTD1-1 (Clontech) in Y187 were used.

For co-immunoprecipitations (co-IPs) and luciferase assays, FLAG-$ASXL2_{720-1370}$ was inserted into pcDNA3 vector (Invitrogen). Myc-WTIP, Myc-NΔWTIP, and Myc-NΔZYXIN (Srichai et al. 2004) were generous gifts from Dr. John R. Sedor (Case Western Reserve University). The WTIPΔLD2,3-HA construct (van Wijk et al. 2009) was a gift from Dr. Sigmar Stricker (Berlin, Germany). The LD2,3-HA construct was generated by inserting WTIP cDNA into pCS2 destination vector (gift from Dr. Hua Jin, University of Illinois at Chicago). The following reporter constructs were used in luciferase assays: RARE-tk-luciferase (gift from Dr. Rene Bernards, Netherlands Cancer Institute) and pSV-β-gal (Promega).

For GST-pulldown assays, GST-NΔWTIP (Srichai et al. 2004) was a gift from Dr. Sedor (Case Western Reserve University). GST-PHD was constructed by inserting the PHD region of ASXL2 (aa1332-1370) into pGEX-6P-1 vector (GE Healthcare). For radiolabeling, $ASXL2_{600-}$

$_{1370}$, ASXL2$_{600-1295}$, and pcDNA3-WTIP (van Wijk et al. 2009) (gift from Dr. Stricker, Berlin, Germany) were used.

The WT1 isoforms WT1a, WT1b, WT1c and WT1d were gifts from Dr. Mark Minden (Princess Margaret Hospital, Toronto, Canada).

2.2 Yeast two-hybrid assays

Yeast two-hybrid screening was performed according to the following flow chart:

Construction of GAL4-DNA-BD-ASXL2 bait plasmids (See Section **2.1**)

↓

Individual transformation of bait plasmids into AH109

↓

Testing of bait constructs for auto-activation and toxicity

Pre-transformed Matchmaker cDNA library (Clontech catalog #638833)

↓

Mating of bait and library cultures

↓

Plating of mated cultures on high-stringency selection plates

↓

Verification of positive phenotype by re-streaking 3X

↓

Isolation of plasmids from yeast

↓

Transformation of plasmids into *E. coli* and DNA extraction

↓

Identification of positive clones via DNA sequencing

↓

Confirmation of protein interaction in yeast by individual transformation of positive clones in Y187 and mating with bait plasmids

2.2.1 Preparation of competent yeast cells

AH109 cells from a frozen yeast stock were streaked onto a YPDA agar plate and incubated upside down at 30°C for 3-5 days until colonies appeared. A single fresh yeast colony was used to inoculate 3 ml YPDA medium in a sterile culture tube and incubated at 30°C with constant shaking at 200 rpm for 8-12 hours. This culture was diluted into 50 ml YPDA medium and incubated with shaking at 30°C until OD_{600} of 0.2-0.5 was achieved. Cells were then harvested by centrifugation at room temperature for 10 minutes at 3000 rpm. The supernatant was discarded and cell pellet was resuspended in 10-20 ml of 1X TE-LiCl. The cell suspension was incubated at 30°C for 55 minutes without shaking. Cells were again harvested by centrifugation at room temperature for 10 minutes at 3000 rpm. The cell pellet was resuspended in an equal volume of 1X TE-LiCl. This volume is usually 150-300 ul. The cells were now ready to be transformed with plasmid DNA.

2.2.2 Small scale transformation of competent yeast cells

Approximately 150 ul of the cell suspension prepared in Section **2.2.1** was used for transformation of 100 ng plasmid DNA and 10 ug carrier sperm DNA and another 150 ul was used for transformation of only 10 ug carrier sperm DNA as control. The cells and plasmid mixture was incubated at 30°C for 30 minutes without shaking. Next, 300 ul of 50% PEG in TE was added to the mixture and incubated at room temperature for 1 hour. The cells were then heat shocked in a 42°C water bath for 15 minutes with occasional swirling. The tubes were then allowed to cool to room temperature and 100ul of 1/10 and 1/100 dilutions were plated on SD/-Trp selection media. The plates were incubated upside down at 30°C until colonies appeared (3-5 days). This procedure was used for individually transforming pGBKT7-ASXL2 and all its truncation constructs into AH109 cells.

2.2.3 Testing bait for auto-activation and toxicity

The pGBKT7-ASXL2 bait construct in AH109 cells was streaked out of glycerol stock onto SD/-Trp selection media plate. Once colonies were formed (3-5days at 30°C), a fresh colony was used to grow in liquid SD/-Trp media overnight. The culture was diluted 1/1000 and plated on SD/-Trp/X-α-Gal and SD/-Ade/-His/-Trp/ X-α-Gal selection plates. After 3-5 days, plates were analyzed for auto-activation. The results are detailed in **Appendix D**.

To test for toxicity, glycerol stocks of pGBKT7 empty vector in AH109 cells and the pGBKT7-ASXL2 construct in AH109 cells were streaked onto SD/-Trp plates and allowed to grow for 3-5 days at 30°C. Fresh colonies were used to grow liquid overnight cultures in SD/-Trp media. The cultures were diluted 1/1000 and plated on SD/-Trp plates and allowed to grow. The colonies of the bait vector were compared with the colonies containing empty pGBKT7 vector. The results are detailed in **Appendix E**.

2.2.4 Control mating experiments

The following control strains were streaked from glycerol stocks onto the appropriate selection media: pGBKT7-53 in AH109 onto SD/-Trp, pTD1-1 in Y187 onto SD/-Leu, pGBKT7 in AH109 onto SD/-Trp, and pACT2 in Y187 onto SD/-Leu. Plates were incubated at 30°C until colonies appeared. For positive control mating, one colony of pGBKT7-p53 and one colony of pTD1-1 was placed in a single 1.5 ml centrifuge tube containing 500 ul of 2X YPDA and vortexed to mix. The mixture was incubated at 30°C overnight with shaking at 200 rpm. The mated culture was diluted 1/1000 and plated on SD/-Ade/-His/-Leu/-Trp/X-α-Gal (QDO). Results were analyzed after 3-5 days. The negative control mating was done in the same manner except using pGBKT7 and pACT2 empty vector colonies. The results are detailed in **Appendix F**.

2.2.5 Two-hybrid library screening using yeast mating

Three individual rounds of library screening were done using pGBKT7-ASXL2$_{1\text{-}1370}$, pGBKT7-ASXL2$_{1\text{-}599}$ or pGBKT7-ASXL2$_{600\text{-}1370}$ as bait. Bait strains in glycerol stocks were streaked onto SD/-Trp plates and allowed to grow at 30°C for 3-5 days. One fresh, large colony was used to inoculate 50 ml of SD/-Trp liquid medium. The culture was incubated at 30°C with shaking at 250 rpm until the OD_{600} was close to 0.8 (~16-20 hours). The cells were then centrifuged at 1000 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 4-5 ml SD/-Trp media.

The library strain used was a pre-transformed human heart Matchmaker cDNA library (Clontech Catalog No. 638833). One ml of the library strain was thawed in a room temperature water bath and combined with the 5 ml bait strain in a sterile 2 L flask. Next, 45 ml of 2X YPDA liquid medium was added to the mixture. The flask was incubated at 30°C for 24 hours, slowly shaking at 50 rpm.

A drop of the culture was checked under a phase contrast microscope for the presence of zygotes. The cells were centrifuged at 1000 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml of 0.5X YPDA liquid medium containing 50 ug/ml kanamycin. The mating mixture was plated onto 60 mm SD/-Ade/-His/-Leu/-Trp/X-α-Gal plates (200 ul solution per plate). Serial dilutions of the mating mixture were also plated on SD/-Trp, SD/-Leu, and SD/-Trp/-Leu plates to later determine mating efficiency and number of clones screened. Plates were incubated at 30°C for 5-8 days.

2.2.6 Calculating the number of clones screened

The number of clones screened was calculated by counting the colonies from the SD/-Trp/-Leu plates and using the following formula:

Number of Screened Clones = CFU/ml X resuspension volume (ml)

CFU= colony forming unit

The results are detailed in **Appendix G**.

2.2.7 Determining the mating efficiency

In order to determine the mating efficiencies, first the viabilities of bait, prey, and diploids were determined using the following formulas:

Viability of Prey library = number of CFU/ml on SD/-Leu

Viability of Bait = number of CFU/ml on SD/-Trp

Viability of Diploids = number of CFU/ml on SD/-Leu/-Trp

Limiting partner = strain with the lower viability (bait or prey)

Mating efficiency = number of CFU/ml of diploids / number of CFU/ml of limiting partner

The results are detailed in **Appendix H**.

2.2.8 Identification and verification of positive phenotype

All positive colonies were collected and categorized into two groups: weak interactors or strong interactors. Strong interactors were healthy, blue colonies that were robustly growing and were very similar to the positive control colonies. Weak interactors were light blue colonies that were sometimes smaller in size than the control colonies. Colonies that were not blue were not counted as positive clones. All positive clones were collected and glycerol stocks were made.

The positive phenotype of only the strong interactors was verified by restreaking onto QDO/X-α-Gal plates three consecutive times.

2.2.9 Isolation of plasmid DNA from yeast

To isolate plasmid DNA from yeast, a single healthy colony of the yeast strain of interest was suspended in 100 ul of extraction medium (2.5 M LiCl, 50 mM Tris-HCl (pH 8.0), 4% Triton-X-100, 62.5 mM Na_2EDTA) in a microcentrifuge tube. Next, 100 ul of 1:1 (v/v) phenol:chloroform and 0.2 g glass beads (0.45-0.50 mm) were added to the tube and the contents were vortexed vigorously for 2 minutes and centrifuged at 16,000 g for 1 minute. The supernatant was transferred to a fresh tube and 0.1 volume of 5 M ammonium acetate (pH 5.2) was added followed by 3-4 volumes of ice-cold ethanol. The tubes were placed at -80°C for 30 minutes and centrifuged at 16,000 g for 30 minutes. The supernatant was discarded and the pellet was air dried until no traces of ethanol were evident. The precipitated DNA was dissolved in 50 ul TE and stored at 4°C.

2.2.10 Transformation of plasmid DNA into *E. coli*

The plasmid DNA isolated from yeast was transformed into DH5α competent cells. About 50 ul of competent cells were thawed on ice and 2 ul of plasmid DNA was added to the tube and gently mixed before being incubated on ice for 30 minutes. Next, the mixture was heat shocked in 42°C water bath for 45 seconds, then placed on ice for 2 minutes. Pre-warmed 950 ul LB broth was added to the mixture and incubated at 37°C at 220-250 rpm for 1 hour. 50-100 ul of the solution was plated on LB plates with the appropriate antibiotics. Plates were incubated for 16-18 hours in a 37°C incubator.

2.2.11 DNA purification and sequencing

DNA was amplified and purified using the QIAprep Spin Miniprep Kit (Catalog No. 27104). All DNA sequencing was conducted by University of Illinois at Chicago's Research Resources Center, DNA Services Facility. The identified clones are listed in **Appendix I**.

2.2.12 Confirmation of protein interactions in yeast

The identified clones were individually transformed into yeast and mating experiments were repeated using full-length ASXL2, N-terminal region or C-terminal region three times along with the negative control. I was not able to finish the confirmation tests on all of the identified clones. However, the results of the tests that were completed are detailed in **Appendix J**.

2.3 Cell culture

2.3.1 HEK293 and HeLa cells

HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone) at 37°C with 5% CO_2.

2.3.2 Primary mouse epicardial cells

Wild type E11.5 mouse hearts were isolated in cold phosphate buffered saline (PBS). At the same time, culture medium, containing DMEM, 10% FBS and 1:500 dilution of primocin (InvivoGen) was warmed in 37°C beads bath along with PBS. Hearts were washed twice with PBS and once with the culture medium before being placed in a 35mm plate that had been pre-

coated with collagen (50ug/ml) for 30 minutes at 37°C with 5% CO_2. Seven to ten hearts was determined to be the best amount per plate. Individual hearts were transferred into the culture plate by first cutting the tip of a 1 ml pipette tip using a UV-autoclaved razor blade and pipetting some PBS a few times. This is done so that the heart doesn't get stuck in the pipette tip. Hearts were carefully transferred to the center of the dish. Next, 500 ul of culture media was gently added to cover the hearts, making sure that the hearts are not dislodged. Plates were placed in 37°C incubator with 5% CO_2. After exactly 24 hours, hearts were removed by either adding more media to dislodge them or carefully suctioned out with a pump. Fresh, pre-warmed culture media was added to the plates and the plates were gently returned to the incubator for another 16-20 hours.

2.3.3 Immortalized mouse epicardial cells

Immortalized mouse epicardial cells (Austin et al. 2008) were cultured in Immorto-media, which was made as follows: 234 ml 1X DMEM (Cell-Gro); 12.5 ml FBS (Sigma); 2.5 ml 100X ITS-G (Invitrogen); 0.5 ml 5000U/ml IFN-y (Pepprotech) and 250 ul 1000X pen/strep. Cells were cultured at 33°C with 5% CO_2 in dishes that were pre-coated with collagen (50ug/ml) in 0.2N acetic acid.

2.4 Transfection for co-immunoprecipitation assays

The day before transfection, HEK293 cells from a confluent 10 cm plate were dissociated using 2 ml of 0.25% Trypsin-EDTA (Invitrogen) at 37°C for 5 minutes and diluted 1:5 in 30 ml final volume of culture media. Ten ml of the solution was used to seed each of three 10 cm culture dishes. The next day, the following components were added in order to an eppendorf tube

for each of the three plates: 1 ml serum-free DMEM, 6 ug total plasmid DNA and 18 ul branched polyethylenimine (PEI) (Sigma-Aldrich). The components were mixed by vortexing briefly and incubated at room temperature for 15 minutes. The DNA/PEI mixture was then added to the cells and culture dishes were returned to the incubator.

2.5 Transfection for luciferase assays

The day before transfection, HeLa cells were dissociated and diluted as described in section **2.4**. 500 ul of the solution was used to seed each well of a 24-well cell culture dish. The next day, the following components were added in order to an eppendorf tube for each well of the 24-well plate: 50 ul Opti-MEM (Life Technologies), 800 ng total plasmid DNA and 4.8 ul branched polyethylenimine (PEI) (Sigma-Aldrich). The components were mixed, incubated, and added to cells as described in section **2.4**.

The day before transfection, HEK293 cells were dissociated and diluted as described in section **2.4**. One ml of the solution was used to seed each well of a 12-well cell culture dish. 500 ul of Wnt 3A conditioned media was added to the appropriate experimental wells. The next day, the following components were added in order to an eppendorf tube for each well. The next day, the following components were added in order to an eppendorf tube for each well of the 12-well plate: 1 ml serum-free DMEM, 2 ug total plasmid DNA and 6 ul branched polyethylenimine (PEI) (Sigma-Aldrich). The components were mixed, incubated, and added to cells as described in section **2.4**.

2.6 Co-Immunoprecipitation

HEK293 cells were harvested 48 hours post-transfection from a 10 cm plate by using 2 ml of 0.25% Trypsin-EDTA (Invitrogen) and incubating at 37°C for 5 minutes. Using a cell scraper, cells were collected in eppendorf tubes and centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was discarded and cell pellet was gently re-suspended in 1 ml low salt lysis buffer (150 mM NaCl; 3% Triton-X 100; 50 mM Tris-Cl, pH 7.4). Protease inhibitor cocktail (Calbiochem) was added to buffer just before use to prevent protein degradation. Cells were incubated for 1 hour on a rotating shaker at 4°C. After that, 50 ul of the solution was removed and saved as whole lysate (wl) and 50 ul SDS sample buffer (Sigma-Aldrich) was added to it and boiled for 5 minutes before storage at -20°C. The remaining cell lysate was centrifuged for 10 minutes at 20,000 g at 4°C. 50 ul of the supernatant was saved as input (inp) for immunoprecipitation (IP) and 50 ul SDS sample buffer (Sigma-Aldrich) was added to it and boiled for 5 minutes before storage at -20°C. The remaining supernatant was incubated with 30 ul anti-FLAG M2 magnetic beads (Sigma) or 30 ul anti-Myc agarose beads (Clontech) and incubated overnight at 4°C on a rotating platform. The next day, beads were separated by either placing the tubes on a magnetic separator or centrifuging at 20,000 g for 10 minutes at 4°C. Immunoprecipitates were washed 3 times in wash buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl). After the last wash, 50 ul SDS sample buffer (Sigma-Aldrich) was added to samples and boiled for 5 minutes to elute the proteins before storage at -20°C. The samples were now ready for SDS-PAGE and immunoblotting using antibodies against the tagged proteins. Gel and antibody conditions are detailed in **Appendix K**.

2.7 GST-pulldown assays

GST and GST-fusion proteins were expressed in *E. coli* and immobilized on Glutathione Sepharose 4 Fast Flow beads (GE Healthcare) according to the manufacturer's instructions. Radiolabeled proteins were obtained in vitro using the TNT Coupled Reticulocyte Lysate system (Promega) in the presence of 20 uCi [^{35}S]-methionine (Perkin-Elmer). TNT reactions were incubated at 30°C for 90 minutes. 2.5 ul of each reaction was saved as input and 20 ul SDS sample buffer was added and boiled for 5 minutes before storing at -20°C. The remaining TNT reaction was incubated with immobilized GST (control) or GST-fusion proteins (experimental) supplemented with protease inhibitor cocktail (Calbiochem) and 100 uM ZnCl (Sigma) rotating overnight at 4°C. After incubation, the beads were washed extensively in wash buffer (1X PBS, pH 7.3; 3% Triton-X 100) and eluted by adding 100 ul SDS sample buffer and boiling for 5 minutes. Proteins were separated via SDS-PAGE and visualized by autoradiography.

2.8 Luciferase reporter assays

HeLa cells were seeded in 24-well plates and transfected with RARE-tk-luc, pSV-β-gal, and constructs for ASXL2, WTIP or both. Total amount of DNA was kept constant using pcDNA3 empty vector. After 48 hours, cells were fed with DMEM containing 5% charcoal-stripped FBS (Life Technologies) and incubated overnight with or without 0.2μM ATRA (Sigma). Cells were lysed using 1X passive lysis buffer (Promega) and luciferase activity was measured according to the Luciferase Reporter Assay (Promega). β-galactosidase activity was assayed to normalize for transfection efficiency and is detailed in **Appendix L**.

HEK293 cells were seeded in 12-well plates and transfected with Top-Flash luciferase reporter, pSV-β-gal, and constructs for ASXL2, WTIP or both. Total amount of DNA was kept constant using pcDNA3 empty vector. After 48 hours, cells were lysed using 1X passive lysis buffer (Promega) and luciferase activity was measured according to the Luciferase Reporter Assay (Promega). β-galactosidase activity was assayed to normalize for transfection efficiency.

