Role of Axin1 scaffold protein in regulating the c-Myc oncoprotein.

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CERTIFICATE OF APPROVAL

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List of Abbreviations

APC	Adenomatous Polyposis Coli
ARF	ADP Ribosylation Factor
B56	PP2A Regulatory B Subunit/B' Family/PR56
BMP	Bone Morphogenic Protein
B-Myc	Brain-Myc
CDK9	Cyclin dependent kinase 9
CK1	Casein Kinase 1
c-Myc	Cellular Myc
DIX	Dishevelled Interaction Domain
DMEM	Dulbecco's Modified Eagle's Medium
Dox	Doxycycline
DVL	Dishevelled
E-Box	Enhancer Box
ERK	Extracellular Receptor Kinase
FBS	Fetal Bovine Serum
GSK3β	Glycogen Synthase Kinase 3β
HAT	Histone Acetyltransferase
HCC	Hepatocellular Cancer
HDAC	Histone deacetylase
HEK293	Human Embryonic Kidney 293 cells
HIPK2	Homeodomain-interacting protein kinase 2
HLH	Helix Loop Helix
LEF	Lymphoid Enhancer Binding Factor

L-Myc	Lung Myc
LRP5/6	Low density Lipoprotein Receptor 5/6
LZip	Leucine Zipper
MB1	Myc Box I
MB2	Myc Box II
MEKK1	Mitogen Activated Protein/Extracellular Regulated Kinase Kinase Kinase
MNT	Max binding protein
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
N-Myc	Neuronal Myc
p53	Tumor Protein 53
PARsylation	Poly ADP Ribosylation
PI3K	Phosphoinositide-3-Kinase
PIAS	Protein Inactivator of Activated STAT
Pin1	Prolyl Isomerase 1
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
RGS	Regulator of G protein Signaling
RTK	Receptor Tyrosine Kinase
SAPK/JNK Kinase	Stress Activated Protein Kinase/Jun N terminal
SCC	Squamous Cell Carcinoma
SCF	Skp/Cullin/F-box
shRNA	Short Hairpin RNA

siRNA	Small Interfering RNA
S-Myc	Suppressor Myc
SV40	Simian Virus 40
T58	Threonine 58
TAD	Transactivation Domain
TCF	T Cell Specific Factor
TGFβ	Transforming Growth Factor β
TRRAP	Transformation/Transcription domain Associated Protein
UTR	Untranslated Region
WNT	Wingless
WT	Wildtype
β-gal	β-Galactosidase
βTRCP	Beta Transducin Repeat Containing Protein

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Abstract

Axin1 is a scaffold protein that regulates multiple signaling pathways including Wnt/β-Catenin, SAPK/JNK, TGFβ, and p53. Axin1 also coordinates a degradation complex for c-Myc. c-Myc is a proto oncogene that belongs to a family of basic helix loop helix transcription factors and it is overexpressed in many cancers. This thesis seeks to investigate the role of Axin1 in regulating the c-Myc oncoprotein in leukemia and also provides insight into the role of Axin1 Exon 7 in binding to c-Myc and regulating its transcriptional activity.

Axin1 has been characterized as a tumor suppressor and it is mutated in hepatocellular, colorectal and other cancers. c-Myc is known to be overexpressed in leukemia. Since Axin1 coordinates a destruction complex for c-Myc, I investigated the role of Axin1 in leukemia. Here, I show that increased Axin1 protein levels are able to decrease c-Myc protein levels in the U937 leukemia cell line and I also show that the U937 leukemia cell line preferentially expresses the variant 2 isoform of Axin1, which is less effective in regulating c-Myc. I also sequenced Axin1 in 26 leukemia patient samples and found many sequence variations of Axin1 and I show that Axin1 protein sequence changes are rare in leukemia.

We have previously shown that Axin1 coordinates a degradation complex for c-Myc consisting of Gsk3β, PP2A, Pin1, Axin1 and c-Myc. Preliminary protein truncation experiments suggested that Exon 7 of Axin1 is important for Axin1 binding to c-Myc. Based on these results, I investigated the role of Axin1 Exon 7

in binding and regulating c-Myc. Here, I show that Exon 7 of Axin1 contains a domain that is conserved across species and deletion of this conserved domain decreases Axin1 binding to c-Myc. Furthermore, I also show that Axin1 in which Exon 7 is deleted is specifically defective in regulating c-Myc while maintaining its activity towards β-Catenin. These findings further expand our understanding of Axin1's role in regulating c-Myc in cancer and they provide tools that may help in discriminating Axin1's role in different pathways.

Chapter One:

Introduction

Axin Background

Axin is a scaffold protein that contains multiple domains. It was originally identified as a gene product that is expressed from the mouse fused locus (Perry et al. 1995). It was later renamed as Axin, due to its ability to suppress Axis formation. Axin was initially identified to have a role in development by aiding in the determination of dorsal ventral axis formation (Zeng et al. 1997; Farr et al. 2000). Homozygous deletion of Axin1 in vertebrate embryos leads to duplication of the axis. Homozygous deletion of Axin1 is embryonic lethal at day E9.5, and in addition to axis duplication, produces truncation of the forebrain and neural tube defects (Perry et al. 1995; Vasicek et al. 1997). Further studies have found that these effects of Axin1 are due to its function as a regulator of the Wnt/β-Catenin pathway, which is involved in axis determination. Axin's role in the Wnt/β-Catenin signaling has been well studied where it serves to maintain low levels of cytoplasmic β-Catenin by aiding its degradation when the Wnt ligand is not present. Recent studies have found that Axin1 affects not only β-Catenin signaling, but also functions in SAPK/JNK, TGFβ, p53 and c-Myc pathways fSalahshor & Woodgett 2005; Arnold et al. 2009). Axin1 differentially regulates these pathways by binding to and coordinating the formation of different complexes of proteins.

The Axin1 gene is reported to encode two variants through alternative splicing (Figure 1.1). The variant 2 is smaller and is missing the coding region of exon 9 (Salahshor & Woodgett 2005). In a yeast two hybrid screen of β-Catenin binding proteins, another homologue of Axin, Conductin, was isolated (Behrens

et al. 1998). Recent studies have found that Conductin (Axin 2) is also a negative regulator of Wnt/β-Catenin pathway and it is also a target gene of β-Catenin, thus forming a negative feedback loop in regulation of Wnt signaling (Lustig et al. 2002; Yan et al. 2001). The *Axin1* gene is conserved in humans, rats, mice, chickens, xenopus and a homologue of Axin1 is also found in Drosophila (Perry et al. 1995; Ikeda et al. 1998; Hedgepeth et al. 1999; Hamada et al. 1999). More recently, an Axin-like protein called PRY-1 was found in *Caenorhabditis elegans* (Korswagen et al. 2002).

Axin Structure

The N-terminal region of Axin1 contains a domain that shares 31% amino acid identity with RGS4 and has been identified to be a Regulator of G protein signaling (RGS) domain (Figure 1.1) (Druey et al. 1996). Notably, Axin1 binds to the Adenomatous Polyposis Coli (APC) gene product through this RGS domain and this is important for its function in regulating Wnt signaling (Karlberg et al. 2010). The C-terminal domain of Axin contains a domain which is homologous to Dishevelled (Dvl) with 37% amino acid sequence similarity (Sussman et al. 1994). This region is termed the DIX domain due to its presence in Dvl and Axin1. The central region of Axin contains binding regions for Gsk3β and β-Catenin, and these regions are present relatively close to each other thus allowing Gsk3β to phoshporylate β-Catenin (Ikeda et al. 1998). The C-terminal region of Axin also has a binding site for the phosphatase, PP2A (Hsu et al.

1999). With these multiple domains and interacting with different proteins, Axin regulates multiple pathways. However, whether Axin1 forms unique protein complexes for each of these pathways that it regulates or whether there is cross talk between different pathways through Axin1 is not understood. Also, whether pathways that use the same components are in some kind of competition for limiting proteins is not clear.