2.9 Immunostaining

2.9.1 Primary embryonic epicardial cells

Primary embryonic epicardial cells were isolated from E11.5 mouse hearts and cultured as described in section **2.3.2**. After 48 h, cells were washed with 1X PBS 3 times for 5 minutes and fixed in 100% methanol for 10 minutes at room temperature. Cells were again washed with 1X PBS as before and blocked with 2.5% normal donkey serum in PBS for 1 hour at room temperature. Next, the cells were stained with anti-WTIP polyclonal antibody (sc-241738; Santa Cruz Biotechnology) at a 1:100 dilution in blocking solution overnight at 4°C. The next day, cells were washed with PBS as before and incubated with Texas-Red-conjugated secondary antibody at a 1:100 dilution for 1 hour at room temperature. Cells were again washed 3 times in PBS before being incubated with Hoescht 33342 nuclear stain (Life Technologies) at a 1:2000 dilution in PBS for 20 minutes at room temperature. Cells were then washed in PBS 3 times before being imaged.

2.9.2 Embryonic heart sections

E18.5 mouse heart frozen sections were fixed in 100% methanol for 10 minutes at -20°C and then washed 3 times in PBS for 5 minutes before being blocked for 1 hour at room

temperature using 0.05% Tween-20 and 5% normal donkey serum in PBS. Next, the sections were stained with anti-WTIP polyclonal antibody (sc-241738; Santa Cruz Biotechnology) at a 1:50 dilution in blocking solution overnight at 4°C. Blocking and all antibody incubations were performed in a humidified chamber on a rocking platform. The next day, cells were washed in PBS with 0.1% Tween-20 5 times for 5 minutes at room temperature and incubated with TRITC-conjugated secondary antibody at a 1:200 dilution for 1 hour at room temperature. Cells were again washed in PBS with 0.1% Tween-20 5 times for 5 minutes at room temperature before being mounted with Vectashield Mounting Media with DAPI (Vector Labs). Slides were sealed, dried and stored at -20°C until imaged.

2.10 Reverse transcription (RT)-PCR

Total RNA was extracted from immortalized mouse embryonic epicardial cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized and amplified from 200ng of total RNA using OneStep RT-PCR Kit (Qiagen) with primers specific to *Asxl2*: forward, 5'-CGAGCACTGATCAACAAGCAC- 3', reverse, 5'-TCTTGTCGAATTCT-CACCTGC- 3' or *Actin* control: forward, 5'-TCACCCACACTGTGCCCATCT- 3', reverse, 5'- TGGTGAAGCTGTAGCCACGCT- 3'. All reactions were run at the same time in a final volume of 25 ul, annealing temperature of 50°C, and 30 cycles.

3. WTIP INTERACTS WITH ASXL2 AND BLOCKS ASXL2-MEDIATED ACTIVATION OF RETINOIC ACID SIGNALING

3.1 Abstract

The Asx-like (ASXL) family proteins are chromatin factors that play dual roles in transcriptional activation and repression. ASXL2 is highly expressed in the heart and is required for proper heart development and function. Here, we identify a novel ASXL2-binding partner, the LIM domain-containing protein WTIP. Genetic and biochemical assays show a direct interaction between ASXL2 and WTIP. In HeLa cells, ASXL2 enhances retinoic acid-dependent luciferase activity, while WTIP represses it. Furthermore, WTIP blocks ASXL2's stimulatory effect on transcription. In addition, we found that ASXL2 and WTIP are expressed in mouse embryonic epicardial cells, a tissue that is regulated by retinoic acid signaling. Together, these results implicate ASXL2 and WTIP in regulation of retinoic acid signaling during heart development.

3.2 Introduction

The *Drosophila* Asx protein is a chromatin factor that plays dual roles in transcriptional activation and repression (Milne et al. 1999). Asx has three mammalian homologs, Asx-like 1, 2 and 3 (ASXL1, ASXL2, and ASXL3). Mutations in ASXL family proteins have been implicated in a wide range of myeloid malignancies (Carbuccia et al. 2009; Boultwood et al. 2010; Chou et al. 2010) and in Bohring-Opitz syndrome (Hoischen et al. 2011; Bainbridge et al. 2013). Using a mutant mouse model, we have previously shown that ASXL2 is highly expressed in the heart (Baskind et al. 2009) and is involved in maintaining proper cardiac function (Lai et al. 2012).

More recently, we demonstrated a requirement for both ASXL1 and ASXL2 during normal heart development (McGinley et al. 2014).

A number of studies have shown that Asx and ASXL proteins regulate chromatin configuration through functional interactions with two histone-modifying complexes, Polycomb repressive deubiquitinase (PR-DUB) (Scheuermann et al. 2010) and Polycomb repressive complex 2 (PRC2) (Lai and Wang 2013; Abdel-Wahab et al. 2012). PR-DUB removes ubiquitin from mono-ubiquitinated histone H2A lysine 119 (uH2A). The deubiquitinase activity of PR-DUB was shown to be required for proper *Hox* gene repression in *Drosophila* (Scheuermann et al. 2010). PRC2 contains histone methyl-transferase activity and methylates lysine 27 of histone H3 (H3K27) (Czermin et al. 2002). Trimethylated H3K27 (H3K27me3) is a well-known mark of gene silencing.

The full-length ASXL2 protein contains several conserved domains separated by sequence-divergent regions. These include the HARE helix-turn-helix (HARE-HTH) predicted DNA-binding domain (aa10-88), the Asx-homology (ASXH) domain (aa215-344), a nuclear receptor (NR) binding motif (aa896-901), and the plant homeodomain (PHD) (aa1335-1366) (Aravind and Iyer 2012; Fisher 2003; Cho et al. 2006). In *Drosophila* Asx and mouse ASXL1, an N-terminal fragment containing HARE-HTH and ASXH was shown to mediate interaction with the histone deubiquitinase Calypso/BAP1 (Scheuermann et al. 2010). Consistent with this finding, our lab has shown that ASXL2 and BAP1 interact *in vivo* to regulate deubiquitination of uH2A (Lai and Wang 2013). We and others have also shown that ASXL1/2 interact with PRC2 and regulate PRC2 recruitment to target loci (Abdel-Wahab et al. 2012; Lai and Wang 2013). However, it is unclear which region of ASXL mediates this interaction.

There is also evidence that ASXL proteins interact with nuclear receptors. For example, ASXL1 has been shown to interact with the retinoic acid receptor RARα via an NR binding box and act as either co-activator or co-repressor of RA signaling in a cell type-dependent manner (Cho et al. 2006; Lee et al. 2010). RA signaling plays important roles during heart development by regulating chamber formation and cardiomyocyte proliferation and maturation (Kastner et al. 1997; Niederreither et al. 2001; Stuckmann et al. 2003). Since our lab has previously shown that ASXL2 is predominantly expressed in the heart (Baskind et al. 2009) and required for heart morphogenesis (McGinley et al. 2014), we hypothesize that ASXL2 may regulate RA signaling.

Here we present a study aimed at a better understanding of the functional mechanism of ASXL2. First, we screened for proteins that interact with ASXL2 outside the N-terminal region. We discovered genetic and biochemical evidence for a direct interaction between ASXL2 and Wilms Tumor 1-Interacting Protein (WTIP), a member of the ZYXIN family of LIM domain-containing proteins (Kadrmas and Beckerle 2004). Secondly, we investigated the effect of ASXL2, with or without WTIP, on RA signaling.

3.3 Results

3.3.1 Genetic interactions between WTIP and ASXL2 in yeast

To identify protein partners that interact with ASXL2 outside of the N-terminus, a yeast two-hybrid screen was conducted using a cDNA corresponding to the C-terminal region ($ASXL2_{600\text{-}1370}$) (Fig. 6A). One of the positive clones isolated encoded a fragment of the human WTIP (Fig. 6B). WTIP contains an N-terminal proline-rich region with putative SH3 binding sites and a C-terminal region with three LIM domains (Srichai et al. 2004). The $ASXL2_{600\text{-}1370}$-

interacting clone, hereafter referred to as $hWTIP_{245-430}$, encoded partial LIM 1 but complete LIM 2 and 3 of WTIP.

Confirmation matings showed that $hWTIP_{245-430}$ strongly interacts with $ASXL2_{600-1370}$, but not $ASXL2_{1-599}$ or full-length protein (Fig. 6C, D). Furthermore, strong interaction was detected with either the PHD finger alone ($ASXL2_{1313-1370}$), or a fragment missing the PHD finger ($ASXL2_{600-1295}$) (Fig. 6C, D), suggesting that the WTIP-ASXL2 interaction is mediated by at least two regions of ASXL2.

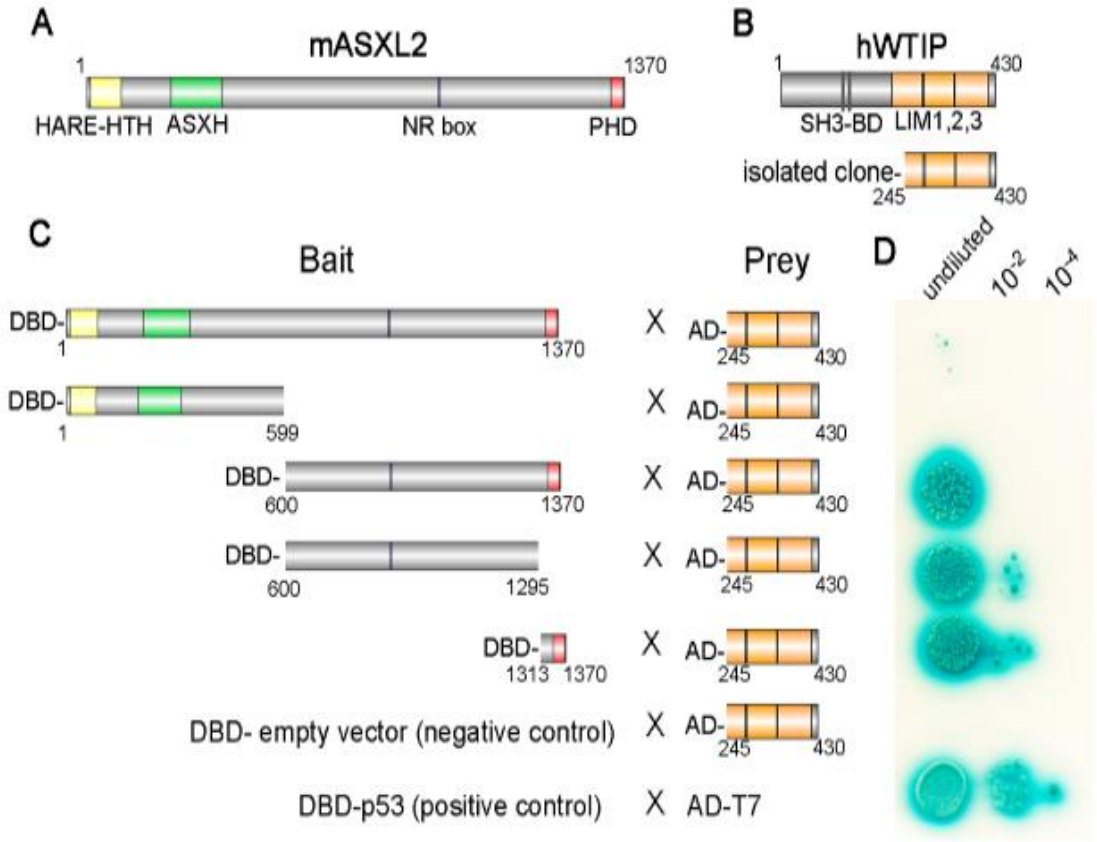


Figure. 6. ASXL2 interacts with WTIP in yeast. (A-B) Schematic representation of ASXL2 (A) and WTIP (B) proteins, showing their conserved domains. (C) Yeast two-hybrid matings using truncations of ASXL2 fused to Gal4-DNA binding domain (DBD) as bait and the isolated WTIP clone fused to GAL4-activation domain (AD) as prey. (D) Serial dilutions of the mating mixtures from (C) were plated on SD/-Ade/-His/-Leu/-Trp/X-α-Gal plates. The WTIP clone interacts with multiple parts on C-terminal ASXL2 but not N-terminal or full-length ASXL2. HARE-HTH: HARE-helix-turn-helix domain; ASXH: *Asx*-homology domain; NR: nuclear receptor box; PHD: plant homeodomain; SH3-BD: SH3 binding domain.

3.3.2 Biochemical characterization of ASXL2-WTIP interaction in mammalian cells

To determine whether ASXL2 and WTIP physically interact in mammalian cells, we performed reciprocal co-IP assays using FLAG-tagged ASXL2 and Myc-tagged mouse WTIP. Myc-WTIP co-IPed with FLAG-ASXL2$_{720\text{-}1370}$ in double transfected cells, but not in single-transfected control cells (Fig. 7).

Next, we examined the specificity of the ASXL2-WTIP interaction. Since WTIP belongs to the ZYXIN family of proteins, members of which share high sequence similarity within the LIM regions (Srichai et al. 2004), we decided to check whether ASXL2 interacted with the LIM domains of ZYXIN. While the LIM domains of WTIP (NΔWTIP) co-IPed with ASXL2$_{720\text{-}1370}$ and vice versa (Fig. 8), the LIM domains of ZYXIN (NΔZYXIN) did not (Fig. 9). This suggests that the ASXL2-WTIP interaction is specific to the LIM domains of WTIP.

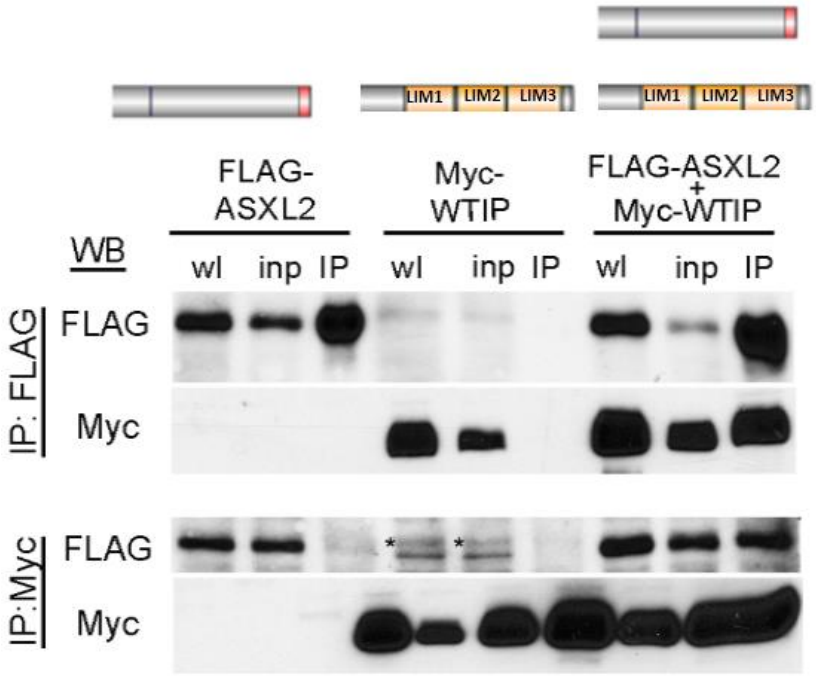


Figure 7. Biochemical characterization of ASXL2 and WTIP interaction. Reciprocal co-IP assays. HEK293 cells were transiently transfected with FLAG-tagged $ASXL2_{720\text{-}1370}$ (left), Myc-WTIP (middle) or both (right) constructs. Proteins were IPed using anti-FLAG or anti-Myc beads, as indicated. Whole lysate (wl), input (inp) and IP fractions were used for SDS-PAGE followed by Western blot (WB) analysis. (* non-specific bands).

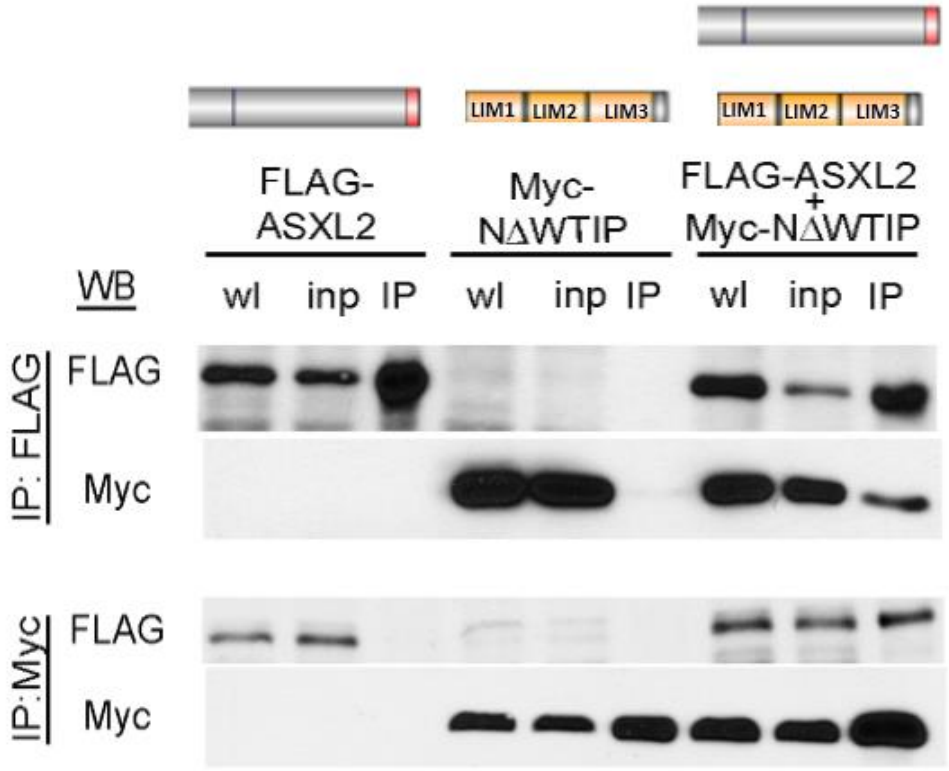


Figure 8. ASXL2 interacts with the LIM domains of WTIP. Reciprocal co-IP assays. HEK293 cells were transiently transfected with FLAG-tagged ASXL2$_{720\text{-}1370}$ (left), Myc-NΔWTIP (middle) or both (right) constructs. Proteins were IPed using anti-FLAG or anti-Myc beads, as indicated. Whole lysate (wl), input (inp) and IP fractions were used for SDS-PAGE followed by Western blot (WB) analysis.

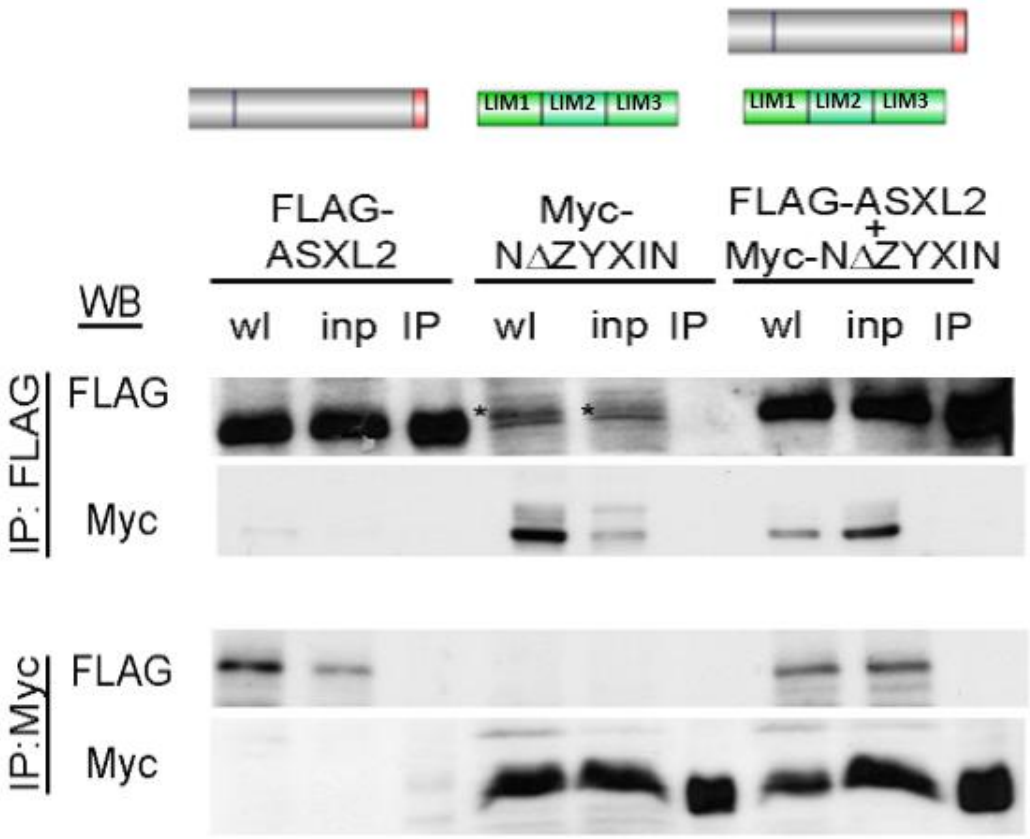


Figure 9. ASXL2 does not interact with the LIM domains of ZYXIN. Reciprocal co-IP assays. HEK293 cells were transiently transfected with FLAG-tagged ASXL2$_{720\text{-}1370}$ (left), Myc-NΔZYXIN (middle) or both (right) constructs. Proteins were IPed using anti-FLAG or anti-Myc beads, as indicated. Whole lysate (wl), input (inp) and IP fractions were used for SDS-PAGE followed by Western blot (WB) analysis. (* non-specific bands).

Since ASXL2 interacts with LIM 2 and 3 of WTIP in yeast two-hybrid assays, we used co-IP assays to biochemically determine the role of the two LIM domains in the interaction. A WTIP fragment containing only LIM 2 and 3 (LD2,3-HA) co-IPed with FLAG-ASXL2$_{720\text{-}1370}$ (Fig. 10), while a truncation missing LIM 2 and 3 (WTIPΔLD2,3-HA) did not (Fig. 11), confirming that these domains are sufficient and necessary for interaction.

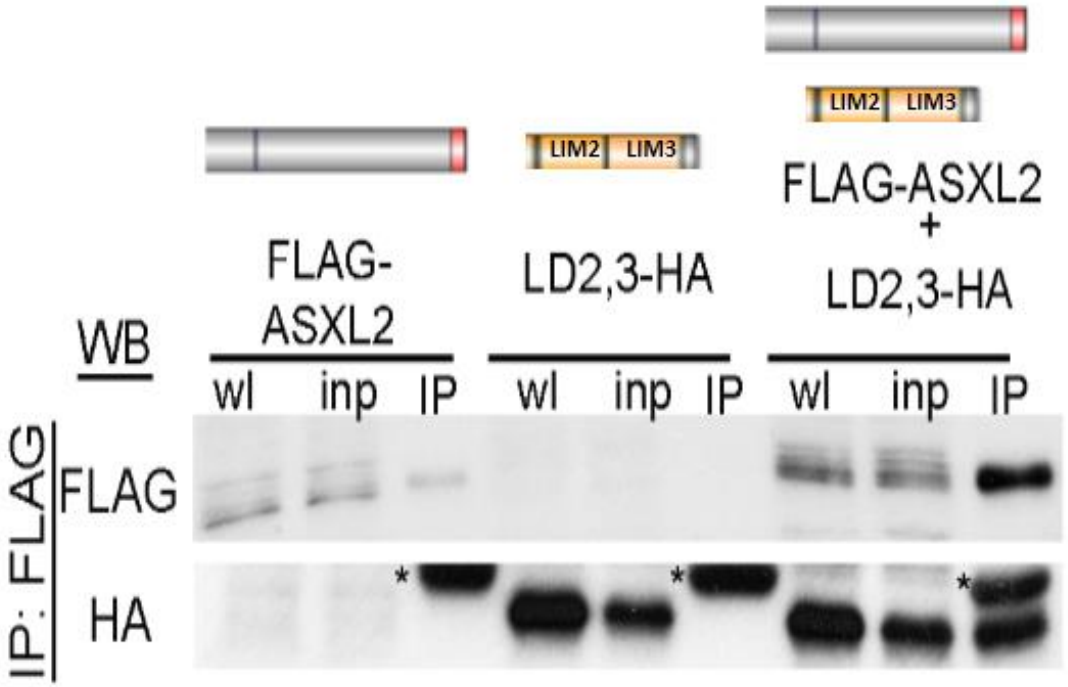


Figure 10. LIM domains 2 and 3 of WTIP are sufficient for interaction with ASXL2.

HEK293 cells were transiently transfected with FLAG-tagged $ASXL2_{720\text{-}1370}$ (left), LD2,3-HA (middle) or both (right) constructs. Proteins were IPed using anti-FLAG beads, as indicated. Whole lysate (wl), input (inp) and IP fractions were used for SDS-PAGE followed by Western blot (WB) analysis. (* non-specific bands).

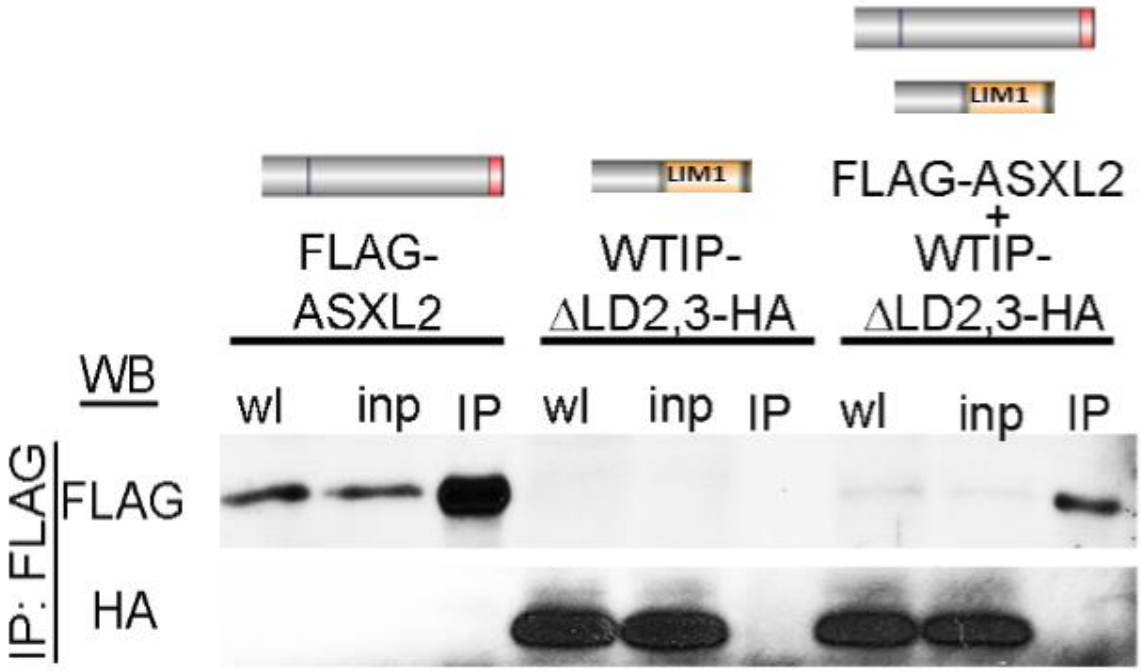


Figure 11. LIM domains 2 and 3 of WTIP are necessary for interaction with ASXL2. HEK293 cells were transiently transfected with FLAG-tagged ASXL2$_{720\text{-}1370}$ (left), WTIPΔLD2,3-HA (middle) or both (right) constructs. Proteins were IPed using anti-FLAG beads, as indicated. Whole lysate (wl), input (inp) and IP fractions were used for SDS-PAGE followed by Western blot (WB) analysis.

3.3.3 ASXL2 and WTIP interact directly *in vitro*

To determine whether ASXL2 and WTIP interact directly, GST-pulldown assays were performed. GST-NΔWTIP immobilized on glutathione-Sepharose beads retained *in vitro*-translated $ASXL2_{600\text{-}1370}$ and $ASXL2_{600\text{-}1295}$ (Fig. 12, top). Furthermore, GST-PHD retained *in vitro*-translated WTIP (Fig. 12, bottom). These results are consistent with results of the yeast two-hybrid assays (Fig. 6C,D). Together, they indicate that the PHD domain of ASXL2 and at least one other region outside the PHD domain directly interact with the LIM region of WTIP.

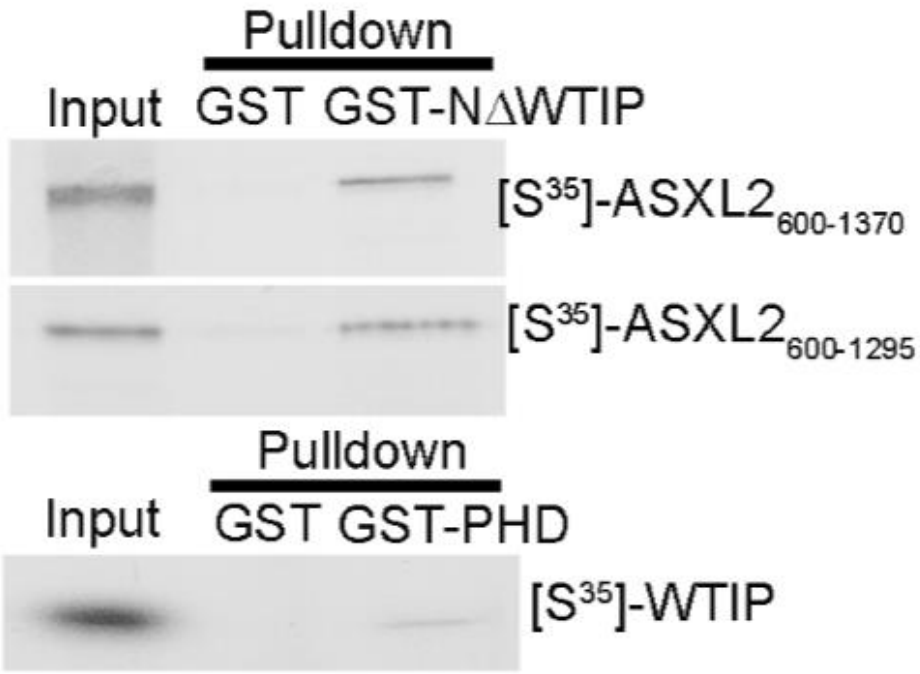


Figure 12. Direct interaction between ASXL2 and WTIP. GST-pulldown assays were conducted to examine direct interaction between GST-NΔWTIP and [S^{35}]-labeled ASXL2 constructs (top) and GST-PHD and [S^{35}]-labeled WTIP (bottom). Input and pulldown fractions were loaded for SDS-PAGE and bands were visualized by autoradiography.

3.3.4 C-terminal ASXL2 enhances ligand-dependent retinoic acid signaling

The effect of ASXL2 on RA signaling was examined using a retinoic acid-response element (RARE)-driven luciferase reporter. $ASXL2_{720\text{-}1370}$ enhanced *all-trans* retinoic acid (ATRA)-induced luciferase activity in HeLa cells in a dose-dependent manner (Fig. 13). Interestingly, full-length ASXL2 had no effect on RA signaling (Fig. 14).

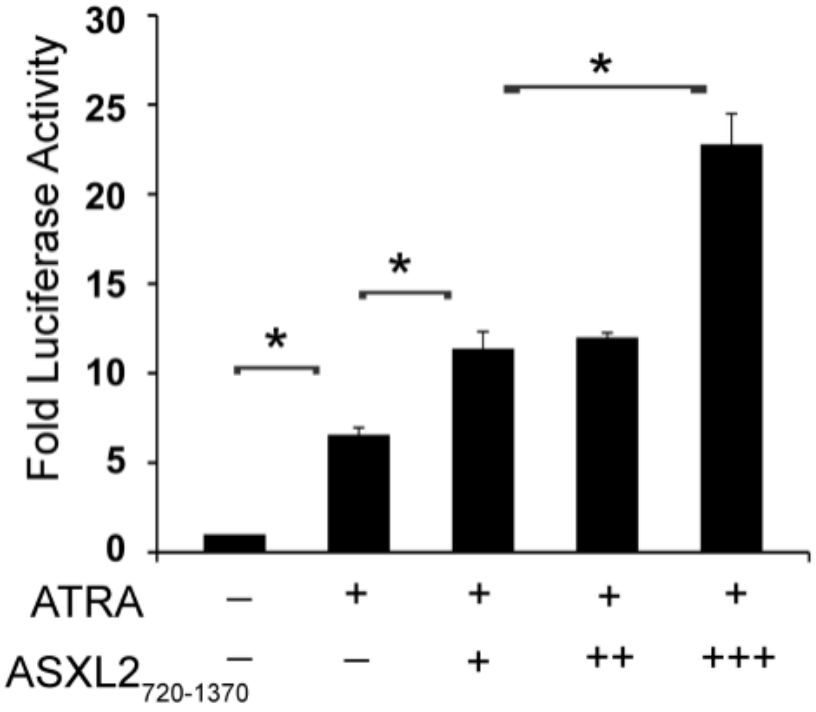


Figure 13. C-terminal ASXL2 regulates RA-dependent luciferase activity. HeLa cells were transfected with RARE-tk-luciferase and pSV-β-gal reporter constructs and increasing amounts of $ASXL2_{720\text{-}1370}$ with or without 0.2μM *all-trans* retinoic acid (ATRA). Luciferase activity was measured and normalized to β-gal activity. Error bars represent standard deviations from at least 3 independent experiments. (* $p<0.05$).

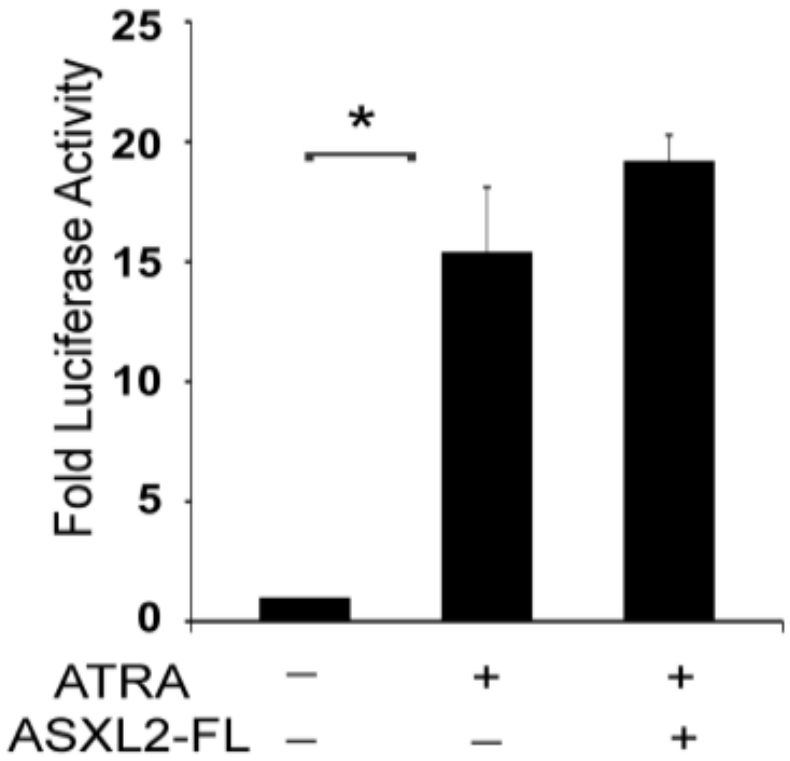


Figure 14. Full-length ASXL2 has no effect on RA-dependent luciferase activity. HeLa cells were transfected with RARE-tk-luciferase and pSV-β-gal reporter constructs and ASXL2-FL with or without 0.2μM *all-trans* retinoic acid (ATRA). Luciferase activity was measured and normalized to β-gal activity. Error bars represent standard deviations from at least 3 independent experiments. (* $p<0.05$).

3.3.5 LIM domains 2 and 3 of WTIP block ASXL2's effect on retinoic acid signaling

WTIP has been shown to interact with RARα in HEK293 cells and repress RARE-dependent luciferase activity in P19 cells (Hou et al. 2010). This prompted us to test the effect of WTIP on the increased RA signaling activity observed with $ASXL2_{720\text{-}1370}$ (Fig. 15). HeLa cells were transfected with constructs for ASXL2, WTIP, or both. Similar to previous results, $ASXL2_{720\text{-}1370}$ alone enhanced ligand-dependent transcriptional activity. Conversely, WTIP alone repressed ATRA-induced transcription. When both ASXL2 and WTIP are present, WTIP blocks ASXL2's effect on luciferase transcription, suggesting that WTIP acts downstream of ASXL2 in the regulation of RA signaling.

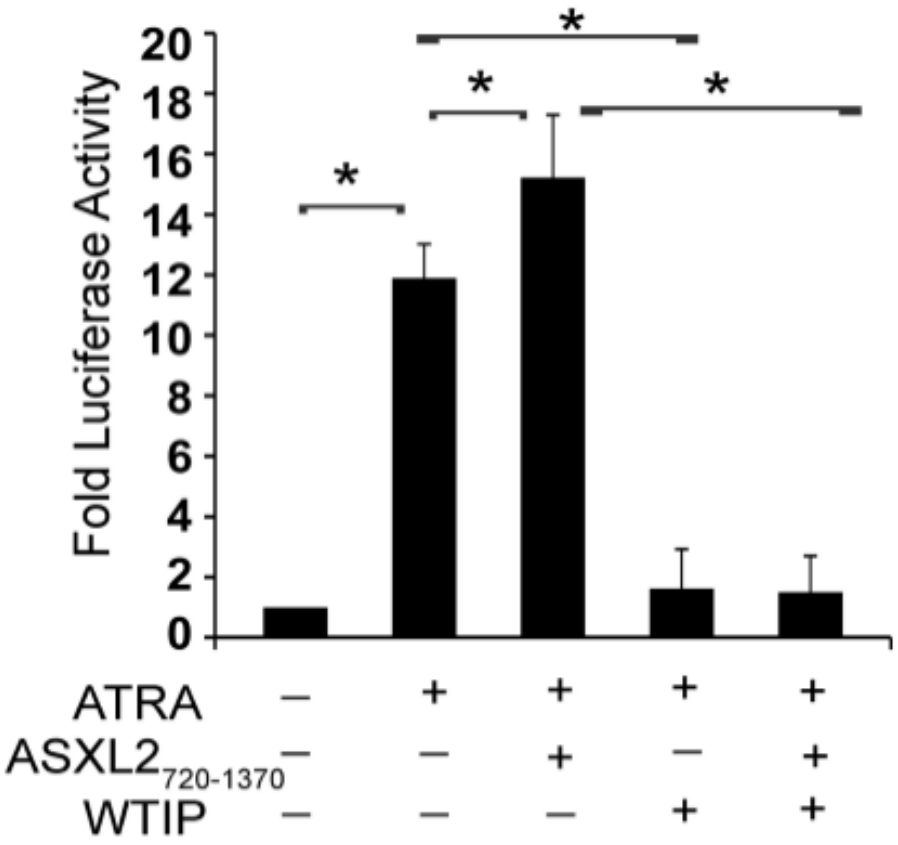


Figure 15. WTIP blocks ASXL2's effect on RA-dependent luciferase activity. HeLa cells were transfected with RARE-tk-luciferase and pSV-β-gal reporter constructs and ASXL2, WTIP or both, with or without 0.2μM *all-trans* retinoic acid (ATRA). Luciferase activity was measured and normalized to β-gal activity. Error bars represent standard deviations from at least 3 independent experiments. (* $p<0.05$).