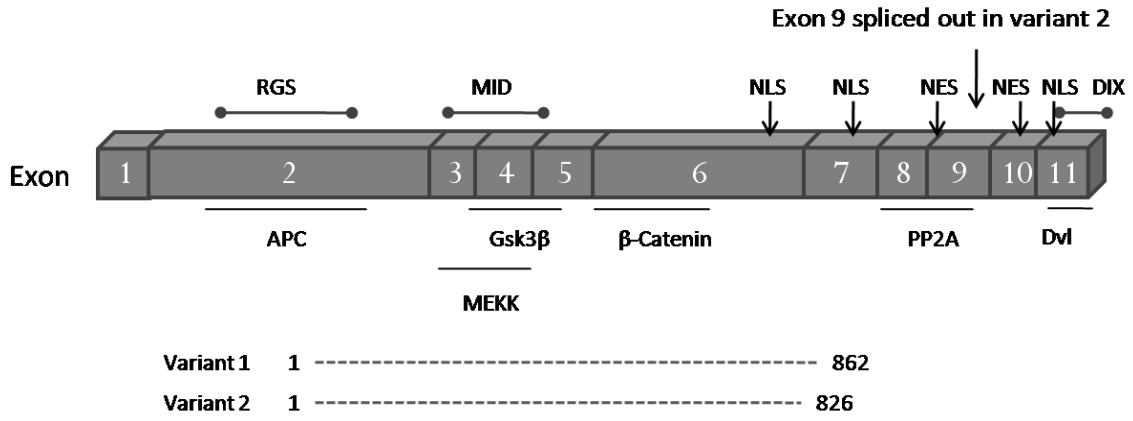

Figure 1.1: Structure of Axin1 scaffold protein.
The Axin1 gene is reported to encode two isoforms. Isoform b (variant 2) has the exon 9 spliced out. The major domains of Axin1 are marked in the schematic with respect to its exons. The relative regions of Axin1 binding to its partner proteins are shown. Axin1 contains multiple nuclear export and import sequences. RGS: Regulator of G protein Signaling domain, MID: MEKK Interacting Domain, NLS: Nuclear Localization Sequence, NES: Nuclear Export Sequence (Adapted from Salahshor and Woodgett 2005, used with permission).

Axin1 has been found to be constitutively and ubiquitously expressed (Zeng et al. 1997). Axin1 is present mostly in the cytoplasm in normal epithelial cells and contains multiple Nuclear Localization Sequence (NLS) and Nuclear Export Sequences (NES), as shown in Figure 1.1 (Cong & Varmus 2004). In contrast, Axin2 expression appears to be cell specific and is upregulated in response to the Wnt signaling thus forming a negative feedback loop (Zeng et al., 1997). Human Axin1's subcellular location appears to be cell specific since it is found in the nucleus in HCT116 and both in the cytoplasm and nucleus in HEK 293 cells (Anderson et al. 2002). Recently, studies have shown that Axin1's location might also have an impact on its function since it has been found to shuttle β-Catenin out of the nucleus (Cong & Varmus 2004; Henderson 2000).

Axin negatively regulates the Wnt/β-Catenin signaling.

The role of Axin1 in the Wnt singling is the most well studied of its functions. Axin1 is a critical regulator of Wnt signaling and injection of Axin1 mRNA is able to prevent aberrant axis formation induced by Wnt in Xenopus but does not prevent axis formation induced by bone morphogenic protein (BMP), indicating that Axin1 is a specific regulator of Wnt signaling (Zeng et al. 1997). In the absence of Wnt, β-Catenin is constitutively degraded by the β-Catenin degradation complex which is coordinated by Axin1 and consists of APC, Gsk3β and CK1 (Figure 1.2). This complex constitutively phosphorylates excess β-Catenin in the cytoplasm that is then recognized by the E3 ubiquitin ligase, Beta Transducin Repeat Containing Protein (βTRCP) and degraded (Bellamy et al. 1995). Wnt signals through its receptors Frizzled (Fz) and Low-density lipoprotein Receptor Protein (LRP5/6). Wnt induces bridging of Fz with LRP5/6 and this leads to phosphorylation of the cytoplasmic tail of LRP5/6 at PPPSPxS (P, Proline; S, Serine: x, variable residue) by CK1γ and Gsk3β (Davidson et al. 2005; Zeng et al. 1997). It is known that bridging of these receptors is sufficient for signaling, since a chimeric protein, with linked Fz and LRP5/6 domains, is able to constitutively activate the pathway even in the absence of Wnt ligand (Tolwinski et al. 2003). Phosphorylation of LRP5/6 is able to recruit Axin1 from the cytoplasm into the membrane. Dvl, which binds to Fz, is important for the initiation of LRP5/6 phosphorylation and might also recruit Axin1 to the membrane, through its DIX domain (Tamai et al. 2004; Mao et al. 2001; Davidson et al. 2005; Zeng et al. 1997). Axin1's recruitment to the membrane

receptor complex and phosphorylation of LRP5/6 by Axin1 bound Gsk3β leads to recruitment of more Axin1 to the membrane, decreasing Axin1 concentration in the cytoplasm (MacDonald et al. 2008; Wolf et al. 2008). This decrease of Axin1 concentration in the cytoplasm leads to accumulation of β-Catenin in the cytoplasm which is then able to translocate into the nucleus and upon binding to the TCF partner protein is able to increase transcription of its target genes.

It is interesting that one of β-Catenin's target genes is Axin2/Conductin, which has been shown to have a similar role of downregulating β-Catenin (Zeng et al. 1997). Thus β-Catenin induction of Axin2 forms a negative feedback loop of Wnt/β-Catenin signaling.

It is important to note that how Wnt is able to decrease β-Catenin degradation in the cytoplasm is not fully understood. It has been suggested that decreased total Axin1 protein levels might also account for decreased β-Catenin degradation since Wnt induces increased turnover of Axin1. Also, it has been suggested that Axin1 along with Dvl might form polymeric complexes through their DIX domains, thereby decreasing Axin1 in the cytoplasm (MacDonald et al. 2009). A recent study has also suggested that Wnt signaling leads to recruitment of the Axin1, Gsk3β complex to the membrane which is then sequestered into multivesicular endosomes thereby decreasing their activity in the cytoplasm (Taelman et al. 2010).

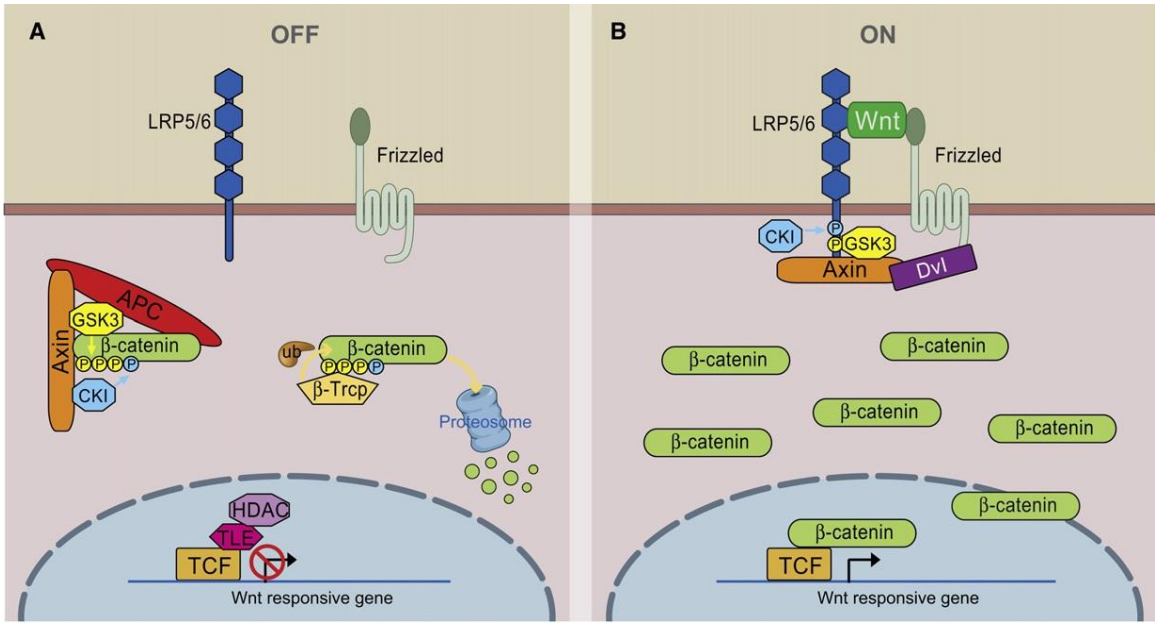

Figure 1.2: Axin1 regulation of the Wnt/β-Catenin pathway.
In the absence of Wnt (left panel), Axin1 along with APC, Gsk3β, and CKI phosphorylates β-Catenin, which is recognized by βTRCP and degraded. In the presence of Wnt (right panel), Axin1 is recruited into the membrane by Dvl along with Gsk3β, thereby decreasing Axin1 concentration in the cytoplasm, allowing β-Catenin to translocate into the nucleus and increase transcription of its target genes. (From MacDonald et al., 2009, used with permission)

Axin1's role in SAPK/JNK signaling pathway

The Stress Activated Protein Kinase/Jun N terminal Kinase (SAPK/JNK) pathway is activated in response to cellular stress. The SAPK/JNK pathway is involved in regulation of cellular proliferation, morphogenesis and cell death (G. L. Johnson & Lapadat 2002). SAPK/JNK pathway can induce apoptosis or cell survival depending upon the cellular context (Leppä & Bohmann 1999; Schaeffer & Weber 1999). The SAPK/JNK pathway is activated in response to stress by GTPases of the Rho family (Rac, Rho, cdc42) (Matsukawa et al. 2004). These then activate the MAP3K (Mitogen Activated Protein Kinase Kinase Kinase) including MEKK1 and MEKK4 (Fanger et al. 1997). These MAP3 Kinases then act on MAP2 Kinases including MKK4 and MKK7. This then leads to activation of SAPK/JNK which phosphorylates c-Jun transcription factors leading to increased transcription of its target genes and apoptosis (Matsukawa et al. 2004).