We next tested which part of WTIP is important for repression of RA signaling. As shown in Figures 16 and 17, LD2,3-HA repressed ATRA-induced transcription while WTIPΔLD2,3-HA did not. These results suggest that LIM 2 and 3 of WTIP are sufficient and necessary for repressing RA signaling activity.

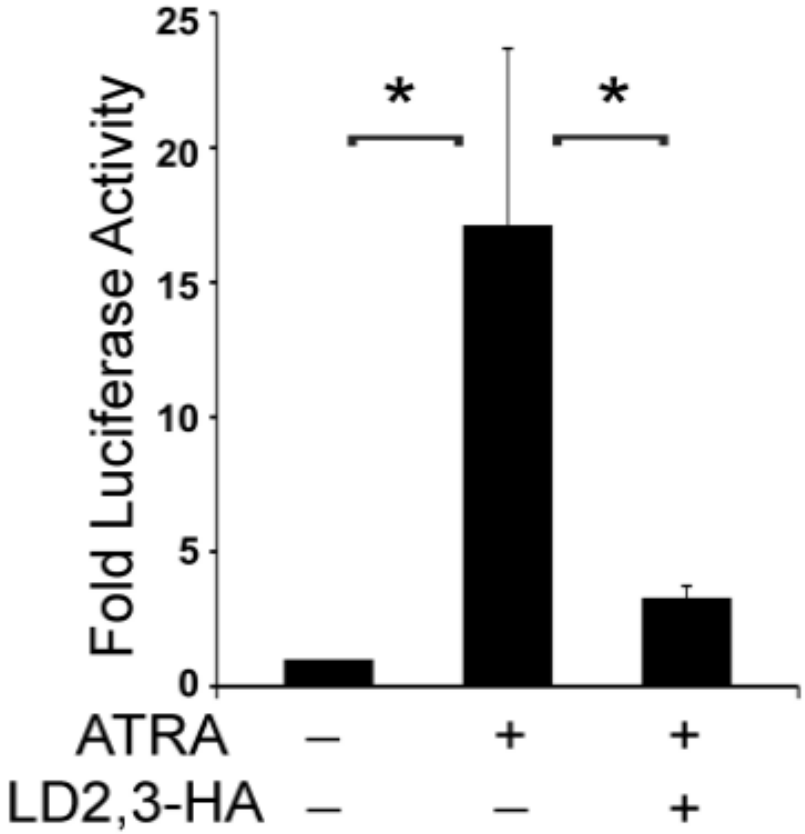


Figure 16. LIM 2 and 3 of WTIP are sufficient for repressing RA signaling activity. HeLa cells were transfected with RARE-tk-luciferase and pSV-β-gal reporter constructs and LD2,3-HA, with or without 0.2μM *all-trans* retinoic acid (ATRA). Luciferase activity was measured and normalized to β-gal activity. Error bars represent standard deviations from at least 3 independent experiments. (* $p<0.05$).

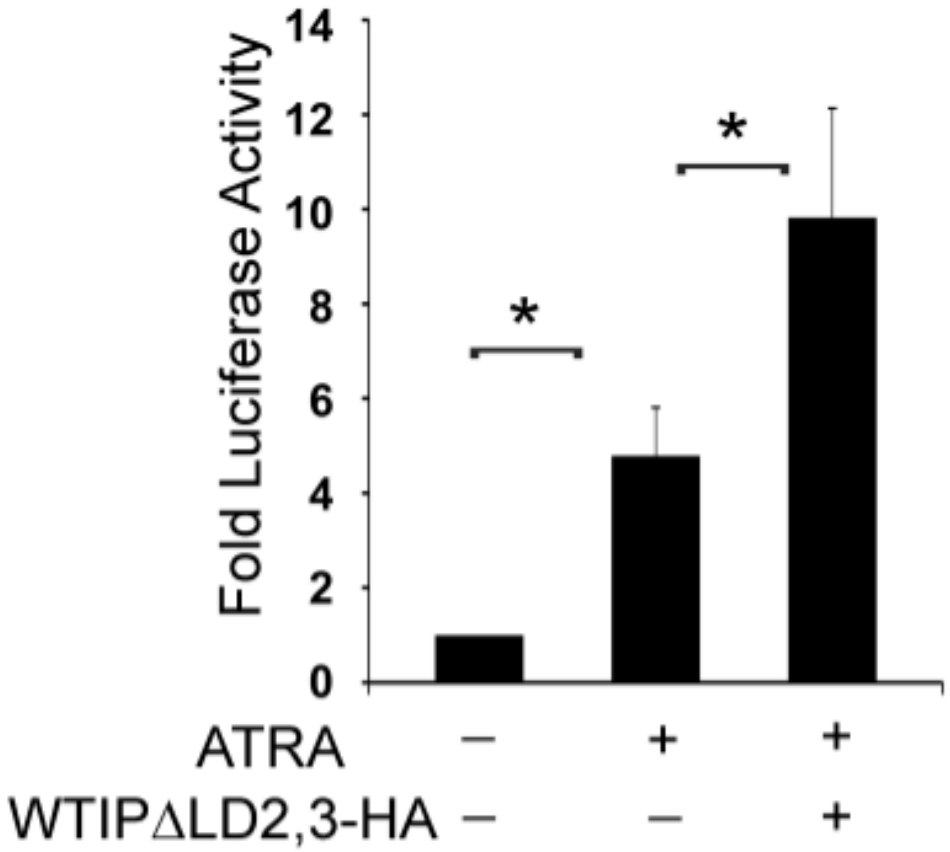


Figure 17. LIM 2 and 3 of WTIP are necessary for repressing RA signaling activity. HeLa cells were transfected with RARE-tk-luciferase and pSV-β-gal reporter constructs and WTIPΔLD2,3-HA, with or without 0.2μM *all-trans* retinoic acid (ATRA). Luciferase activity was measured and normalized to β-gal activity. Error bars represent standard deviations from at least 3 independent experiments. (* $p<0.05$).

3.3.6 Mouse embryonic epicardial cells express WTIP and ASXL2

WTIP is known to interact with WT1, an important transcription factor that regulates epithelial-to-mesenchymal transition (EMT) in multiple tissues (Hohenstein and Hastie 2006). In the developing heart, WT1 is expressed specifically in the epicardium and regulates multiple signaling pathways, including RA signaling (Zeng et al. 2011; von Gise et al. 2011).We asked whether WTIP is also expressed in the epicardium by immunostaining primary epicardial cells isolated from E11.5 mouse embryos. We detected positive WTIP expression in these cells (Fig. 18). Interestingly, WTIP localizes to the cytoplasm and peri-nuclear regions but not in the nuclei.

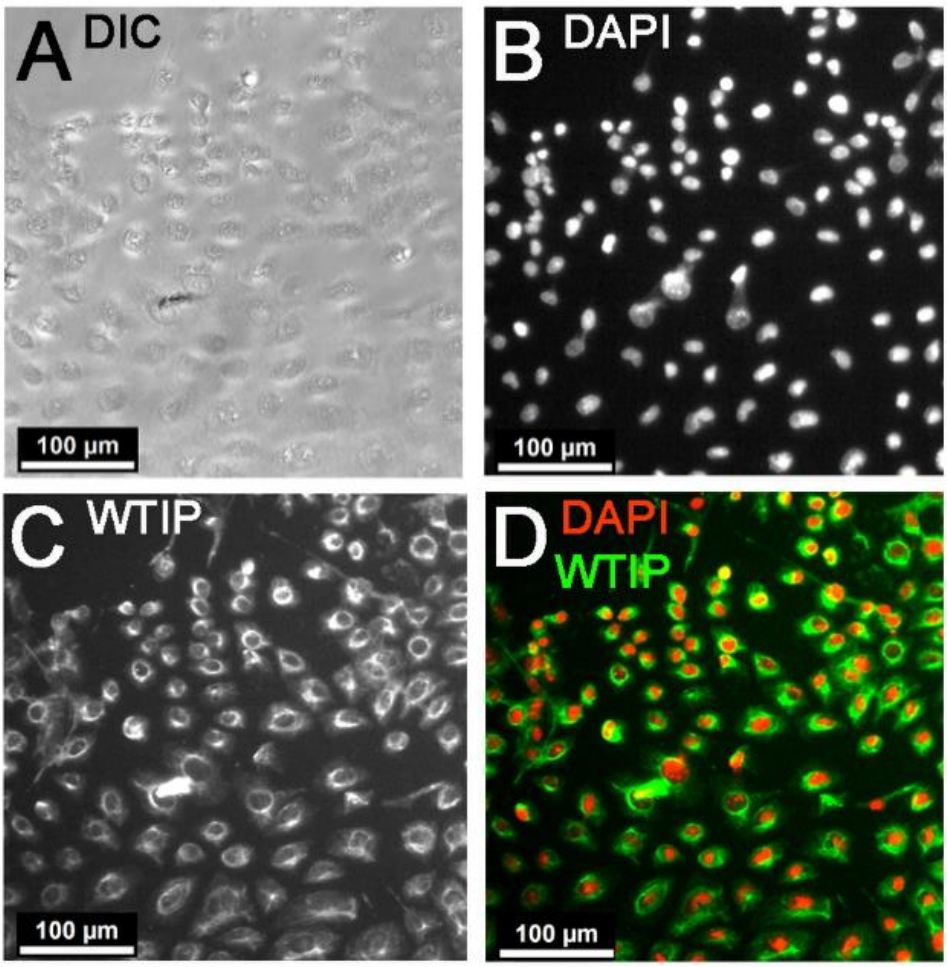


Figure. 18. WTIP expression in embryonic epicardial cells. Primary epicardial cells cultured from E11.5 mouse hearts (A-D) were stained with WTIP antibody. Shown are DIC (A), DAPI (B), WTIP (C), and merged (D) images.

We have previously shown that *Asxl2* is broadly expressed throughout the developing heart (Baskind et al. 2009; McGinley et al. 2014), however we have not specifically examined whether it is expressed in the epicardium. No existing ASXL2 antibodies detect endogenous levels of ASXL2 protein in immunostainings, therefore, we used RT-PCR to examine *Asxl2* expression in an immortalized epicardial cell line (Austin et al. 2008). A substantial amount of the *Asxl2* transcript (227 bps) was detected in these cells in addition to the positive control *Actin* transcript (129 bps) (Fig. 19).

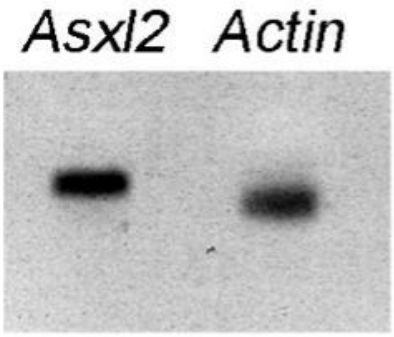


Figure 19. *Asxl2* expression in mouse epicardial cell line. Total RNA was extracted from immortalized mouse embryonic epicardial cells using TRIzol reagent (Invitrogen). The cDNA was synthesized and amplified from 200ng of total RNA using OneStep RT-PCR Kit (Qiagen) with primers specific to *Asxl2*: forward, 5'-CGAGCACTGATCAACAAGCAC- 3', reverse, 5'-TCTTGTCGAATTCT-CACCTGC- 3' or *Actin* control: forward, 5'-TCACCCACACTGTGCCCATCT- 3', reverse, 5'- TGGTGAAGCTGTAGCCACGCT- 3'. All reactions were run at the same time in a final volume of 25 ul, at an annealing temperature of 50°C, and for 30 cycles.

3.3.7 WTIP expression in E18.5 mouse epicardium

Next we examined WTIP expression on frozen E18.5 heart sections (Fig. 20). WTIP is expressed in both epicardium and myocardium. Cells in the myocardium exhibit strong nuclear localization of WTIP. In contrast, in the epicardium, WTIP is distributed in a diffused pattern without apparent nuclear localization.

Together, our results show that *Asxl2* and WTIP are both expressed in mouse embryonic epicardial cells, suggesting functional roles for these proteins in the epicardium.

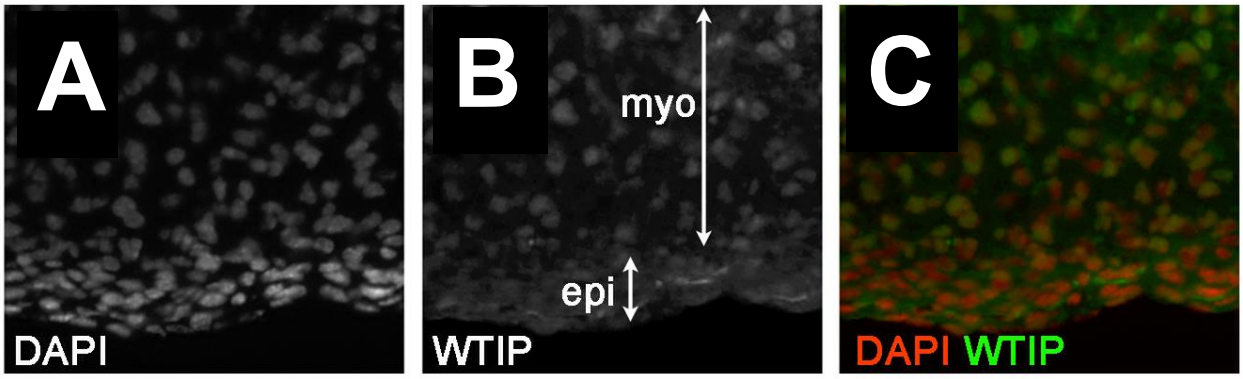


Figure 20. WTIP expression in embryonic mouse heart sections. (A-C) E18.5 mouse heart sections were stained with WTIP antibody. Shown are DAPI (A), WTIP (B), and merged (C) images. epi: epicardium; myo: myocardium.

3.4 Discussion

3.4.1 Structure and function of ASXL family proteins

Mammalian ASXLs are large proteins ranging from 1370 to 2259 amino acids. Sequence analyses have identified several short conserved regions interspersed with long stretches of divergent sequences (Fisher et al. 2006; Aravind and Iyer 2012; Katoh and Katoh 2003, 2004). ASXL proteins do not have enzyme domains or sequence-specific DNA binding domains, suggesting that they likely function as co-factors for other proteins or as molecular scaffolds for multi–protein assemblies. Indeed, recent studies have identified several protein partners for ASXL family members, including the histone deubiquitinase BAP1 (Scheuermann et al. 2010), the histone methyltransferase complex PRC2 (Lai and Wang 2013), and the nuclear receptors RARα (Cho et al. 2006), PPARγ (Park et al. 2011), and LXRα (Park et al. 2014). Here, we report the identification of another ASXL protein partner, WTIP. Using luciferase assays, we show that ASXL2 enhances, while WTIP inhibits, RA-induced transcription in HeLa cells. Furthermore, WTIP is able to block the stimulating effect of ASXL2 on RA signaling.

3.4.2 Potential mechanism of ASXL2 in retinoic acid signaling

The ability of ASXL2 to enhance RA signaling in HeLa cells is consistent with previous reports that ASXL2 facilitates nuclear receptor-mediated signaling, namely PPARγ regulation in adipocytes (Park et al. 2011) and LXRα regulation in hepatocytes (Park et al. 2014). In 3T3-L1 cells, ASXL2 has been shown to positively regulate signaling during adipogenesis by recruiting H3K9ac and H3K4me3, which are activating histone modifications, and the H3K4 methyltransferase MLL1 (Park et al. 2011). In HeLa cells, ASXL2 may be utilizing a similar mechanism to enhance RA signaling by

recruiting activating histone marks to target gene promoters. Whether this is indeed the mechanism awaits future study.

3.4.3 Functional differences between full-length and C-terminal ASXL2

Interestingly, while the C-terminus of ASXL2 is able to both interact with WTIP and activate RA signaling, the full-length ASXL2 does neither (Fig. 6D and Fig. 14). One possibility is that the N-terminus of ASXL2 has an auto-inhibitory effect that interferes with the functions of the C-terminal region. This type of auto-regulatory mechanism has been well-characterized in several transcription factors and signaling proteins (Pufall and Graves 2002). The inhibition may be relieved in the presence of a protein-binding partner that induces conformational changes. Alternatively, full-length ASXL2 may require specific partners to fold properly and such partners may be lacking in yeast and HeLa cells. Consistent with this scenario, analysis of ASXL2 sequence using PONDR-FIT (Xue et al. 2010), a program that predicts intrinsically disordered proteins (IDPs), identified long stretches of disordered regions. An important feature of IDPs is that the whole or part of the protein transitions to a more ordered structure upon binding the right partner (Espinoza-Fonseca 2009).

3.4.4 ASXL2 and WTIP interaction may regulate retinoic acid signaling during heart development

The co-expression of ASXL2, WTIP, and WT1 in embryonic epicardial cells raises the possibility that functional interactions between these proteins may be part of the mechanism regulating RA signaling during heart development. Consistent with their roles in transcriptional regulation, both ASXL2 and WT1 are nuclear proteins (Lai et al.

2012; Moore et al. 1999). On the other hand, WTIP has been shown to shuttle between the nuclei and cytoplasm (Srichai et al. 2004; Kim et al. 2010). Various studies have established that RA signaling has to be tightly regulated as too much or too little production can result in heart defects (Niederreither et al. 1999; D'Aniello et al. 2013). One possibility is that WTIP is poised in the perinuclear region so that it can quickly translocate into the nucleus and repress transcription when RA signaling activity has passed a certain threshold. This regulation could be part of a negative-feedback mechanism that keeps RA fluctuations in the growing heart under control.

Curiously, at E18.5, WTIP localizes to nuclei in the myocardium, but not in the epicardium or in primary epicardial cells (Fig. 18, 20). This difference in localization suggests a mechanism for differential regulation of RA signaling within the heart. At this developmental stage, which is close to birth, RA signaling may not play an active role in the epicardium and therefore, does not need to be closely monitored/regulated. We expect ASXL2 to enhance RA signaling in the epicardium, where WTIP is cytoplasmic. On the other hand, the activating effect of ASXL2 would be blocked by nuclear WTIP in the myocardium.

Finally, it is conceivable that the spatial pattern of WTIP localization is regulated by developmental signals, providing further means to dynamically regulate RA signaling in the heart. In the myocardium, WTIP may be actively repressing RA signaling and this may be a developmental cue for other pathways to take effect such as the Wnt signaling or fibroblast growth factor (FGF) signaling pathways, both of which are important during heart development.