It has been shown that overexpressed Axin1 is able to induce apoptosis by activating the SAPK/JNK pathway (Y Zhang et al. 1999; Y Zhang et al. 2000). Importance of Axin1 in the induction of apoptosis through the SAPK/JNK pathway is also indicated by the Axin1 mutations found in cancers which are resistant to apoptosis (Neo et al. 2000; Clevers 2000). Axin1 is different from other scaffold proteins of the MAPK pathways in that, it directly interacts with MEKK4 instead of interacting with MKK4/7, like other scaffold proteins JIP and MP1. Axin1 binds to MEKK4 through a domain termed MID (MEKK4 interacting domain), which is flanked by its APC and Gsk3β binding domain (Figure 1.1) (Y Zhang et al. 1999). Axin1 is known to dimerise through a dimerization domain in

its C-terminus. This C-terminal dimerization domain and also Axin1's ability to downregulate β-Catenin have been shown to be required for Axin1 to activate the JNK pathway (Figure 1.3) (Neo et al. 2000; Hsu et al. 1999). Recently, other studies have found that Axin1 also interacts with MEKK1 through a domain that is within its PP2A binding region (Wen Luo et al. 2003). Interestingly, MEKK1 and MEKK4 compete for binding to Axin1 even though their binding regions do not overlap. The reason for this is currently not known. One more additional player in the Axin1 regulation of JNK pathway is the sumoylation proteins. In a yeast two hybrid screen, three E3 sumoylation enzymes have been isolated namely PIAS1, PIASxb, PIASy through binding to Axin1 (H.-L. Rui et al. 2002). The extreme C-terminal end of Axin1 has the amino acid sequence KVEKVD, which contains two contiguous sumoylation sites. Removing either of the sites decrease Axin1's effect on JNK pathway and removing both sites (AxinΔC6) totally abolishes Axin1's effect on JNK pathway (H.-L. Rui et al. 2002) indicating that sumoylation forms an integral part of Axin1's activity on the JNK pathway.

Also it is interesting to note that Axin1's ability to activate JNK pathway is antagonized by Gsk3β and CK1α but not by APC and β-Catenin and that β-Catenin is involved in Axin1's regulation of the JNK pathway (Y Zhang et al. 2001). How Axin1 is switched between acting on the Wnt signaling pathway or the JNK pathway and how Axin1's regulation of both these pathways interplay is not known.

Axin1 regulates the TGFβ signaling

TGFβ signaling forms an important regulator of several cellular functions including proliferation, migration and apoptosis (Massagué 1998). Axin1 also functions in the TGFβ signaling pathways by regulating the Smad3 protein (Furuhashi et al. 2001) (Figure 1.3). In the presence of TGFβ, TGFβ type 1 receptor phoshporylate Smad3 (Heldin et al. 1997). Axin1 is shown to bind to Smad3 at its C-terminal which overlaps with Axin1's Dvl binding domain (Furuhashi et al. 2001). Interestingly, Dvl does not compete with Smad3 for Axin1 binding. Axin2 is also shown to bind Smad3 (Furuhashi et al. 2001). In the absence of TGFβ, Axin1 is found associated with Smad3 in the cytoplasm. However, in the presence of TGFβ, Axin1 acts as an adapter protein for Smad3 allowing it to be efficiently phoshporylated by the TGFβ type 1 receptor. Upon phosphorylation, Smad3 is released from both TGFβ receptor 1 and Axin1 and is thus able to translocate into the nucleus and increase transcription of its target genes. Thus, Axin1 performs a positive role in the TGFβ signaling pathway in contrast to the Wnt pathway where it performs a negative role.

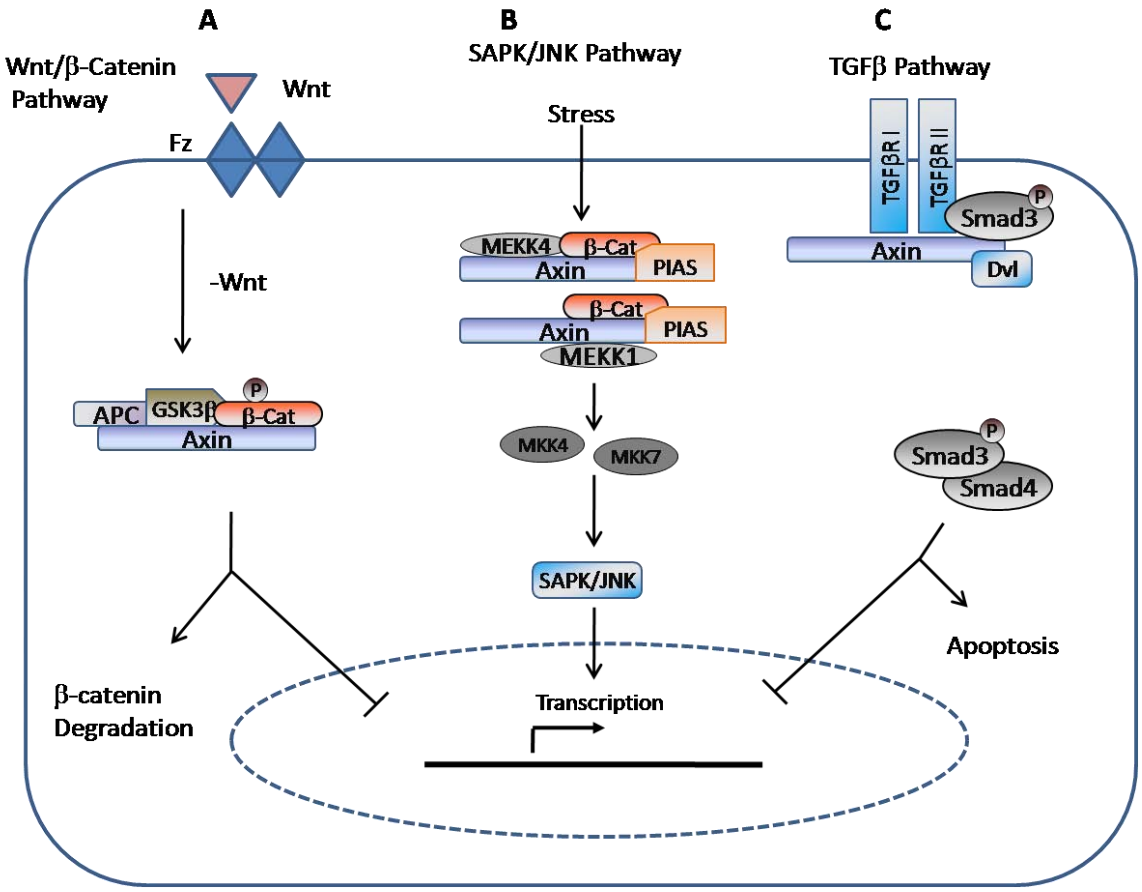

Figure 1.3: Axin1 regulates multiple signaling pathways.
Axin1 binds to multiple different proteins and is able to regulate different pathways. (A) In the absence of Wnt Axin1 binds to APC (not shown here) Gsk3β and β-Catenin and phosphorylates β-Catenin leading to its degradation. (B) Under stressed conditions Axin1 stimulates apoptosis by activating the SAPK/JNK pathway by binding with MEKK1. (C) In the presence of TGFβ, Axin1 helps to phoshporylate Smad3, which then upregulates its target gene expression. (Adapted from Salahshor and Woodgett, used with permission)

Axin1 also functions in the p53 pathway where it acts as a positive regulator. Recently, Li et al., have found that Axin1 performs a structural role in coordinating the formation of a ternary complex consisting of Axin1, HIPK2 and p53 and thus aids in phosphorylation and activation of p53 in response to stress

(Q. Li et al. 2007) (Figure 1.4). Recent evidence suggests that the death domain associated protein (Daxx), which signals from the FasL to induce apoptosis, also regulates the p53 pathway (X. Yang et al. 1997; Ohiro et al. 2003). Daxx has been found to activate the p53 pathway under stressed conditions and downregulate p53 pathway under non-stressed conditions. While under non-stressed conditions, Daxx stabilizes Mdm2, an E3 ubiquitin ligase for p53, thereby downregulating p53 levels (Tang et al. 2006). But under stressed conditions, Daxx is able to increase activating phosphorylation (Serine 46) of p53. This was later found to be due to Daxx binding to the Axin1, HIPK2, p53 ternary complex and increasing HIPK2 activity on p53 (Y. Rui et al. 2004). p53 binding to Axin1 seems to occur through two different mechanisms. p53 can bind to Axin1 directly and also indirectly through HIPK2. However, the precise mechanism of p53 binding to the Axin1 complex is currently controversial (Lin & Q. Li 2007).

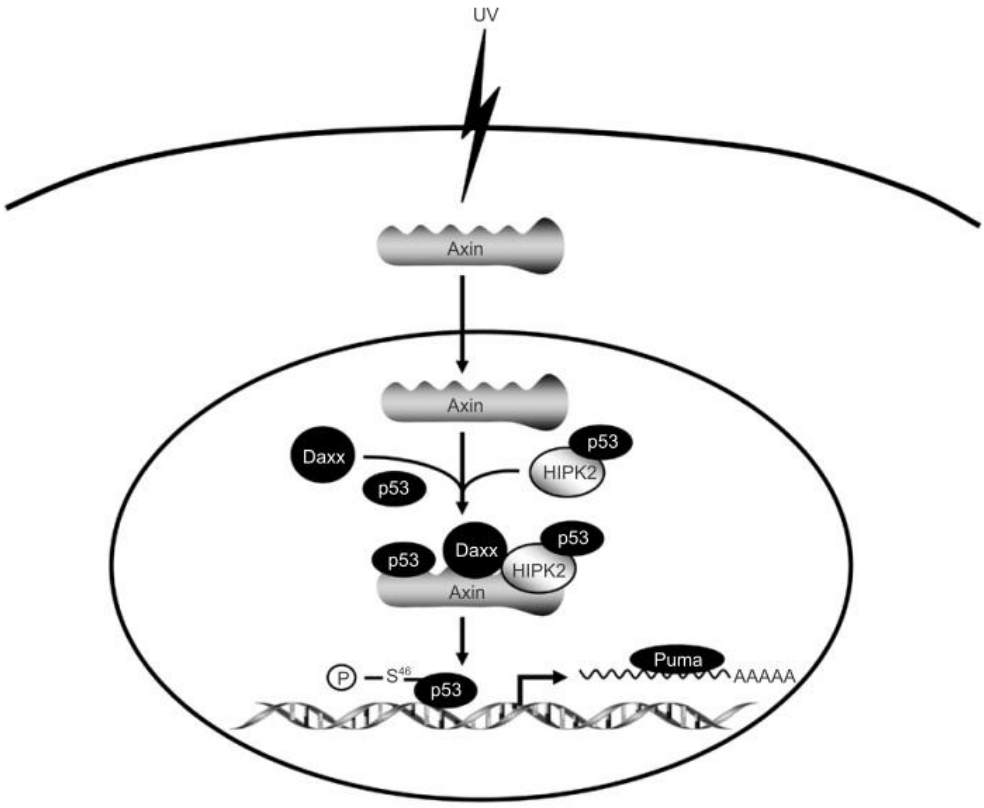

Figure 1.4: Axin1 increases p53 phosphorylation under UV stress by forming a ternary complex.
Under stressed conditions Axin1 translocates into the nucleus where it forms a complex with p53, Daxx and HIPK2. Daxx binding to the Axin1, HIPK2 and p53 increases HIPK2 activity on p53 which phosphorylates p53 at S46 and activates it to induce apoptosis. Axin1's effect on p53 seems to increase p53's activity on specific target genes. (From Lin et al., 2007, used with permission)

Axin1 was also found to localize to the centrosome and the DIX domain is necessary for its centrosomal localization (Alexandrova & Sokol 2009). Knockdown of Axin1 decreased the localization of γ tubulin ring complex proteins

to the centrosome (Fumoto et al. 2009). Furthermore, Axin1, which is deficient in localizing to the centrosome, was more efficient in regulation of the Wnt/β-Catenin pathway. But Axin1's function in the centrosome is not well understood.

Axin1 is regulated by different mechanisms

Axin1 has been shown to be the rate limiting member of Wnt/β-Catenin signaling and is expressed at low levels compared to other components (E. Lee et al. 2003). This suggests that small changes in Axin1 levels might have major effects on the activity of the Wnt/β-Catenin pathway, thus indicating the importance of regulation of Axin1 levels.

Axin1 levels are regulated by phosphorylation, intra cellular localization and Poly Adenosine diphosphate Ribosylation (PARsylation). Axin1 phosphorylation by Gsk3β increases its protein stability (Yamamoto et al. 1999; Z.-H. Gao et al. 2002) whereas dephosphorylation of Axin1 by PP1 decreases Axin1's stability (Wen Luo et al. 2007). Also, the adapter protein, Dab2, by preventing PP1 binding to Axin1, increases Axin1 phosphorylation and thereby stabilizes Axin1 protein levels (Jiang et al. 2009). Phosphorylation is also known to affect Axin1's affinity to its partner proteins. Phoshporylated Axin1 can bind to β-Catenin more efficiently than dephosphorylated Axin1 and Wnt signaling induces dephosphorylation of Axin1 thus decreasing Axin1's affinity towards β-Catenin. Axin1 protein is also regulated by PARsylation (Yue Zhang et al. 2011; S.-M. A. Huang et al. 2009). More specifically, Axin1 is PARsylated by PARP5

(Tankyrase) and this PARsylated Axin1 is then recognized by the PARsylation dependent E3 ubiquitin ligase, RNF146 and is subsequently degraded by the ubiquitin proteasome system. Recently, two small molecule inhibitors of tankyrase, XAV939 and IWR, have been shown to increase Axin1 protein levels by decreasing its PARsylation and subsequently its degradation (S.-M. A. Huang et al. 2009; Chen et al. 2009).

Intracellular localization is another method of regulation of Axin1 function. Axin1 is found mostly in the cytoplasm and contains multiple nuclear import and export signal sequences (Figure 1.1). Axin1 has been found to act as a chaperon for β-Catenin by transporting it out of the nucleus (Cong & Varmus 2004). Wnt signaling induces Axin1 localization from the cytoplasm into the membrane (Figure 1.2). This membrane bound Axin1 is sufficient for Wnt signaling in drosophila (Tolwinski 2009). The LRP5/6 protein is able to recruit Axin1 into the membrane and this decreases Axin1 concentration in the cytoplasm and increases β-Catenin induced transcription (Tolwinski et al. 2003). A recent study has found that Wnt signaling leads to sequestration of the Axin1 Gsk3β complex into multivesicular endosomes thereby preventing its activity in the cytoplasm (Taelman et al. 2010). Taken together this suggests that Axin1's effect on different pathways can be regulated by multiple different mechanisms.