4. POTENTIAL ROLE FOR ASXL2-WTIP INTERACTION IN THE REGULATION OF WNT SIGNALING

4.1 Introduction

We have discovered that the ETP protein ASXL2 directly interacts with the ZYXIN family member WTIP (Chapter 3). WTIP was initially discovered due to its interaction with the transcriptional regulator WT1 (Srichai et al. 2004). The *WT1* gene is best known for its role during embryonic kidney development and in the childhood kidney cancer Wilms' tumor. However, it is also essential for the development of several other organs including the spleen, central nervous system, and the heart (Wagner et al. 2003). WT1 has at least 36 different isoforms and is involved in regulating cell differentiation, proliferation, and apoptosis. It can act as a tumor suppressor or an oncogene, depending on the cellular context (Huff 2011).

During embryonic heart development, WT1 is confined to the epicardium and regulates the epithelial-to-mesenchymal transition (EMT) (Martínez-Estrada et al. 2010). Epicardial cells undergo the EMT process to give rise to cells that make up the coronary vasculature and cardiomyocytes. Studies using knockout mice showed that WT1 is required for normal epicardial EMT (von Gise et al. 2011). Furthermore, it was observed that WT1 regulates epicardial EMT through multiple signaling pathways, including canonical Wnt signaling, non-canonical Wnt signaling, and retinoic acid signaling (von Gise et al. 2011).

Existing research suggests that WTIP may regulate Wnt signaling activity independent of WT1 as well. In Cos-1 cells, WTIP was observed to repress canonical

Wnt signaling activity in a dose-dependent manner (van Wijk et al. 2009). Furthermore, WTIP repressed Wnt-induced secondary axis formation in the *Xenopus* embryo (van Wijk et al. 2009).

Based on these observations, and the fact that ASXL2 and WTIP are expressed in epicardial cells (Chapter 3), we hypothesized that ASXL2-WTIP interaction may play a role in Wnt signaling activity, either through or independently of WT1.

4.2 Results

4.2.1 Testing the effect of WT1 isoforms on Wnt signaling activity

Of the four major WT1 isoforms (WT1a, b, c, and d), the –KTS isoforms (WT1a and WT1b) lack a three amino acid insertion that inhibits DNA binding. Only these isoforms are able to function efficiently in transcriptional regulation (Kramarzova et al. 2012). Additionally, WT1b isoform contains exon 5, which encodes an activation domain (Kramarzova et al. 2012). In HeLa cells, this isoform enhanced the transcriptional activity of a WT1-dependent promoter and WTIP inhibited that effect (Srichai et al. 2004). Since the Wnt signaling pathway is a major target of WT1, we decided to test whether WT1b is able to induce Wnt signaling in HeLa cells. If this tested positive, we would then test the effect of WTIP, ASXL2, and both proteins on the WT1-induced Wnt signaling activity.

The effect of WT1b on Wnt signaling activity in HeLa cells was examined using a TopFlash luciferase reporter. When Wnt3A conditioned media was used, there was enhanced Wnt signaling activity (Fig. 21). However, increasing amounts of WT1b (100ng-300ng) were unable to induce Wnt signaling (Fig. 21).

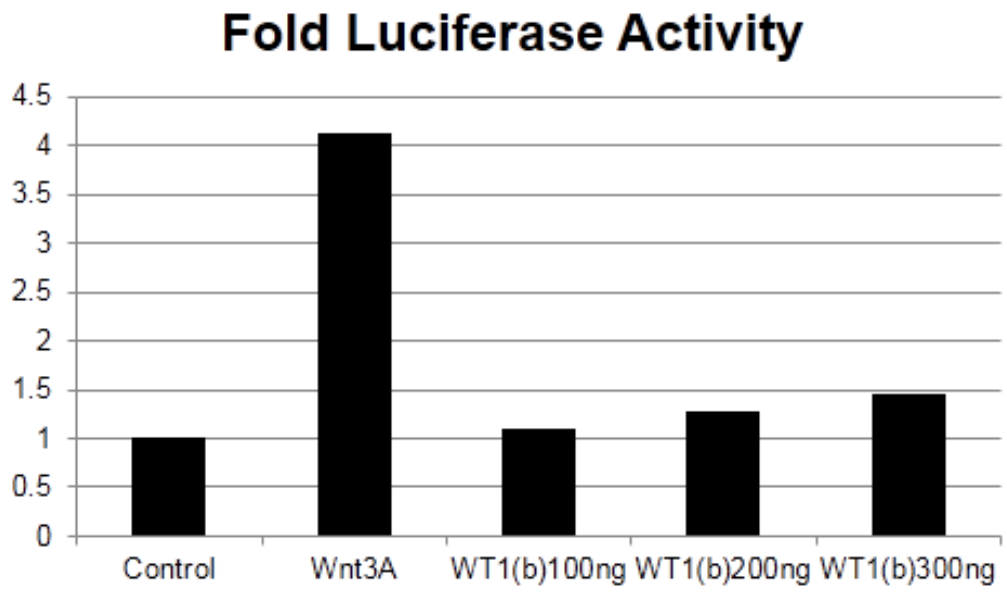


Figure 21. Effect of WT1b on Wnt signaling. HeLa cells were transfected with TopFlash luciferase and pSV-β-gal reporter constructs and increasing amounts of WT1b. Wnt3A conditioned media was used for positive control. Luciferase activity was measured and normalized to β-gal activity. (n=1)

We next tested the effect of all four WT1 isoforms (a,b,c and d) on Wnt signaling activity in HeLa cells using the same scheme as described above (Fig. 22). This time, two different positive controls were used, Stemolecule CHIR99021 (a GSK3β inhibitor), and Wnt3A conditioned media. Wnt3A conditioned enhanced Wnt signaling activity the most. WT1b isoform was also able to induce transcription more than CHIR99021, but less than Wnt3A media. Interestingly, WT1c,d were also able to induce transcription, despite containing the KTS amino acids insertion which inhibits DNA binding.

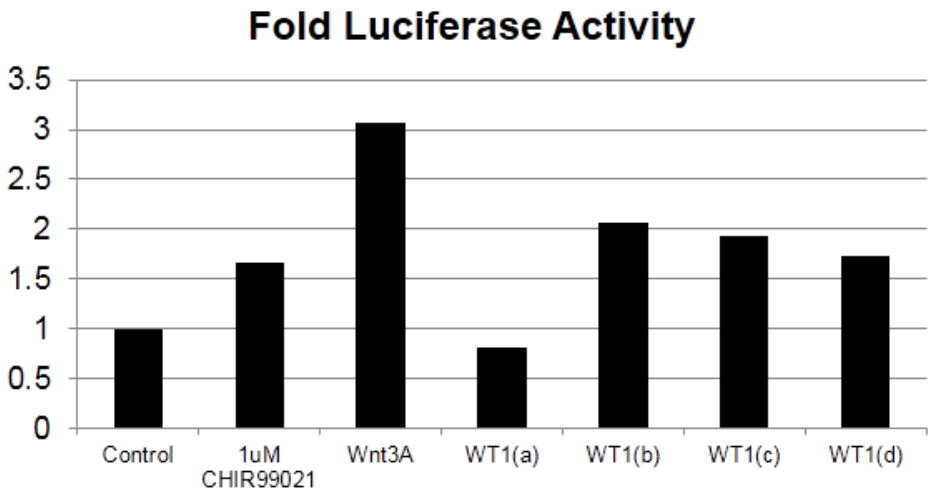


Figure 22. Effect of four WT1 isoforms on Wnt signaling. HeLa cells were transfected with TopFlash luciferase and pSV-β-gal reporter constructs and four different WT1 isoforms (500ng). CHIR99021 and Wnt3A conditioned media were used for positive control. Luciferase activity was measured and normalized to β-gal activity. (n=1)

4.2.2 Testing the effect of ASXL2 and WTIP on WT1b-induced Wnt signaling

The results from Figure 22 indicated that WT1b is able to enhance, albeit only moderately, Wnt signaling. We next investigated the effect of ASXL2 and WTIP on the WT1b-dependent Wnt signaling activity. HeLa cells were transfected with WT1b and $ASXL2_{720\text{-}1370}$, WTIP, or both (Fig. 23) and luciferase activity was compared to the Wnt3A control. This time, we did not detect enhancement of Wnt signaling with WT1b. However, there was a slight decrease in transcriptional activity in the presence of WTIP. This suggests that WTIP may play a repressive role in Wnt signaling. ASXL2 did not have an effect on transcription. More experiments need to be conducted and with the positive control working properly in order to make any strong conclusions.

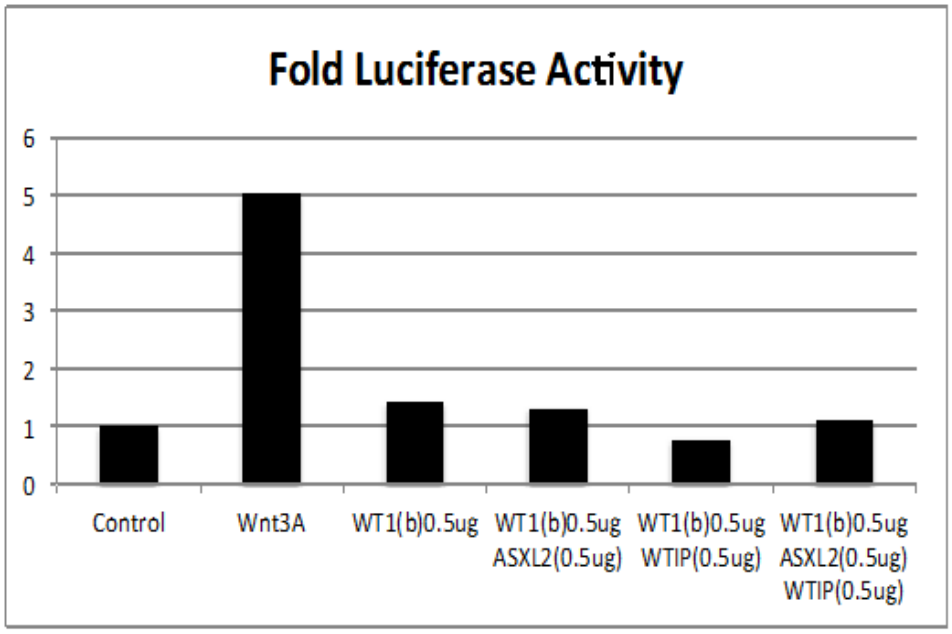


Figure 23. Effect of ASXL2 and WTIP on WT1b-induced Wnt signaling. HeLa cells were transfected with TopFlash luciferase and pSV-β-gal reporter constructs and WT1b, with or without $ASXL2_{720\text{-}1370}$ and WTIP. Wnt3A conditioned media was used for positive control. Luciferase activity was measured and normalized to β-gal activity. (n=1)

4.2.3 Testing the effect of WTIP and ASXL2 on Wnt signaling independent of WT1

In order to test our second hypothesis, that ASXL2-WTIP interaction regulates Wnt signaling activity independent of WT1, we first checked to see if WTIP alone is able to repress transcription of Wnt-dependent luciferase reporter in HEK293 cells (Fig. 24). Wnt3A conditioned media enhanced transcriptional activity of the reporter, but the presence of WTIP inhibited that effect, suggesting that WTIP negatively regulates Wnt signaling.

Since $ASXL2_{720\text{-}1370}$ was shown to enhance RA signaling activity and WTIP inhibited that effect (Chapter 3), we were curious to see whether these two proteins had a similar effect on Wnt signaling. Interestingly, we observed that $ASXL2_{720\text{-}1370}$ and WTIP both repress Wnt-dependent transcription (Fig. 25). However, WTIP-mediated repression was more pronounced that ASXL2-mediated repression. This result is interesting as it suggests that ASXL2 plays opposite roles in transcriptional regulation in RA signaling and Wnt signaling pathways. Nevertheless, these experiments need to be repeated in order to confirm these preliminary results.

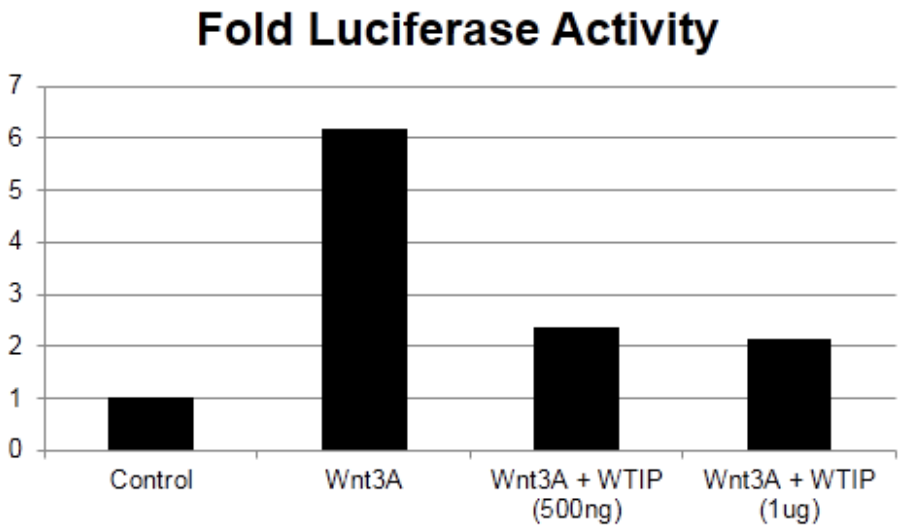


Figure 24. WTIP represses Wnt signaling activity in HEK293 cells. HEK293 cells were transfected with TopFlash luciferase and pSV-β-gal reporter constructs and Wnt3A, with or without WTIP. Luciferase activity was measured and normalized to β-gal activity. (n=1)

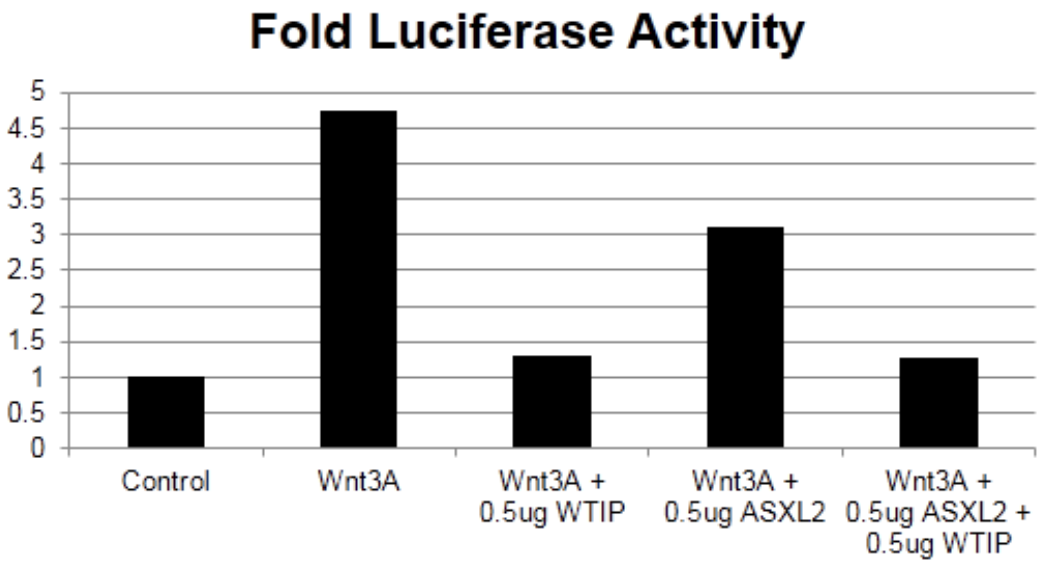


Figure 25. WTIP and ASXL2 negatively regulate Wnt signaling activity in HEK293 cells. HEK293 cells were transfected with TopFlash luciferase and pSV-β-gal reporter constructs and Wnt3A, along with WTIP, $ASXL2_{720\text{-}1370}$, or both. Luciferase activity was measured and normalized to β-gal activity. (n=1)

4.3 Discussion

Based on existing research, we initially hypothesized that WT1 would be able to enhance Wnt signaling in HeLa cells and that would provide us a system in which to test the role of ASXL2 and WTIP in this signaling pathway. While we did observe such an effect of WT1 on Wnt signaling in this cell line, the effect was moderate and not robust enough for further testing of the effect of ASXL2 and WTIP (Fig. 21 and Fig. 23). This assay needs to be repeated and tested in multiple different cell lines. Perhaps, a mouse embryonic epicardial cell line would be best since WT1 is known to be a marker for the epicardium and has been shown to regulate Wnt signaling in this tissue (von Gise et al. 2011).

On two different occasions (Fig. 24 and Fig. 25) we observed that WTIP inhibits Wnt signaling activity in HEK293 cells, independently of WT1. This is consistent with existing research showing that WTIP acts as a transcriptional repressor in the Wnt pathway (van Wijk et al. 2009). This, in addition to our results showing that WTIP negatively regulates RA signaling (Chapter 3), suggests that WTIP's general function may be to inhibit transcription in multiple signaling pathways. The molecular mechanism through which WTIP carries out this function requires further testing.

5. GENERAL DISCUSSION

5.1 ZYXIN family proteins and their roles in the developing heart

The biological functions of ZYXIN family proteins seem to be dependent on their cellular localization. For example, when not in the nucleus, the protein Migfillin has been found to be localized to cell-extracellular matrix focal adhesion points where it interacts with actin filaments and adhesion proteins to orchestrate proper cell morphology (Tu et al. 2003). Similarly, the protein ZYXIN is predominantly localized to focal adhesions and is required for the accumulation and localization of certain focal adhesion proteins (Garvalov et al. 2003; Drees et al. 2000). When localized to the nucleus, these proteins have been shown to associate with TFs and regulate gene transcription (Kadrmas and Beckerle 2004). Three proteins from this family, Ajuba, Migfillin, and ZYXIN have been shown to be involved in heart development through their roles in transcriptional regulation.

Ajuba was shown to interact with the transcription factor Isl1 and repress its transcriptional activity in cell-based assays (Witzel et al. 2012). Isl1 is a LIM/homeodomain TF and is a marker for cardiac progenitors of the secondary heart field (SHF), which gives rise to the right ventricle and outflow tract of the heart (Cai et al. 2003; Dyer and Kirby 2009). Ajuba knockdown in Zebrafish embryos resulted in an expansion of the SHF progenitor cells and an increased number of cardiomyocytes at the arterial and venous poles (Witzel et al. 2012). Furthermore, the Isl1-Ajuba complex repressed Isl1 expression in the developing Zebrafish heart in a RA-dependent manner. It was therefore concluded that Ajuba plays a critical role during heart development in regulating the SHF by linking RA signaling to Isl1 function.

During heart development, ZYXIN was shown to play a regulatory role in the epithelial-to-mesenchymal transition (EMT) process. During the EMT process, cells undergo a series of morphological and molecular changes resulting in a loss of cell-cell contacts and loss of N-cadherin and adhesion molecules on the cell surfaces. The cell shape changes as the cytoskeleton is getting reorganized so that cells can acquire a more motile phenotype in order to migrate (Son and Moon 2010). During heart development, one of the processes during which EMT occurs is during formation of the atrioventricular canal (AVC), which gives rise to the heart valves and septa (Sakabe et al. 2005). Interestingly, ZYXIN is expressed in the AVC of E13.5 mouse heart and is required for TGF-β1-induced EMT (Mori et al. 2009). Furthermore, Twist1, a key TF that promotes EMT, requires Zyxin for induction of actin reorganization and cell migration (Mori et al. 2009). Thus, these results implicate Zyxin as a key player in the EMT process of the developing AVC.