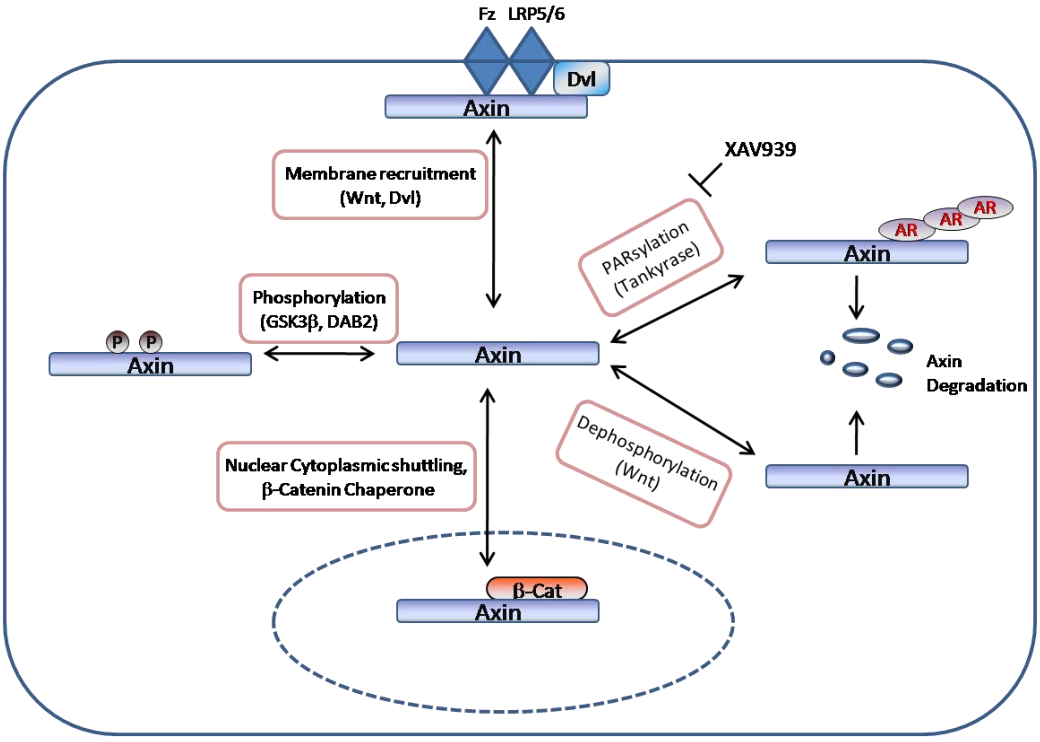

Figure 1.5: Schematic of Axin1 regulation by different mechanisms
Axin1's function on different pathways is regulated by multiple mechanisms including phosphorylation, recruitment to the membrane, nuclear cytoplasmic shuttling, dephosphorylation and PARsylation. The proteins that affect these processes are given in parenthesis. XAV939 by inhibiting tankyrase prevents Axin1 PARsylation. P = phosphorylation, AR = ADP Ribose.

Axin1 coordinates a degradation complex for c-Myc

c-Myc belongs to a family of transcription factors that includes N-Myc, L-myc, S-myc, and B-myc. c-Myc has many cellular functions like regulation of cellular proliferation, differentiation and apoptosis. c-Myc has been extensively studied for the past 30 years and is generally accepted to be a major oncoprotein, which is deregulated in multiple cancers (Meyer & Penn 2008). c-Myc deregulation in cancers can be due to gene amplification, overexpression or

increased protein stability. Two conserved phosphorylation sites in c-Myc, Threonine 58 and Serine 62 form a major regulatory mechanism of c-Myc protein stability (reviewed in (R. C. Sears 2004)). Our lab has shown that phosphorylation at these sites are coordinated by the scaffold protein Axin1 (Arnold et al. 2009). Specifically, Axin1 has been shown to associate with endogenous c-Myc along with Gsk3β, PP2A and Pin1. Overexpressed Axin1 is able to increase c-Myc association with Gsk3β and PP2A and conversely knocking down Axin1 is able to increased c-Myc protein levels. This increase in c-Myc protein levels, due to Axin1 knockdown, is associated with decreased T58 phosphorylation and increased S62 phosphorylation and increased c-Myc half-life. Overexpressed Axin1 is able to increase c-Myc ubiquitination and decrease c-Myc expression and this is independent of c-Myc mRNA. Truncation experiments have suggested that exon 7 of Axin1 is important for its binding to c-Myc. Furthermore Gsk3β and PP2A binding to Axin1 increases Axin1 association to c-Myc suggesting that the complex formation is a coordinated process. The c-Myc degradation pathway is explained in much more detail later in this chapter.

Taken together, it is apparent that Axin1 acts as a scaffold protein and by interacting with multiple proteins plays a vital role in regulating many major cellular pathways.

Axin1's role in cancer.

Axin1 is a scaffold protein that regulates Wnt/β-Catenin, c-Myc, SAPK/JNK, p53 and the TGFβ pathways. Axin1 has generally been accepted to play a tumor suppressor role in cancer (Salahshor & Woodgett 2005). Axin1 mutations have been found in many cancers and Axin1 deregulation also occurs through deletions, or by downregulation. Hyperactivity of the Wnt/β-Catenin signaling pathway is implicated in many hepatocellular cancers (HCC) and colorectal cancers (CRC) (Miyoshi et al. 1998; de La Coste et al. 1998; Webster et al. 2000; W. Liu et al. 2000). Wnt signaling is upregulated in HCCs mainly through mutations in β-Catenin. HCCs in which β-Catenin is wild type show Axin1 mutations. Mutations of Axin1 in HCCs are small deletions, insertions, or non sense mutations that lead to truncation of Axin. One study found that Axin1 is mutated in three of four HCC cell lines and 5 of 87 primary HCCs (Satoh et al. 2000). In addition, introduction of Axin1 using an adenoviral vector induces apoptosis in a few of these cell lines. Mutations in both Axin1 and Axin2 were detected in ovarian endometrioid adenocarcinoma which show increased nuclear β-Catenin accumulation (R. Wu et al. 2001). In esophageal cancer, decreased Axin1 expression is found to correlate with increased invasion, lymph node involvement and lymphatic invasion (A. F.-Y. Li et al. 2009; Nakajima et al. 2003). Furthermore, while Axin1 is found in the cytoplasm in normal stratified squamous epithelium of the esophagus it showed mixed localization in the case of esophageal adenocarcinomas (de Castro et al. 2000). Another tumor in which deregulations of Wnt pathway are common is Medulloblastoma. Axin1 mutations

have been found in sporadic cases of medulloblastoma (Baeza et al. 2003). These are mainly point mutations, and deletions. The DIX domain and the APC binding domain of Axin1 are commonly mutated in medulloblastoma.

Colorectal cancer is well known for its deregulated Wnt signaling pathway. Genetic mutation in the APC gene causes familial adenomatous polyposis which leads to colorectal cancer with 95% penetrance (Kinzler & Vogelstein 1996). In addition to β-Catenin mutation, Axin1 mutations are also commonly found. These are mainly between exons 2 – 5 which affect Axin1 binding to Gsk3β, β-Catenin or APC (Webster et al. 2000; Shimizu et al. 2002; Jin et al. 2003). Furthermore, missense mutations in exon 8, which leads to premature termination, thereby removing Axin1's dimerization domain are also seen (W. Liu et al. 2000). Axin1 mutations are also found in HCT-8, HCT-15, DLD1 colon cancer cell lines (Webster et al. 2000). Axin1 mutations have also been suggested to correlate with β-Catenin accumulation in prostate cancer (Yardy et al. 2009).

We have previously shown that the SupB15 leukemia cell line carries an in-frame deletion of Axin1 cDNA in the exon 5 region, which affects Axin1 binding to Gsk3β, and is also defective in regulating c-Myc (Malempati et al. 2006). Taken together, these findings suggest that Axin1 deregulation aids in the tumorigenesis of many different types of cancer.

c-Myc background

The c-Myc gene was originally identified 30 years ago as a cellular homologue of v-myc which was the transforming protein in the avian myelocytomatosis virus MC29 (Vennstrom et al. 1982). Since then it has been intensively studied and is now known for its function as a transcription factor that regulates a multitude of cellular functions such as cell growth, proliferation, apoptosis, differentiation, metabolism and neoplastic transformation (Meyer & Penn 2008). c-Myc is widely expressed during development and also in rapidly proliferating cells (Downs et al. 1989; Malynn et al. 2000). c-Myc has since been found to regulate approximately 15% of all genes (Patel et al. 2004) and has been identified as an oncogene which is deregulated in almost all human cancers (Pelengaris et al. 2002). The c-Myc gene is highly conserved from drosophila to humans and shows functional similarity between species.

c-Myc family of transcription factors

c-Myc is the well studied member in the family of non redundant transcription factors which include N-myc, L-myc, S-myc and B-myc. c-Myc being a transcription factor contains a DNA binding domain and an transactivation domain (TAD) as shown in Figure 1.6. The C-terminal region of c-Myc contains its TAD which enables Myc to recruit various coactivators when bound to DNA. This region also contains two regions called Myc Box I and II which are highly conserved across species and play an important part in regulating c-Myc protein

levels. The C-terminus of Myc contains a basic helix loop helix leucine zipper motif (bHLHZ), which allows for DNA binding (Landschulz et al. 1988; Blackwood & Eisenman 1991; R. L. Davis et al. 1990). c-Myc binds to its partner protein Max, another bHLHZ protein, through its HLHZ motifs. Upon dimerization of c-Myc and Max, the zippered HLHZ domains are able to recognize DNA through the basic domains at sequences called E-Boxes, which have the consensus of CACGTG (Blackwell et al. 1990; Blackwood & Eisenman 1991). c-Myc, upon binding to DNA at these E-Box sequences, then recruits co activators like transformation/transcription domain-associated protein (TRRAP), GCN5, TIP60 and these Histone Acetyl Transferases along with ATPase and Helicase cause acteylation of histones and opening of the chromatin, thereby increasing transcription of target genes (McMahon et al. 1998; McMahon et al. 2000; Wood et al. 2000; Frank et al. 2003). c-Myc also recruits CDK9, which can directly phoshporylate RNA polymerase II and increase transcription of its target genes (Eberhardy & Farnham 2002).

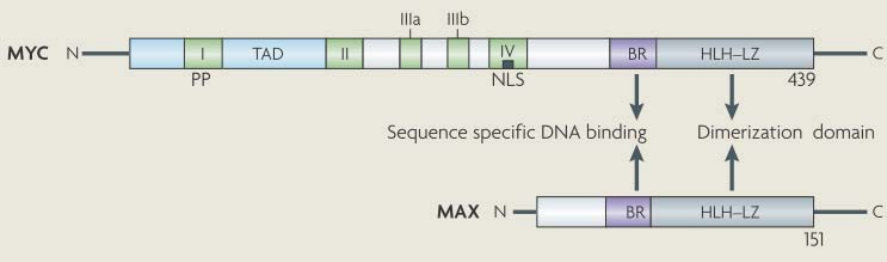

Figure 1.6: Structure of c-Myc and its partner protein MAX.
c-Myc contains a transactivation domain (TAD) in its C-terminal (light blue) and the basic region (purple) and the helix-loop-helix-Leucine Zipper region(dark blue). Its partner protein MAX also has the bHLHZ motif. The conserved Myc boxes I to IV are also shown (green). (From Meyer and Penn, 2008; used with permission)

While c-Myc binding along with Max to E-Box elements increases transcription, c-Myc can also lead to repression of transcription. While it is not fully understood how c-Myc is able to repress transcription it is known that c-Myc and Max heterodimer binds to MIZ1 and represses MIZ1 activation of its target genes (Figure 1.7). Interestingly, in this case the Myc-Max-Miz1 heterotrimer binds to initiator (INR) elements in the DNA instead of E-Box sequences.

c-Myc's transactivation function is also antagonized by the MAD and MNT group of proteins. The Mad family of proteins can bind to MAX and thus competes with c-Myc for Max binding (Ayer & Eisenman 1993). This Mad-Max complex however recruits histone deacetylase (HDAC) through SIN3 adapter proteins and thus leads to closed chromatin structure and repressed transcription (Figure 1.7). In this context it is important to note that while Max is expressed constitutively expression of Myc and Mad group of proteins are tightly regulated (Ayer et al. 1993; Hassig et al. 1997; Hurlin et al. 1997; Ayer et al. 1995).

Cellular functions of c-Myc

c-Myc is a transcription factor that regulates multiple cellular functions such as cell cycle regulation, cellular proliferation, induction of apoptosis, regulation of differentiation, cell growth, angiogenesis and cellular metabolism (Figure 1.7). c-Myc has been shown to bind to and potentially regulate approximately 15% of cellular genes (Patel et al. 2004). c-Myc, while being pro proliferative and inducing cell growth and angiogenesis, can also induce

apoptosis under certain conditions through a process called oncogene induced apoptosis.

One of the cellular functions of c-Myc is its regulation of cell cycle. Rat fibroblasts in which both the c-Myc alleles are deleted, exhibit a long G1 phase in the cell cycle (Mateyak et al. 1997). c-Myc is shown to control progress of the cell cycle into the S phase from G1. This is done by activating cyclin D2 (C Bouchard et al. 2001), cyclin E (Pérez-Roger et al. 1997) cyclin dependent kinase 4 (cdk4) (Hermeking et al. 2000) and E2F1, E2F2 and E2F3α (M. R. Adams et al. 2000; Leone et al. 1997; R Sears et al. 1997)and by repressing the cyclin dependent kinase inhibitors, p15 and p21 (Staller et al. 2001; S. Wu et al. 2003). Also, overexpression of Myc in quiescent cells can induce entry into the cell cycle and proliferation. c-Myc is also a crucial regulator of cellular differentiation, as it has been shown to be one of the four factors able to reprogram differentiated cells into the pluripotent state (Takahashi & Yamanaka 2006).

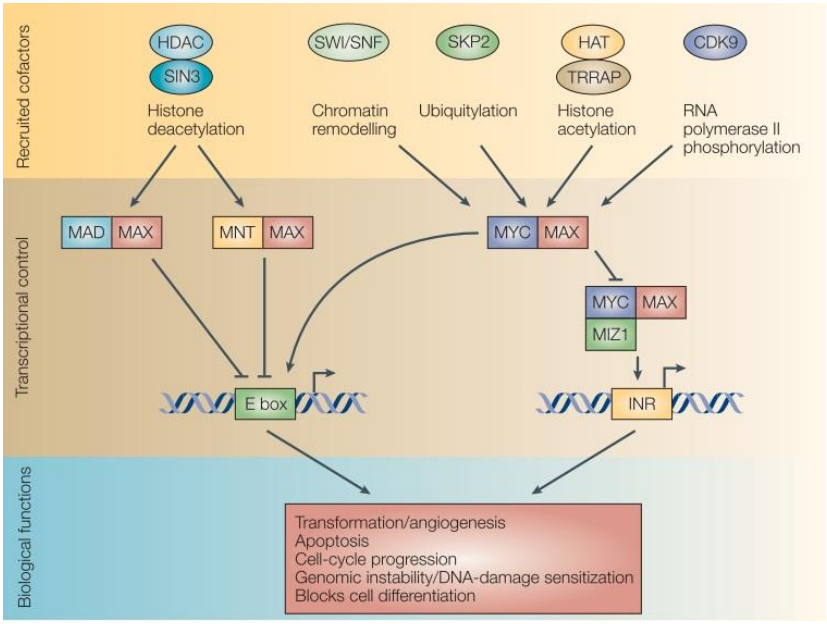

Figure 1.7: The Myc-Max network.
c-Myc hetero-dimerises with MAX and binds to DNA sequences called E-box and upregulates its transcriptional activity by recruiting activators like SWI/SNF, SKP2, TRRAP, and CDK9. Alternatively, Myc-MAX dimer when bound to MIZ1 can repress MIZ1 activation on INR elements. Moreover, Myc is antagonized by MAD and MNT family members which compete with Myc for MAX and bind to E-Box elements and recruit repressive factors like HDAC and SIN3. Myc's activity on E-Box and INR elements produces many of Myc's effects on cell function. (From Patel et al., 2004, used with permission)

While c-Myc major function is pro proliferative and aids in cellular growth and protein and ribosomal synthesis it can also induce apoptosis under specific contexts (Pelengaris et al. 2002; G I Evan et al. 1992; Schreiber-Agus et al. 1995). The apoptosis function of c-Myc seems to be linked to its ability to induce cell cycle progression. c-Myc sensitizes cells to apoptosis during the cell cycle

and in the absence of appropriate survival signals can induce cells to undergo apoptosis. This is considered to be a failsafe mechanism in order to eliminate cells with aberrant activation of c-Myc. This induction of apoptosis by c-Myc can occur through either p53 dependent or p53 independent mechanisms (Meyer & Penn 2008; Chi V Dang et al. 2005). c-Myc activates ARF, which can then activate p53 that then upregulates its target genes leading to apoptosis. Inactivation of the p53/ARF pathway is shown to increase c-Myc induced tumorigenesis in mice (Caroline Bouchard et al. 2007; Finch et al. 2006; Alt et al. 2003; Schmitt et al. 1999). c-Myc can also induce apoptosis by p53 independent mechanisms, namely, by activating Bax (Dansen et al. 2006). It has been suggested that c-Myc causes conformational changes in Bax, which then leads to cytochrome p450 release from the mitochondria which then activates downstream effector caspases that ultimately execute apoptosis (Annis et al. 2005; Soucie et al. 2001). c-Myc mutants in cancer and also v-myc have been shown to be defective in inducing apoptosis thereby contributing to tumorigenesis (Hemann et al. 2005).

c-Myc is also a major regulator of cellular growth. Cell growth and cell cycle are tightly regulated and increased cell growth is often coupled to cell cycle progression. c-Myc increases cell growth and cell size by upregulating ribosomal RNA and also increasing synthesis of ribosomal proteins leading to assembly of new ribosomes (Kim et al. 2000; Gomez-Roman et al. 2003; Grandori et al. 2005). c-Myc also increases cell size by upregulating translation initiation factors and tRNA biogenesis, thereby leading to increased protein synthesis (Gomez-

Roman et al. 2003; Kim et al. 2000). Deletion of Myc in wing imaginal disc cells of Drosophila leads to cells that are significantly smaller in size and conversely overexpression of myc in these cells results in larger cells. c-Myc also increases transcription of nucleolin and nucleophosmin, resulting in increased RNA processing (Schlosser et al. 2003; Ginisty et al. 1998; Maggi et al. 2008).