Lastly, the protein Migfillin was also shown to be involved in heart development. Migfillin is expressed in the embryonic mouse heart and showed direct interaction with the homeodomain TF Nkx2.5 (Akazawa et al. 2004). During early heart development, Nkx2.5 directs looping of the heart and cardiomyocyte differentiation (Lyons et al. 1995). Migfillin was observed to be a transcriptional co-activator for Nkx2.5 activity and nuclear accumulation of Migfillin enhanced cardiac differentiation (Akazawa et al. 2004). This unveiled yet another role for LIM proteins in heart development, as transcriptional co-activators. Additionally, it was observed that Migfillin translocates to the nucleus in response to increased Ca^{2+} levels. Since Ca^{2+} signaling is important in the process of cardiac hypertrophy (Frey et al. 2000), this suggests that Migfillin may be key in linking cytoplasmic events to gene regulation during heart disease.

Adding to this growing list, our research has identified a novel role for the ZYXIN family member WTIP in heart development. We show that WTIP is expressed in the epicardium and myocardium during heart development and is a transcriptional repressor of RA signaling activity. It is feasible that similar to Ajuba and Migfillin, WTIP is involved in transcriptional regulation perhaps via interactions with TFs such as WT1. The presence or absence of other nuclear factors such as ASXL2 may further fine-tune this regulation by recruiting histone-modifying complexes such as PcG/TrxG to target regions. Additionally, similar to Migfillin, WTIP may be involved in sensing external cues and physiological changes and translocating to the nucleus to relay that message into altered gene activity.

Similar to Zyxin, WTIP may also be involved in cell structure integrity and cytoskeletal organization in the heart during the EMT process. Since the epicardial cells also undergo the EMT process and we observed WTIP to be expressed in the epicardium, WTIP may be involved in regulating the EMT process in this tissue. It may accomplish this via transcriptional regulation through interactions with TFs and other nuclear proteins or via cytoskeletal organization though interactions with actin filaments and adhesion proteins.

Overall, accumulating evidence suggests various important roles for ZYXIN family of LIM proteins in cardiac development. These proteins are expressed in various regions of the growing heart and regulate different processes, including EMT, cell differentiation, and morphogenesis. Further research on this family is needed as these proteins may serve as diagnostic or therapeutic tools in identifying and treating various conditions of the developing heart.

5.2 Is ASXL2 a molecular hub for protein interactions?

Although my thesis work focuses on the interaction of ASXL2 with WTIP, our overall yeast two-hybrid screening results indicate that ASXL2 may serve as a molecular scaffold for various protein interactions. Among the other interacting partners of ASXL2 are two PcG proteins, BAP1 and EPC1. This result is consistent with ASXL2's repressive functions as it suggests that ASXL2 interacts with other PcG proteins to carry out its inhibitory effects on transcription. Work from our lab has already shown that ASXL2 interacts with BAP1 in the mouse heart and regulates its enzymatic activity (Lai and Wang 2013). EPC1 directly interacts with EZH2 and is thought to recruit PcG complexes to target region in proliferating cells (Attwooll et al. 2005). However, it should be noted that the EPC1 results in yeast confirmation trials (**Appendix J**) were inconclusive since the negative control matings also resulted in positive clones. This experiment needs to be repeated before any conclusions can be drawn.

We also confirmed in yeast that ASXL2 interacts with ZMIZ1. The *Drosophila* homolog of ZMIZ1 genetically interacts with the TrxG gene *brahma* and is required for proper Hox gene expression (Gutiérrez et al. 2003). It is possible that ASXL2 carries out its activator functions through interactions with Trx group members.

We also pulled two de-ubiquitinating enzymes in the yeast two-hybrid screen, (USP48 and USP33), several cytoplasmic proteins (ACTN2, FLNC, CNRIP1, TRAF2) and the cardiac transcriptional regulator FHL2. FHL2 is highly expressed in the heart and acts as a transcriptional co-activator/repressor for several transcription factors including β-catenin, androgen receptor, CREB and SRF (Müller et al. 2000; Wei et al. 2003; Fimia et al. 2000). This

is also in line with existing research showing ASXL2 association with the nuclear receptors PPARγ and LXRα (Park et al. 2011, 2014).

Overall, our results indicate that ASXL2 has several protein binding partners with varying functions (Fig. 26). But how is it possible for ASXL2 to bind to so many different proteins and carry out such diverse roles? The answer could lie in the structure of the ASXL2 protein itself. Several pieces of evidence from our lab show that ASXL2 may be an intrinsically disordered protein (IDP) (Chen, thesis). One of the unique features of IDPs is that they are naturally unfolded or unstructured, yet still able to perform key biological functions (Dyson and Wright 2005). Upon binding to their protein partner, IDPs go through an induced folding process which helps them fold into more stable secondary and tertiary structure (Dunker et al. 2008). This unique characteristic could be key in allowing ASXL2 to bind to different partners under different physiological conditions and to regulate different biological processes.

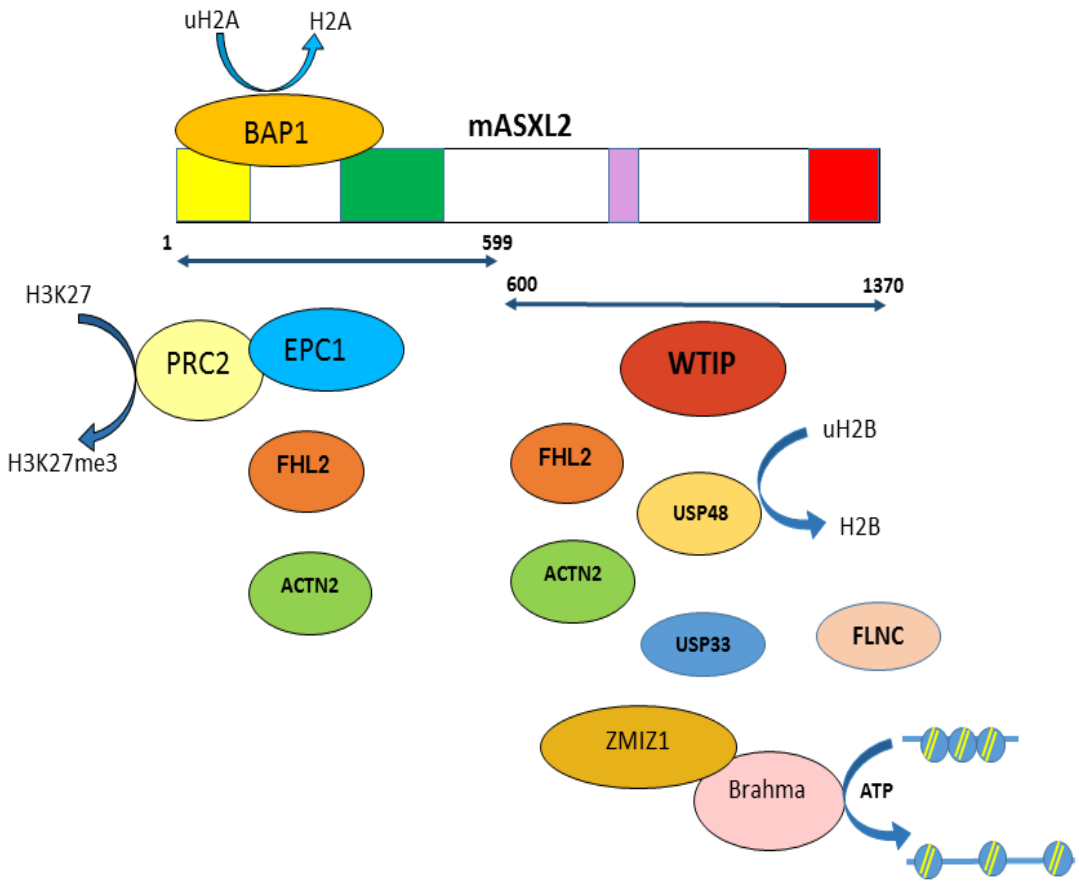


Figure 26. **ASXL2: A molecular hub of protein interactions**. Shown are the different binding partners of ASXL2 discovered in my yeast two-hybrid screen using N-terminal (aa1-599) or C-terminal (aa600-1370) fragments of ASXL2. BAP1 is a PcG member with deubiquitinating activity and has previously been shown to bind ASXL1 in the region containing wHTH and ASXH domain. WTIP is a LIM-domain protein that functions as a transcriptional repressor for WT1 activity. EPC1 is a PcG member that binds core protein EZH2 of the PRC2 complex and is responsible for tri-methylating H3K27. ZMIZ1 *Drosophila* homolog interacts with the TrxG

Brahma complex, involved in ATP-dependent chromatin remodeling. USP48 and USP33 are deubiquinating enzymes. FHL2 is a cardiac transcriptional regulator that showed interaction with both ASXL2 fragments. ACTN2 also showed interaction with both fragments and is a structural protein expressed in skeletal and cardiac muscles. FLNC is another structural protein localizing to skeletal and cardiac muscle sarcomeres.

5.3 Molecular mechanism of WTIP repression

Our results show that WTIP functions as a transcriptional repressor in RA signaling in HeLa cells and potentially Wnt signaling pathways in HeLa and HEK293 cells. This finding is consistent with existing studies that have shown that WTIP represses RA-dependent transcription in P19 cells (Hou et al. 2010), WT1-dependent transcription in 3T3 and HeLa cells (Srichai et al. 2004), and also canonical Wnt signaling activity in *Xenopus* embryos (van Wijk et al. 2009). However, the underlying molecular mechanisms through which WTIP represses gene expression are largely undefined. Mechanism of transcriptional repression include recruitment of co-factors or histone-modifying complexes to target DNA, inhibiting binding of RNA polymerase to promoter, inhibiting transcription initiation, and inhibiting clearance of RNA polymerase from promoter (Rojo 2001). Evidence suggests that WTIP likely utilizes the mechanism involving recruitment of other proteins and complexes to target region.

One possibility of how WTIP functions is that similar to Ajuba and several other LIM proteins, WTIP is an adaptor protein, bringing together multiple different proteins to act on chromatin. The ZYXIN family protein Ajuba was shown to form a multi-protein complex with Snail and PRMT5, bind target promoter region, and repress gene activity (Hou et al. 2008). PRMT5 is a protein arginine methyltransferase and methylates TFs and histone proteins. Its activity is linked to gene repression (Fabbrizio et al. 2002). Existing research already shows that WTIP interacts with the TF WT1 (Srichai et al. 2004) and our work shows that it interacts with ASXL2. The domain organization of WTIP suggests that it has the potential to bind SH3 domains and PDZ domains of other proteins as well. Thus, through these multiple protein interactions, WTIP may recruit histone-modifying complexes and other mediator proteins to target chromatin and carry out repression (Fig. 27). Theoretically, the above-proposed

mechanism suggests that WTIP could recruit co-repressors as well as co-activaors to target chromatin. However, to date, there is no evidence showing WTIP involvement in transcriptional activation.

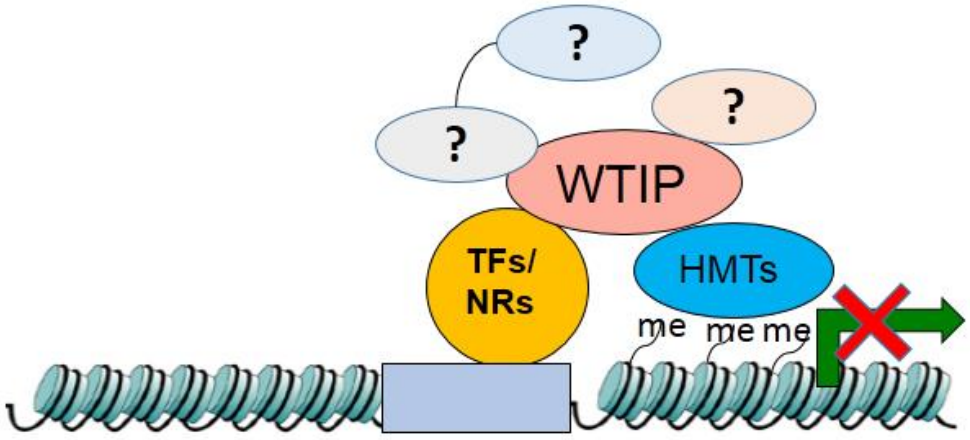


Figure 27. WTIP mechanism of action. WTIP repressive functions likely involve interactions with multiple proteins including transcription factors (TFs), nuclear receptors (NRs), histone methyltranferases (HMTs) and other co-factors (?) and recruitment of these molecules to target chromatin to modify histones and thereby repress gene expression.

5.4 ASXL2: A link between the sarcomere and the nucleus?

Striated muscle cells, including cardiac myocytes, are made up of bundles of myofibrils that contain repeating units called sarcomeres. Sarcomeres are the basic contractile units of the myocyte and are made up predominantly of thick filaments called myosin and thin filaments called actin (Frank et al. 2006). In addition, there are two other filament complexes containing the proteins titin and nebulin and numerous other contractile factors associated with various compartments of the sarcomere, making its architecture even more elaborate. Interactions between thick and thin filaments and the associated proteins allow the sarcomere to extend and contract, serving as the basis for muscle contraction. In recent years, the sarcomere is being more and more appreciated as a highly dynamic structure that is not only capable of generating force, but also capable of allowing the muscle to respond to external stimuli and respond to changes in physiological conditions (Boateng and Goldspink 2008).

The lateral borders of each sarcomere are demarcated by Z-discs. Z-discs serve as a key interface between the contractile apparatus of the sarcomere and the cytoskeleton (Boateng and Goldspink 2008). However, the Z-discs are more than just a structural anchor for the sarcomere. With the discovery of more and more proteins that are associated with the Z-discs, it is becoming clear that it is an important mediator of signal transduction that relays biological messages to the nucleus to regulate gene transcription in the cardiac muscle (Frank et al. 2006). It has been observed that the Z-disc regulates proper myocardial function by sensing changes in cell strain and mediating signaling cascades to maintain cellular homeostasis (Pyle and Solaro 2004).

Among the various molecules that interact with the Z-disc are structural and scaffolding proteins, signaling proteins, ubiquitin ligases, phosphatases, and proteins that shuttle between the Z-disc and nucleus (Frank et al. 2006; Pyle and Solaro 2004). Three of the known Z-disc proteins

also interact with ASXL2, as observed from my yeast two-hybrid experiments: alpha actinin 2 (ACTN2), four and a half LIM domains 2 (FHL2), and filamin C. FHL2 is a cardiac transcription factor that also localizes to the Z-disc (Johannessen et al. 2006). FHL2 is known to shuttle between the cytoskeleton and the nucleus and is most abundantly expressed in the heart (Chan et al. 1998; Ng et al. 2002). FHL2 acts as a transcriptional regulator for several transcription factors including β-catenin, androgen receptor, SRF (Serum Response Factor) and Hand1 (Wei et al. 2003; Müller et al. 2000; Philippar et al. 2004; Hill and Riley 2004). Since FHL2 has been observed to localize to multiple cellular compartments including the cytoplasm, nucleus, focal adhesion points, and Z-discs, it is thought to be involved in the communication between these different regions within the cell (Gautel 2008).

ACTN2 is the cardiac-specific isoform of the alpha actinin family and a major component of the Z-disc (Stromer and Goll 1972). It is a muscle specific actin binding protein that is highly expressed in the heart muscle and localizes to the Z-disc to help anchor myofibrillar actin filaments. One study has shown that in some forms of congenital myopathy, high levels of ACTN2 are found in the nuclei of muscle cells in the form of nuclear aggregates (Domazetovska et al. 2007). ACTN2 has also been observed to enhance the transcriptional activity of nuclear receptors (Huang et al. 2004). Filamin C is the striated-muscle-specific isoform of the filamin protein family and has actin-binding and cross-linking properties. It plays a role in myofibril assembly and repair and also in its attachment to the membrane (Molt et al. 2014).

ACTN2 and FHL2 are Z-disc proteins that have also demonstrated the ability to shuttle in and out of the nucleus. It is possible that these sarcomeric proteins monitor cardiomyocyte performance at the Z-disc, where they are predominantly localized, and relay the message to the

nucleus. In the event of cardiac stress, they shuttle into the nucleus and play a role in gene transcription by interacting with nuclear factors. One such factor may be the epigenetic regulator ASXL2. ASXL2 may be a central player in receiving the signal from the sarcomeric proteins and in the transfer of that information to other factors such as histone-modifying PcG complexes and chromatin remodeling TrxG complexes. If true, this hypothesis will shed new light on the questions surrounding the elaborate protein composition at the sarcomeric Z-disc, why some of those proteins display shuttling properties to the nucleus, where ASXL2 fits in the picture, and how it all ties back to cardiomyocyte function. Further research in this area may unravel ASXL2 as a key communicator of cardiac physiological signals between the sarcomere and the nucleus and may become a target molecule in new cardiovascular therapies.

APPENDICES

APPENDIX A. MAMMALIAN CO-ACTIVATION SCREEN

A1. Introduction

In parallel with the yeast two-hybrid screen, I conducted a GAL4-DNA binding domain-based high-throughput co-activation screen to identify transcriptional partners for ASXL2. This system was adapted from one developed by the Lin lab at the University of Michigan (Li et al. 2008). The Lin lab generated a transcription factor open reading frame collection (TFORC) by compiling a list of human TF and cofactor genes. The main reason why this TFORC seemed useful to us was because at that time, the homolog ASXL1 was shown to act as a transcriptional co-activator for retinoic acid receptor (Cho et al. 2006). Since nothing was known about ASXL2's function in the mammalian system at the time, we thought this assay would be a good starting point to learn more about its functional role. We hypothesized that ASXL2 might function as a transcriptional co-activator or co-repressor for some of these transcription factors.

A2. Materials and methods

In my adapted co-activation assay, mammalian BOSC cells were seeded in 96-well plates and transiently transfected with the UAS-luciferase reporter and individual GAL-TF plasmids in the presence of empty vector pcDNA3 control or pcDNA3-ASXL2 using Lipofectamine 2000 reagent. 30 hours after transfection, the cells were lysed with Glo-lysis buffer and the luciferase activity was measured following the instructions of Steady Glo reagent (Promega). A detailed protocol is included in **Appendix B**.

A3. Results

I finished screening 1025 transcription factors out of the 1388 total, in two different trials, to test the effect of ASXL2 on transcriptional activity. The results were analyzed by calculating fold differences (luciferase activity of experimental group / luciferase activity of control group). In analyzing the results, I was looking for consistency and 2-fold or greater difference in luciferase activity, in order to consider the result as being meaningful. Out of the 1025 TFs screened, 60 of them resulted in a 2-fold or higher increase in transcriptional activity, in both trials. These results are summarized in **Table I**.

Table II: Mammalian co-activation screen results

Plate Position	TF	TRIAL 1	TRIAL 2	Description
GALDBD-101/D08	TES	2.2	2.7	testis derived transcript (3 LIM domains), transcript variant 1
GALDBD-102/B02	TSC22D1	3.4	2.3	transforming growth factor beta 1 induced transcript 4, transcript variant 2
GALDBD-102/B04	CRIP2	3.0	2.2	cysteine-rich protein 2
GALDBD-102/B11	SOX30	3.6	3.4	SRY (sex determining region Y)-box 30, transcript variant 1
GALDBD-102/B12	ZNF451	4.0	2.1	Zinc finger protein 451
GALDBD-102/C09	LPXN	2.3	4.5	leupaxin
GALDBD-102/C10	GMEB1	5.3	2.1	glucocorticoid modulatory element binding protein 1, transcript variant 2
GALDBD-102/D01	BATF	2.3	6.4	basic leucine zipper transcription factor, ATF-like
GALDBD-102/D05	ING4	2.0	2.0	inhibitor of growth family, member 4, transcript variant 1
GALDBD-102/D09	IRF4	2.2	2.0	interferon regulatory factor 4
GALDBD-102/E01	POLR2I	4.6	8.4	polymerase (RNA) II (DNA directed) polypeptide I, 14.5kDa
GALDBD-102/E11	STAT5A	3.1	6.3	signal transducer and activator of transcription 5A
GALDBD-102/G06	PDLIM3	2.0	2.4	PDZ and LIM domain 3
GALDBD-103/A02	DCUN1D1	2.1	2.2	RP42 homolog
GALDBD-103/A05	PLEKHF2	2.0	2.0	pleckstrin homology domain containing, family F (with FYVE domain) member 2
GALDBD-103/A07	FHL1	2.5	2.2	four and a half LIM domains 1
GALDBD-103/A10	BFAR	2.8	2.3	bifunctional apoptosis regulator
GALDBD-103/B01	HOP	2.7	2.2	homeodomain-only protein
GALDBD-103/C07	FHL5	2.5	2.5	four and a half LIM domains 5
GALDBD-103/D04	MLX	3.1	2.4	transcription factor-like 4, transcript variant 2
GALDBD-103/D12	RNF103	3.4	2.1	ring finger protein 103
GALDBD-103/E11	ZNF202	4.0	2.8	zinc finger protein 202
GALDBD-103/E12	RHOBTB1	2.1	2.1	Rho-related BTB domain containing 1
GALDBD-103/F01	FOXP1	2.3	3.0	forkhead box P1
GALDBD-103/F04	CNOT7	4.2	4.2	CCR4-NOT transcription complex, subunit 7
GALDBD-103/F09	COPS2	2.1	3.8	thyroid receptor interacting protein 15
GALDBD-103/F10	HSF1	2.6	2.3	heat shock transcription factor 1

GALDBD-103/G02	CSRP2	2.2	2.2	cysteine and glycine-rich protein 2
GALDBD-103/G09	TGFB1I1	2.5	3.6	transforming growth factor beta 1 induced transcript 1
GALDBD-103/G10	PHF1	2.5	2.6	PHD finger protein 1, transcript variant 2
GALDBD-104/A10	RING1	3.8	3.8	ring finger protein 1
GALDBD-104/B05	NDRG4	4.4	2.0	NDRG family member 4
GALDBD-104/C09	NR1H3	2.1	2.5	nuclear receptor subfamily 1, group H, member 3
GALDBD-104/C10	ZDHHC6	3.0	3.4	zinc finger, DHHC domain containing 6
GALDBD-104/D06	THEX1	3.1	2.7	3' exoribonuclease
GALDBD-104/D10	KLHDC5	2.1	2.5	KIAA1340 protein
GALDBD-104/E12	ZBTB25	2.6	2.3	zinc finger protein 46 (KUP)
GALDBD-104/G06	SYAP1	3.9	2.3	synapse associated protein 1, SAP47 homolog (Drosophila)
GALDBD-104/H09	COPS4	2.1	2.0	COP9 constitutive photomorphogenic homolog subunit 4 (Arabidopsis)
GALDBD-105/D08	TRIM26	2.1	2.1	tripartite motif-containing 26
GALDBD-105/D09	DCP1A	3.0	3.8	transcription factor SMIF
GALDBD-105/F02	ZNF259	2.5	2.3	zinc finger protein 259
GALDBD-105/F07	RORA	2.5	2.6	RAR-related orphan receptor A, transcript variant 1
GALDBD-106/C08	REPIN1	2.8	2.8	replication initiator 1
GALDBD-107/B10	USF2	3.0	44.1	upstream transcription factor 2, c-fos interacting, transcript variant 1
GALDBD-107/B11	ZNF393	3.0	14.2	zinc finger protein 393
GALDBD-107/C11	CREB3L4	5.0	3.4	cAMP responsive element binding protein 3-like 4
GALDBD-107/D01	C6orf113	2.1	2.0	chromosome 6 open reading frame 113
GALDBD-107/E09	ZDHHC12	3.1	2.4	zinc finger, DHHC domain containing 12
GALDBD-107/E10	POGZ	4.6	3.8	pogo transposable element with ZNF domain, transcript variant 3
GALDBD-107/E11	WDFY2	2.7	2.0	WD repeat and FYVE domain containing 2
GALDBD-107/F07	FHL2	6.0	6.0	four and a half LIM domains 2, transcript variant 2
GALDBD-107/F08	ZNF364	2.7	2.4	zinc finger protein 364
GALDBD-107/F12	ARNT	2.6	2.3	aryl hydrocarbon receptor nuclear translocator
GALDBD-107/H10	LMX1B	3.9	2.2	LIM homeobox transcription factor 1, beta
GALDBD-108/A01	LEREPO4	2.6	2.4	likely ortholog of mouse immediate early response, erythropoietin 4
GALDBD-108/F04	FBXL19	2.2	2.0	F-box and leucine-rich repeat protein 19

GALDBD-108/G10	NFKB1	2.3	2.2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
GALDBD-108/H10	NFATC3	4.8	4.7	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3, transcript variant 1
GALDBD-111/C04	WWTR1	2.2	2.0	transcriptional co-activator with PDZ-binding motif (TAZ)
GALDBD-111/H01	GABPB2	0.4	0.3	GA binding protein transcription factor, beta subunit 2, 47kDa, transcript variant gamma-1

In this screen, the transcription factor that displayed the greatest fold difference in transcriptional activation consistently in the presence of ASXL2 was FHL2, with a 6-fold increase (Fig. 28). This result was exciting because FHL2 was identified in the yeast two-hybrid screen as an interactor of ASXL2 as well. FHL2 is a protein that is highly expressed in the heart and has known roles in cardiac regulation (Stathopoulou et al. 2014; Renger et al. 2013). However, it is not essential for cardiac development and function in mice (Chu et al. 2000).

In addition to FHL2, two PcG proteins were also identified in the co-activation screen, PHF1 and RING1 (Fig. 28). PHF1 belongs to the PRC2 complex and RING1 belongs to the PRC1 complex of PcG proteins. This result was consistent with our data which showed that loss of *Asxl2* disrupts PcG activity based on *Asxl2*$^{-/-}$ skeletal phenotype and the reduction in H3K27me3 in the mutant heart (Baskind et al. 2009). It suggested that ASXL2 interacts with other PcG proteins. What was intriguing was that these proteins in the presence of ASXL2 showed enhanced transcriptional activity while PcG activity in general is associated with repression.

One other transcription factor that drew our attention was a calcineurin-nuclear factor of activated T cells (NFAT) family member NFATc3 (Fig. 28). NFATc3 is implicated in hypertrophic signaling since upregulation of calcineurin results in nuclear translocation of NFATc3 and mediates pathways leading to the development of cardiac hypertrophy (Wilkins et al. 2002; Heineke and Molkentin 2006).

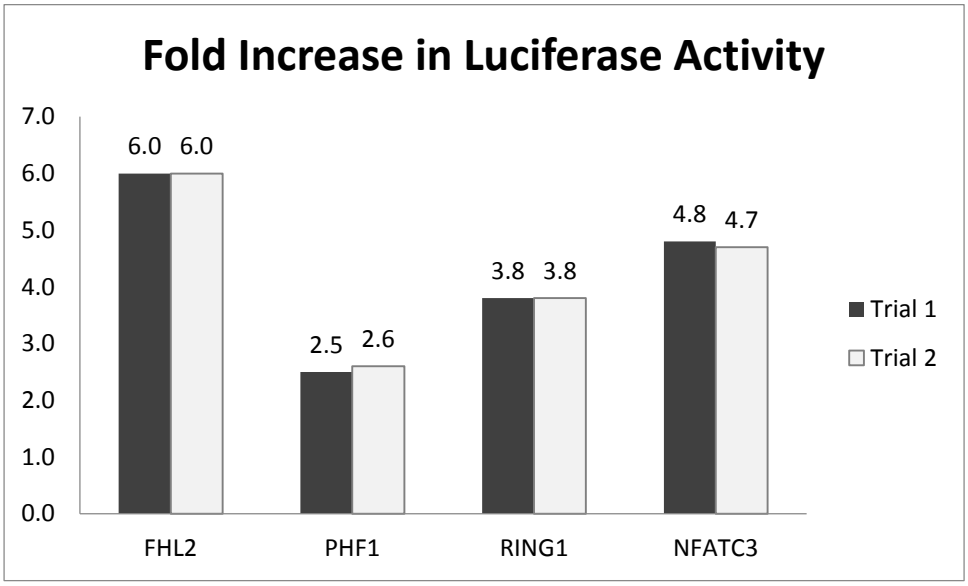


Figure 28. Transcription factors identified from co-activation screen. FHL2, PHF1, RING1, and NFATc3 were four TFs identified from our library screening that showed increased transcriptional activity in the presence of ASXL2 and seemed to be good candidates for further testing. Results from two independent trials are shown.

I conducted confirmation trials for FHL2 and PHF1 and the results are depicted in Figure 29. Although both factors showed at least a 2-fold increase in transcriptional activity in the presence of ASXL2, FHL2 showed a lower fold-increase than before while PHF1 showed a higher fold-increase than before (compare Fig. 28 and Fig. 29).

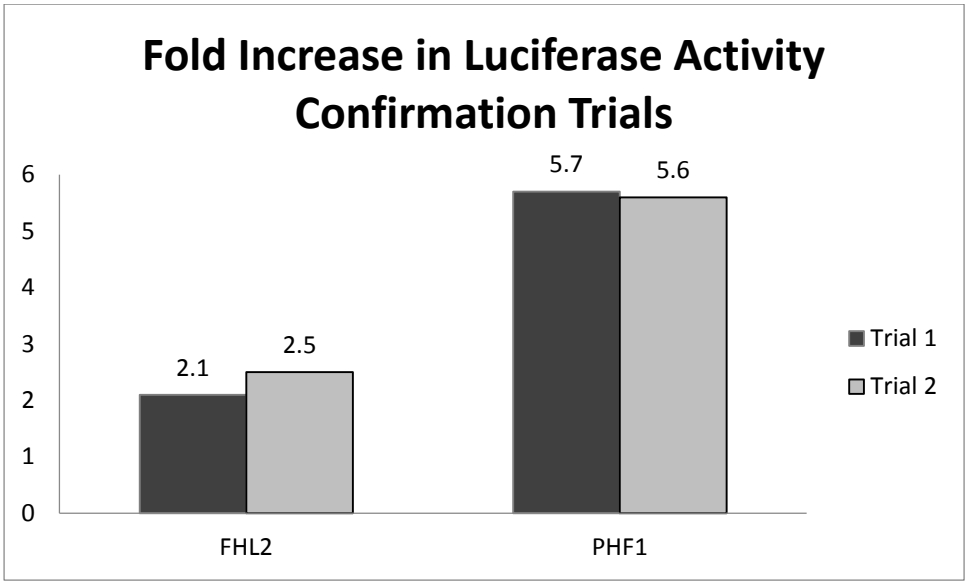


Figure 29. Confirmation trials for PHF1 and FHL2. PHF1 and FHL2 again showed at least a 2-fold increase in transcriptional activity in the presence of ASXL2. Results from two independent confirmation trials are shown.

The results for PHF1, RING1, and NFATc3 were intriguing and I conducted further luciferase-based experiments using these factors in conjunction with ASXL2. The experimental design for these tests is detailed in Appendix C and the results are highlighted in Figures 30, 31, and 32. We found that PHF1 on its own is a transcriptional repressor, and ASXL2 relieves some of that repression. However, these results were not statistically significant as there was high standard deviation. It appears as though RING1 does not have an effect on transcription, however, in the presence of ASXL2, the luciferase activity increased dramatically. Again, these results were not statistically significant and need to be repeated with less error in order to draw meaningful conclusions. NFATc3 on its own showed a dramatic increase in transcriptional activity and the presence of ASXL2 did not change that effect.

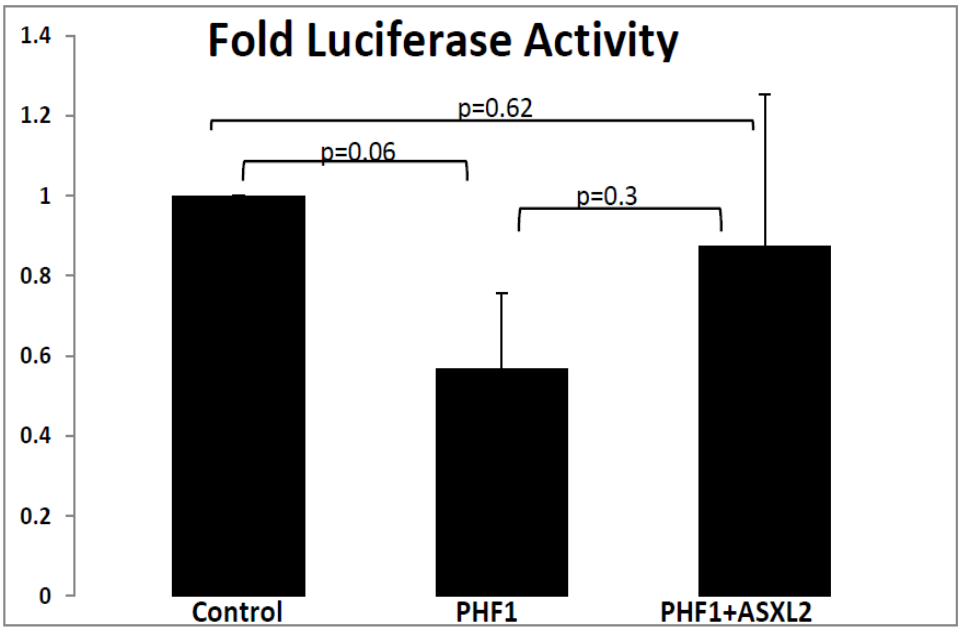


Figure 30. Effect of PHF1 and ASXL2 on transcriptional activity. Luciferase assays were conducted in 293T cells testing the effects of PHF1 alone and PHF with ASXL2 on transcription. Luciferase readings were normalized to β-gal assay readings. Error bars represent standard deviation from three independent experiments.

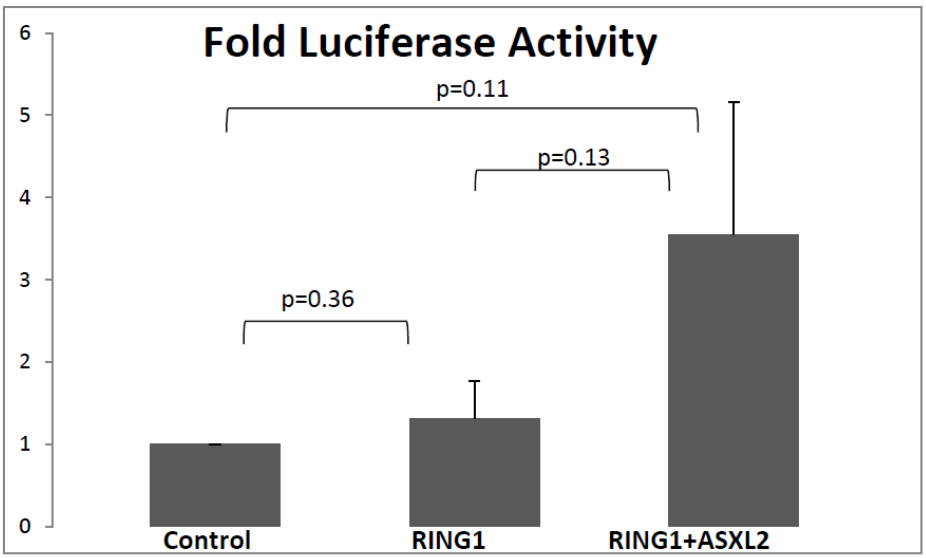


Figure 31. Effect of RING1 and ASXL2 on transcriptional activity. Luciferase assays were conducted in 293T cells testing the effects of RING1 alone and RING1 with ASXL2 on transcription. Luciferase readings were normalized to β-gal assay readings. Error bar represents standard deviation from three independent experiments.

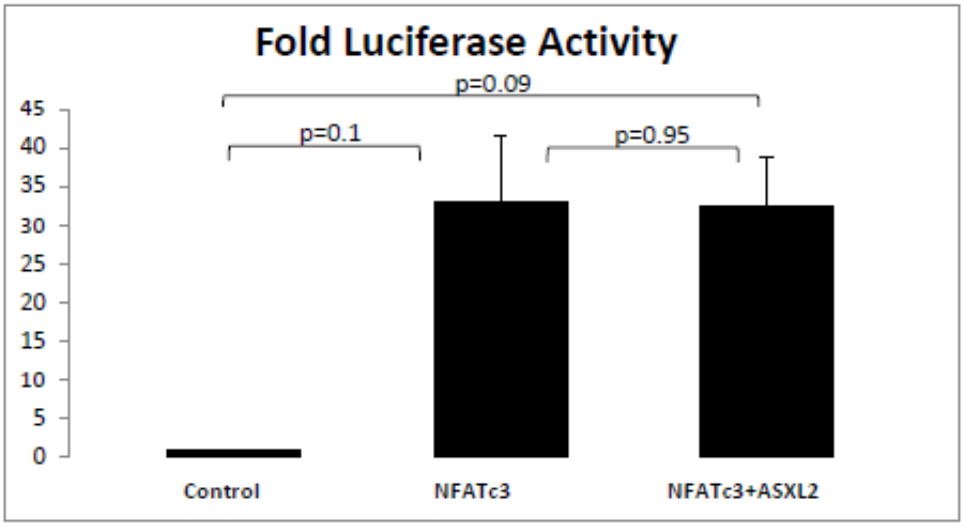


Figure 32. Effect of NFATc3 and ASXL2 on transcriptional activity. Luciferase assays were conducted in 293T cells testing the effects of NFATc3 alone and NFATc3 with ASXL2 on transcription. Luciferase readings were normalized to β-gal assay readings. Error bars represent standard deviation from two independent experiments.

A4. Discussion

Our mammalian co-activation screen resulted in numerous TFs and co-factors that were potential ASXL2 binding partners. Four of the factors that were interesting were FHL2, PHF1, RING1, and NFATc3. FHL2 seemed to be a good candidate due to its high expression in the heart and and its known roles in cardiac regulation. However, it is known to bind to numerous proteins and we wondered whether our results were due to non-specific binding of FHL2 with ASXL2. Nonetheless, we haven't done further testing with this candidate protein to support that claim.

PHF1 and RING1 were interesting candidates due to their role as PcG members. Although these proteins may be involved in transcriptional repression on their own, their involvement with ASXL2 seems to relieve that repression. Additional analysis is needed to fully understand ASXL2's role in transcriptional regulation.