Recent studies have identified that c-Myc also regulates cellular metabolism. Interestingly, c-Myc's role in metabolism aids in c-Myc's activity as an oncogene. During oncogenesis tumor cells often show a marked change in metabolism with a propensity to switch from mitochondrial oxidative phosphorylation to using ATP from glycolysis. This happens even in the presence of sufficient amounts of oxygen to support mitochondrial respiration. This phenomenon was first suggested by Warburg in 1956 and is called the Warburg effect (WARBURG 1956). c-Myc aids in this process by upregulating genes involved in glycolysis. Specifically c-Myc upregulates lactate dehydrogenase which converts pyruvate derived from glycolysis to lactate (Shim et al. 1997; Osthus et al. 2000). c-Myc also increases cellular utilization of glutamine by transcriptionally repressing microRNA miR23a and miR-23b which results in increased mitochondrial glutaminase synthesis (P. Gao et al. 2009).

Regulation of c-Myc

Since c-Myc regulates 15% of all genes and is overexpressed in a majority of cancers, its regulation has been studied extensively (Patel et al. 2004). c-Myc

is regulated at multiple levels including transcription, mRNA stability, protein translation and post translational modifications.

c-Myc is expressed at low levels in quiescent cells. Upon mitogen stimulation c-Myc mRNA levels increase and peak at about 2 hours post serum stimulation and then decline to about 50% of peak level, to be maintained at this level for cellular growth and proliferation. This increase is due to increased c-Myc transcription and also increased mRNA stability (Jones & Cole 1987). The c-Myc mRNA contains an AU rich region in the 3' untranslated region (UTR) which plays a negative role in controlling its stability (Jones & Cole 1987; Yeilding et al. 1996; Brewer & Ross 1988). It has been shown that deletion of the 3' UTR increases stability of c-Myc mRNA. The 3' UTR region also has binding sites for RNA binding proteins such as HuR and micro RNA's such as Let-7 (Wong et al. 2011; Sampson et al. 2007). Binding of HuR or Let-7 can then recruit miRNA silencing complex and thus decrease c-Myc mRNA levels. Interestingly, Let-7 is repressed by c-Myc thus forming a positive feedback loop for c-Myc expression (Chang et al. 2009). Furthermore, c-Myc mRNA also has an internal ribosomal entry site (IRES) in the 5' UTR which allows for cap independent translation (Kobayashi et al. 2003; Stoneley et al. 2000). It has been found that activation of phosphoinositide 3 Kinase Akt-mTOR signaling leads to increased translation of c-Myc mRNA through this IRES site.

The c-Myc degradation pathway

c-Myc is also regulated at the post translational level which regulates its protein stability. c-Myc protein is mainly regulated through control of its degradation by the ubiquitin mediated degradation pathway through the 26S proteasome (Yada et al. 2004). Normally the half life of the c-Myc protein is relatively short in the range of 20 to 30 minutes, indicating that c-Myc is degraded soon after it is synthesized (Hann & Eisenman 1984). Our lab has shown that this degradation is regulated by two conserved phosphorylation sites, Threonine 58 and Serine 62, which are located in the Myc box I region (Rosalie Sears et al. 2000). Phosphorylation at S62 stabilizes c-Myc while phosphorylation at T58 decreases c-Myc protein stability. Moreover, phosphorylation at T58 requires prior phosphorylation at S62. c-Myc's protein levels increase at early G1 phase and then quickly decline and are maintained at low levels at late G1 (R. C. Sears 2004). This bell shaped increase and decrease of c-Myc protein is due to coordinated synthesis and degradation of c-Myc protein. Work in our lab has suggested that phosphorylation at the conserved residues T58 and S62 form a part of this regulation of c-Myc protein levels.

The coordinated phosphorylation and dephosphorylation of c-Myc is performed by many players such as Gsk3β, PP2A, Pin1, Axin1 and the E3 ubiquitin ligase F-box protein, Fbw7 (Arnold et al. 2009). As shown in Figure 1.8, in early G1 phase, when there is increased Ras activation along with PI(3)K activation, downstream of Receptor Tyrosine Kinases, there is increased activation of the ERK kinases [Mitogen–activated Protein (MAP) kinases] and

cyclin dependent kinases. This causes c-Myc phosphorylation at residue S62 (Seth et al. 1992; Pulverer et al. 1994; Lutterbach & Hann 1994; Noguchi et al. 1999; R Sears et al. 1999). At this stage, c-Myc is stabilized because of simultaneous activation of PI3K/AKT pathway by RAS and by RTK, leading to suppression of Gsk3β activity (Cross et al. 1995). This concerted activation of ERK and suppression of Gsk3β keeps c-Myc singly phoshporylated at the S62 residue. Then in late G1 phase, when Ras and PI3K activities subside, the suppression of Gsk3β activity is released, leading to phosphorylation of c-Myc at the T58 residue by Gsk3β (Lutterbach & Hann 1994; Pulverer et al. 1994). It is important to note that phosphorylation at T58 by Gsk3β requires prior S62 phosphorylation, since Gsk3β is a processive kinase and requires a priming phosphorylation 4 amino acids carboxy terminal to its target site (Chu et al. 1996). This c-Myc, which is phoshporylated at both S62 and T58, is then recognized by the Pin1 prolyl isomerase which catalyses the conversion of proline 63 from *cis*- to *trans*- conformation (Yeh et al. 2004). The dual phoshporylated c-Myc which is in the *trans* conformation is then able to be recognized by the confirmation sensitive PP2A protein phosphatase, which dephosphorylates c-Myc at the S62 site leading to c-Myc being singly phoshporylated at the T58 site in this region (Yeh et al. 2004; Arnold & R. C. Sears 2006). PP2A is a heterotrimeric complex composed of a structural A subunit, a catalytic C subunit and a regulatory B subunit which allows substrate specificity. Our lab has shown that the regulatory B subunit, B56α, is important for targeting PP2A towards c-Myc (Arnold & R. C. Sears 2006). Furthermore,

work in our lab has found that mutating Threonine 58 to Alanine leads to failure to dephosphorylate the S62 site, since PP2A binding is inhibited, and thus produces a stable from of c-Myc. Wild type c-Myc which is singly phoshporylated at T58 is recognized by the SCF FBW7 ubiquitin machinery that poly-ubiquitinates c-Myc and marks it for degradation by the 26S proteasome complex (Welcker et al. 2004; Yada et al. 2004).