NFATc3 was picked due to its involvement in cardiac hypertrophy, however, the presence of ASXL2 did not have any effect on NFATc3 transcriptional activity. It is possible that these proteins interact in a different cellular context or that they don't interact at all and the initial screening results were falsely positive due to technical errors.

APPENDIX B. CO-ACTIVATION SCREEN PROTOCOL

(For 96-well plate)

1.) Gelatin coat 2 white-walled 96-well plates (~50ul gelatin for at least 15 minutes).

2.) Seed 100 ul of $4x10^5$cells/ml concentration of BOSC cells into each well the night before transfection (Use only antibiotics-free media).

3.) The next morning, replace old media with 100 ul of fresh antibiotics-free media. Do this at least 1 hr before transfection to ensure healthy cells.

4.) DILUTE LIPOFECTAMINE: 0.77 ul Lipofectamine 2000 / well
5 ul OptiMEM / well

Divide in 12 wells and dilute for 20-30 minutes.
In the meantime, dilute DNA.

5.) DILUTE DNA: CONTROL EXPERIMENTAL

Opti-MEM-5 ul/well
UAS-LUC-10 ng/well
Divide evenly.

Add pcDNA3-100 ng/well

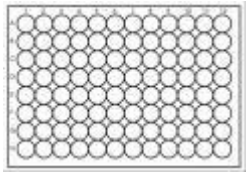
Add pcDNA3-ASXL2 100 ng/well

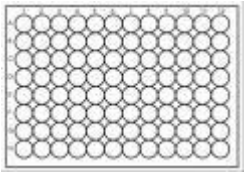
Divide evenly in 1 row.

Divide evenly in 1 row.

From this 1 row add evenly to control plate.

From this 1 row add evenly to exp. plate.





Add 2ul of TFs from the Library to each well of each plate.

6.) From the 1 row of diluted Lipofectamine 2000, add 5.5 ul/well to each of the two diluted DNA rows.

7.) Incubate diluted DNA and Lipofectamine 2000 for 30 min.

8.) Add ~10 ul of the transfection mixture to the cells.

9.) 30 hrs after transfection, measure luciferase activity following instructions of Steady Glo reagent (Promega).

APPENDIX C. EXPERIMENTAL DESIGN FOR PHF1, RING1, and NFATc3 EXPERIMENTS

Performed in duplicates in a 24-well plate.

1. Seed 1.9X10^5 293T cells per well in 0.5 ml DMEM w/ 10% FBS(w/o antibiotics) 20 hrs before transfection.

2. Feed cells with 0.25ml fresh media w/ 10% FBS(w/o antibiotics) 1 hr before transfection.

3. Transfection Mixture **per well**:

	UAS-LUC	**pCMX-Gal4**	**pGAL-PHF1**	**pGal4-RING1**	**pGal4-NFATc3**	**pcDNA3**	**pcDNA3-Asxl2**	**pSV-βgal**	**Total DNA**	**PEI**	**Opti-mem**
Tube 1	0.2ug	0.4ug				0.4ug		0.05 ug	1.05ug	3ul	200ul
Tube 2	0.2ug		0.4ug			0.4ug		0.05 ug	1.05ug	3ul	200ul
Tube 3	0.2ug		0.4ug				0.4ug	0.05 ug	1.05ug	3ul	200ul
Tube 4	0.2ug			0.4ug		0.4ug		0.05 ug	1.05ug	3ul	200ul
Tube 5	0.2ug			0.4ug			0.4ug	0.05 ug	1.05ug	3ul	200ul
Tube 6	0.2ug				0.4ug	0.4ug		0.05 ug	1.05ug	3ul	200ul
Tube 7	0.2ug				0.4ug		0.4ug	0.05 ug	1.05ug	3ul	200ul

Mix the above, vortex 1 sec, incubate at room temp. at least 15 min.

3. Add mixture to wells.

4. 4-6 hrs later, add 0.25 ml fresh media(complete).

5. 48 hrs later, conduct luciferase assay using Steady Glo reagent (Promega) and record activity.

6. Luciferase activity is normalized to β-gal activity.

APPENDIX D. TESTING BAIT FOR AUTO-ACTIVATION IN YEAST

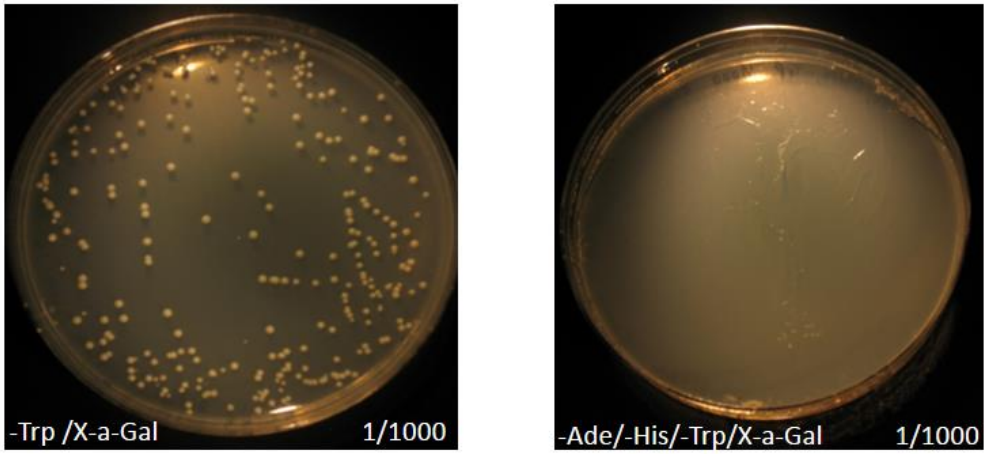


Figure 33: The pGBKT7-ASXL2 bait construct does not self-activate in yeast. The pGBKT7-ASXL2 bait construct was cultured overnight and plated on the indicated yeast drop-out media. There was no self-activation of X-α-Gal or Ade and His reporters.

APPENDIX E. TESTING BAIT FOR TOXICITY IN YEAST

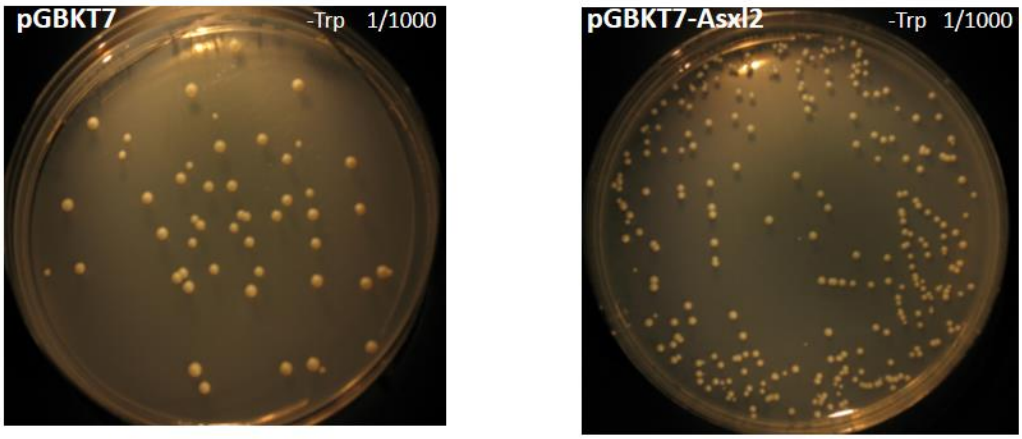


Figure 34: The pGBKT7-ASXL2 bait construct is not toxic in yeast. The pGBKT7-ASXL2 bait construct was cultured overnight and plated on the indicated yeast drop-out media. The colonies, albeit smaller in size compared to the empty vector colonies, were healthy and actively growing.

APPENDIX F. CONTROL MATING EXPERIMENTS IN YEAST

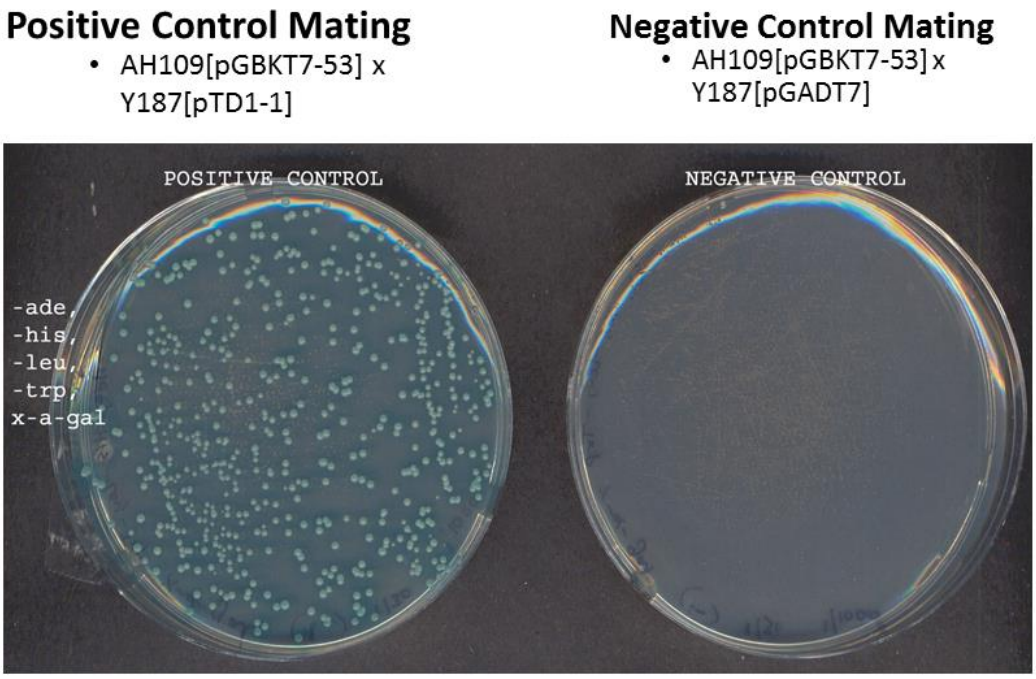


Figure 35: Positive and negative control matings in yeast. The pGBKT7-53 and pTD1-1 cultures were mated overnight and plated on the indicated yeast drop-out media. Healthy, actively growing colonies that were blue in color were considered positive clones. The pGBKT7-53 and pGADT7 mating was also plated on the same drop-out media and served as the negative control.

APPENDIX G. NUMBER OF CLONES SCREENED

The number of clones screened was calculated as described in section **2.2.6**.

Number of clones screened using Full-length ASXL2 as bait:

3.9×10^7 CFU/ml X 13 ml = 5×10^8 clones

Number of clones screened using N-terminal ASXL2 (aa1-599) as bait:

8.15×10^6 CFU/ml X 12.5 ml = 1×10^8 clones

Number of clones screened using C-terminal ASXL2 (aa600-1370) as bait:

10×10^6 CFU/ml X 12.5 ml = 1.3×10^8 clones

APPENDIX H. MATING EFFICIENCY

The mating efficiency was calculated as described in section **2.2.7**.

Mating efficiency using Full-length ASXL2 as bait:

$(3.9 \text{ X } 10^7 / 7.6 \text{ X } 10^7) \text{ X } 100 = 51 \%$

Mating efficiency using N-terminal ASXL2 (aa1-599) as bait:

$(5.5 \text{ X } 10^6 / 2.5 \text{ X } 10^7) \text{ X } 100 = 22 \%$

Mating efficiency using C-terminal ASXL2 (aa600-1370) as bait:

$(7.6 \text{ X } 10^6 / 2.2 \text{ X } 10^7) \text{ X } 100 = 34 \%$

APPENDIX I. YEAST TWO-HYBRID SCREENING RESULTS

TABLE III: YEAST TWO-HYBRID SCREENING RESULTS

	Clones screened	**Mating Efficiency**	**Strong**	**Weak**	**Clones Identified (strong interactors)**
Full Length Asxl2	5 x 10^8	51%	16	>10	8- FHL2 (FL-1,3,4,5,6,11,15,16) 4-ACTN2 (FL-2,9,12,14) 1-BAP1 (FL-10) 1-WTIP (FL-13) 1-rearranged l-myc fusion seq. (FL-7) 1-chr 6 genomic contig seq. (FL-8)
N-term-Asxl2 (aa1-599)	1 x 10^8	22%	12	8	7-BAP1 (N-1,2,6,8,9,10,12) 2-EPC1 (N-3,4) 2-CNRIP1 (N-5,7) 1-FHL2 (N-11)
C-term-Asxl2 (aa600-1370)	1.3 x 10^8	34%	24	>10	7-ACTN2 (C-2,4,5,7,10,14,17) 4-FHL2 (C-3,6,12,13) 5-FLNC (C-8,15,16,19,22) 1-TRAF2 (C-1) 1-USP48 (C-9) 1-USP33 (C-21) 1-WTIP (C-20) 1-ZXDC1 (C-11) 1-ZMIZ1 (C-18) 2-hypothetical protein LOC100506727 (C-23,24)

APPENDIX J. CONFIRMATION OF PROTEIN INTERACTIONS IN YEAST

TABLE IV. CONFIRMATION TRIALS OF PROTEIN INTERACTIONS IN YEAST

	Trials	Full-Length Asxl2	N-term. Asxl2	C-term. Asxl2	vector(pGBKT7)
ACTN2	T1	+++	++	+++	-
	T2	+++	++	+++	-
	T3	+++	++	+++	-
BAP1	T1	++	+++	-	-
	T2	++	+++	-	-
	T3	++	+++	-	-
EPC1	T1	+	+++	-	+++
	T2	+	+++	-	+++
	T3	+	+++	-	+++
FHL2	T1	+++	++	+++	-
	T2	+++	++	+++	-
	T3	+++	+	+++	-
FLNC	T1	++	-	+++	++
	T2	++	-	+++	+
	T3	++	-	+++	++
TRAF2	T1	+	-	+++	-
	T2	++	-	+++	-
	T3	++	-	+++	-
USP33	T1	++	+++	++	-
	T2	+	+	+++	-
	T3	++	++	+++	-
USP48	T1	+	-	+++	-
	T2	+	-	+++	-
	T3	-	-	+++	-
WTIP	T1	+	-	+++	-
	T2	+	-	+++	+
	T3	+	-	+++	-
ZMIZ1	T1	+++	+++	+++	-
	T2	+	++	+++	-
	T3	++	+	+++	-

NOTE: + represents intensity of the interaction based on the blue color of the colonies.
– indicates no interaction/no blue colonies present

APPENDIX K. WESTEN BLOT CONDITIONS FOR CO-IMMUNOPRECIPITATIONS

TABLE V. WESTERN BLOT CONDITIONS FOR CO-IMMUNOPRECIPITATIONS

1° ANTIBODY (dilution)	**COMPANY** (Catalog #)	**GEL %**	**2° ANTIBODY** (dilution)
Rabbit-α-FLAG (1:2000)	Sigma-Aldrich (F7425)	7%	Donkey-α-rabbit-HRP (1:10,000)
Rabbit-α-C-Myc (1:1000)	Santa Cruz Biotechnology (SC-789)	10%	Donkey-α-rabbit-HRP (1:10,000)
Mouse-α-HA.11 (1:1000)	Covance (MMS-101P)	10%	Goat-α-mouse-HRP (1:10,000)
Rabbit-α-HA (1:50)	Santa Cruz Biotechnology (SC-805)	12%	Donkey-α-rabbit-HRP (1:5000)

APPENDIX L. β-GALACTOSIDASE ASSAY

Z buffer (per 50 ml):

0.43 g $Na_2HPO_42H_2O$ (0.06M)

0.24 g NaH_2PO_4 (0.04M)

0.5 ml 1M KCl (0.01M)

0.123 g $MgSO_47H_2O$ (0.001M)

Adjust pH to 7.0

Store at 4°C

β-galactosidase assay solution with ONPG:

Prepare 180 ul solution per 50 ul cell lysate.

144 ul Z buffer

36 ul 4mg/ml ONPG (o-Nitrophenyl-β-D-galactopyranosidase)

1 ul BME (β-mercaptoethanol)

Mix the assay solution with 50 ul cell lysate and incubate at 37°C for 30 mins.

Measure β-galactosidase activity using a plate reader.

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Licensed content author	Farida F. Khan,Yanyang Li,Arjun Balyan,Q. Tian Wang
Licensed content date	Available online 24 July 2014
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Licensed content issue number	n/a
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Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
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Total	0.00 USD

APPENDIX N. PLASMID INFORMATION
TABLE VI. PLASMID INFORMATION

Plasmid	Alternate name	Resistance	Original vector	From	Species
pCMV-tag-myc-WTIP	Myc-WTIP	Kanamycin	Stratagene (pCMVtagmyc)	Sedor Lab	human
pCMV-tag-mycNΔ-WTIP	Myc-NΔWTIP	Kanamycin	Stratagene (pCMVtagmyc)	Sedor lab	mouse
pCMV-tag-mycNΔ-ZYXIN	Myc-NΔZYXIN	Kanamycin	Stratagene (pCMVtagmyc)	Sedor Lab	mouse
pGST-NΔ-WTIP	GST-NΔWTIP	Ampicillin	GE Healthcare (pGEX)	Sedor Lab	mouse
pcDNA3-Flag-CT-Asxl2	FLAG-ASXL2$_{720-1370}$	Ampicillin	Invitrogen (pcDNA3)	Wang Lab	mouse
pcDNA3-HA-wtipΔlim2,3	WTIPΔLD2,3-HA	Ampicillin	Invitrogen (pcDNA3)	Stricker Lab	mouse
pcDNA3-HA-WTIP	HA-WTIP	Ampicillin	Invitrogen (pcDNA3)	Stricker Lab	mouse
HA-Lim2,3	LD2,3-HA	Ampicillin	pCS2-HA3	Hua Lab	mouse
pACT2		Ampicillin	Clontech	Wang lab	
pGBKT7-53		Kanamycin	Clontech	Wang lab	
pTD1-1		Ampicillin	Clontech	Wang lab	
pGBKT7		Kanamycin	Clontech	Wang lab	
pGBKT7-Asxl2	FL-ASXL2	Kanamycin	Clontech	Wang lab	mouse
pGBKT7-N-Term-Asxl2	ASXL2$_{1-599}$	Kanamycin	Clontech	Wang lab	mouse
pGBKT7-C-Term-Asxl2	ASXL2$_{600-1370}$	Kanamycin	Clontech	Wang lab	mouse
pGBKT7-C-Term-Asxl2ΔPHD	ASXL2$_{600-1295}$	Kanamycin	Clontech	Wang lab	mouse
pGBKT7-PHD	ASXL2$_{1313-1370}$	Kanamycin	Clontech	Wang lab	mouse
pcDNA3		Ampicillin	Invitrogen	Wang Lab	
pcDNA3-mWT1-a		Ampicillin	Invitrogen	Minden Lab	mouse
pcDNA3-mWT1-b		Ampicillin	Invitrogen	Minden Lab	mouse
pcDNA3-mWT1-c		Ampicillin	Invitrogen	Minden Lab	mouse
pcDNA3-mWT1-d		Ampicillin	Invitrogen	Minden Lab	mouse
TopFlash-luc		Ampicillin	Randall Moon	Merrill Lab	
pSV-β-gal		Ampicillin	Promega	Schmidt Lab	
RARE-tk-luc		Ampicillin		Bernards Lab	

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