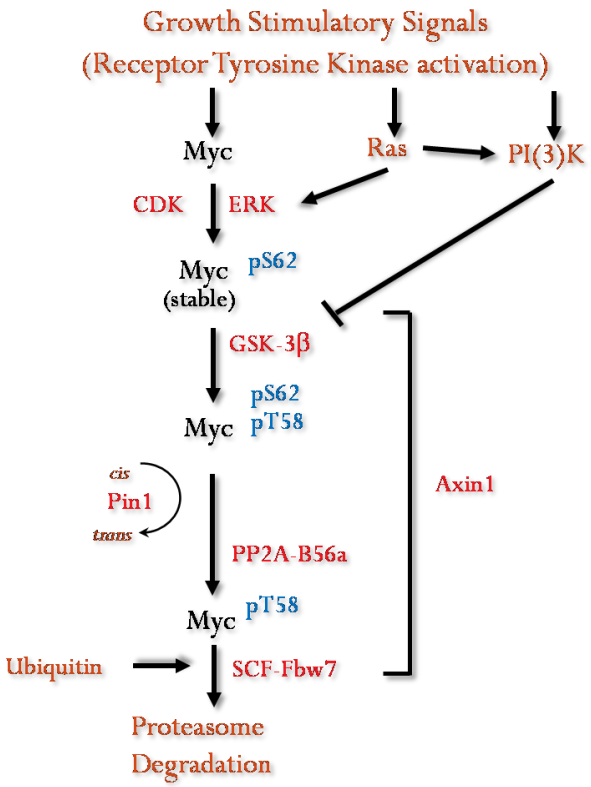

Figure 1.8: Schematic showing the c-Myc S62/ T58 degradation pathway.
In response to growth stimulatory signals, RAS and PI3K/AKT pathways are activated which activates ERK and CDK, and suppresses Gsk3β leading to c-Myc phosphorylation at S62. In late G1, Ras and PI3K activity decreases and Gsk3β phosphorylates Myc at T58. This form of Myc is then dephosphorylated at S62 by PP2A after isomerisation by Pin1. Myc that is phoshporylated at T58 is ubiquitinated by SCF-Fbw7 ubiquitin ligase and degraded.

Furthermore, work in our lab has shown that the multiple proteins that are involved in this pathway are coordinated by the scaffold protein Axin1 (Arnold et al. 2009). Specifically, Axin1 binds to endogenous c-Myc, Gsk3β, PP2A, and Pin1. Axin1 is able to increase c-Myc binding to Gsk3β and PP2A and knocking down Axin1 shows the opposite effect, suggesting that Axin1 coordinates c-Myc interaction with its regulators. Consistently, knocking down Axin1 increases c-Myc protein levels, which is correlated with decreased T58 phosphorylation, increased S62 phosphorylation, and also increased c-Myc protein stability. Furthermore, ectopically expressed Axin1 decreases c-Myc protein levels, which is correlated with increased ubiquitination and degradation of c-Myc. Taken together, it is clear that c-Myc protein stability is regulated by a complex set of proteins that come together to form a multiprotein complex which is coordinated on the scaffold protein Axin1 (Figure 1.9). Moreover, it is interesting to note that this complex is distinct from the Axin1 degradation complex since we have shown that immunoprecipitation with c-Myc does not pull down APC but pulls down Axin1 (Figure 1.10) (Arnold et al. 2009). This is consistent with the different localization of β-Catenin and c-Myc as c-Myc is mainly found in the nucleus and β-Catenin in the cytoplasm. Furthermore, it has been recently suggested that degradation of transcription factors might happen directly at the promoters, thus adding further level of control in regulation of transcription (Geng et al. 2012). In this regard, it is interesting to note that the c-Myc degradation complex coordinated by Axin1 might be present at the promoter of c-Myc's target genes, as we find that chromatin immunoprecipitation of Axin1 is able to pull down E2F2

promoter sequences which has been previously shown to be a c-Myc target gene (Arnold et al. 2009; R Sears et al. 1997)

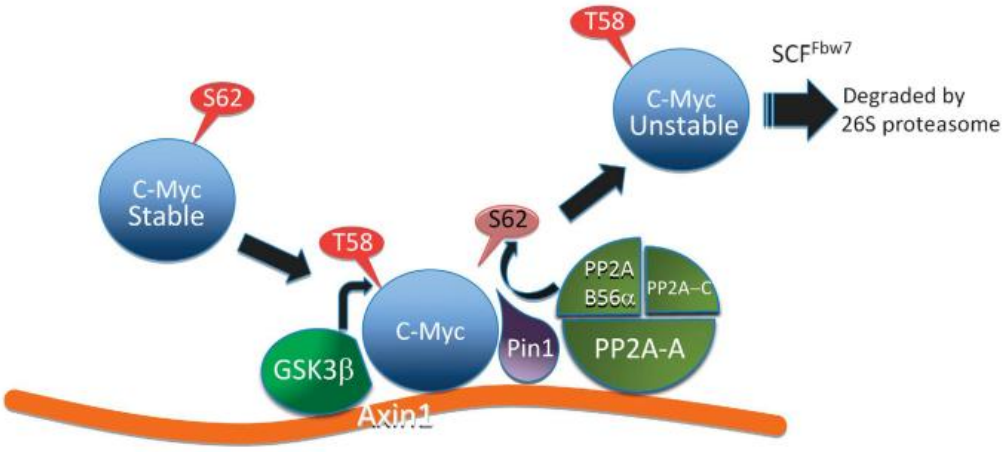

Figure 1.9: Axin1 coordinates the c-Myc destruction complex.

C-Myc which is phoshporylated at S62 is able to bind to Axin1. Axin1 bound Gsk3β then phosphorylates c-Myc at T58. Then, the prolyl isomerase Pin1 isomerizes proline 63 from *cis* to *trans* conformation which allows PP2A to dephosphorylate c-Myc at S62. C-Myc which is singly phoshporylated at T58 is recognized by SCF^{FBW7} and degraded by the proteasome.

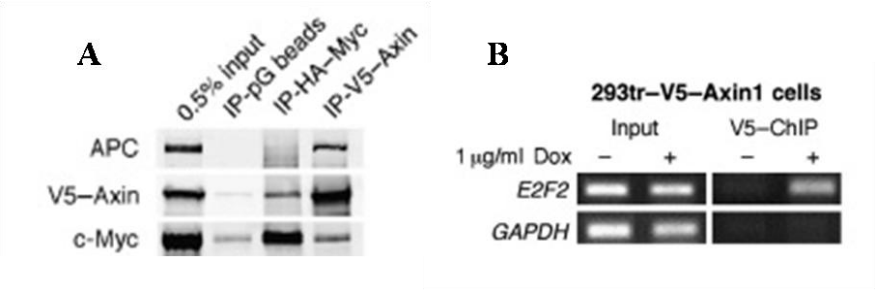

Figure 1.10: c-Myc degradation complex coordinated by Axin1 is distinct from the β-Catenin degradation complex and is present at the promoters.
(A) APC is not present in the c-Myc degradation complex coordinated by Axin1. 293 cells were co-transfected by HA-c-Myc and V5-Axin1. c-Myc or Axin1 was then immunoprecipitated by HA or V5 antibodies. The immunoprecipitated proteins were then analyzed by western blot. (B) Axin1 is present at the promoter sequence of the c-Myc target gene, E2F2. 293tr-V5-Axin1 cells were treated with Dox and V5 antibody was used to immunoprecipitate Axin1. The bound DNA fragments were then amplified using PCR specific for E2F2 promoter sequence. (Data published in Arnold et al., 2009)

c-Myc in cancers

c-Myc potentially regulates approximately 15% of all genes and it is overexpressed in the majority of cancers such as breast, colon, cervical, non small cell lung cancer, osteoblastoma and glioblastoma (Nesbit et al. 1999). C-Myc overexpression in cancers can occur through multiple mechanisms such as gene amplification, translocations and increased protein stability (Meyer & Penn 2008) (Figure 1.11). Homozygous deletion of c-Myc in mice is embryonic lethal and overexpression of c-Myc leads to tumorigenesis in multiple tissues (A. C. Davis et al. 1993; Meyer & Penn 2008). In Burkitt's lymphoma, chromosome 8 is translocated with chromosomes 14, 2, or 22, which contain the immunoglobulin light and heavy chain gene (Dalla-Favera et al. 1982; Taub et al. 1982). This causes the c-Myc gene to be translocated into the highly expressed immunoglobulin locus causing overexpression of c-Myc. It is interesting to note that tumor cells in Burkitt's lymphoma have high mitotic index (Boxer & C V Dang 2001). This model of overexpressed c-Myc also produces clonal lymphomas in the B cell compartment in the Eμ-Myc mouse (J. M. Adams et al. 1985). Also diffuse large B cell lymphoma and multiple myeloma have translocations of c-Myc in approximately 15% of cases. While mutations of Ras are common occurrence in cancers c-Myc mutations are rare except in Burkitt's lymphoma and some AIDS associated lymphomas (Bhatia et al. 1993; Clark et al. 1994). Here, the mutations cluster around the T58 phosphorylation residue and prevent phosphorylation at T58. c-Myc can also be upregulated transcriptionally due to deregulation of its upstream players. In AML, translocation that lead to oncogenic

fusion proteins such as PML-RARα, AML1-ETO has been shown to induce c-Myc expression levels (Müller-Tidow et al. 2004; Rice et al. 2009).

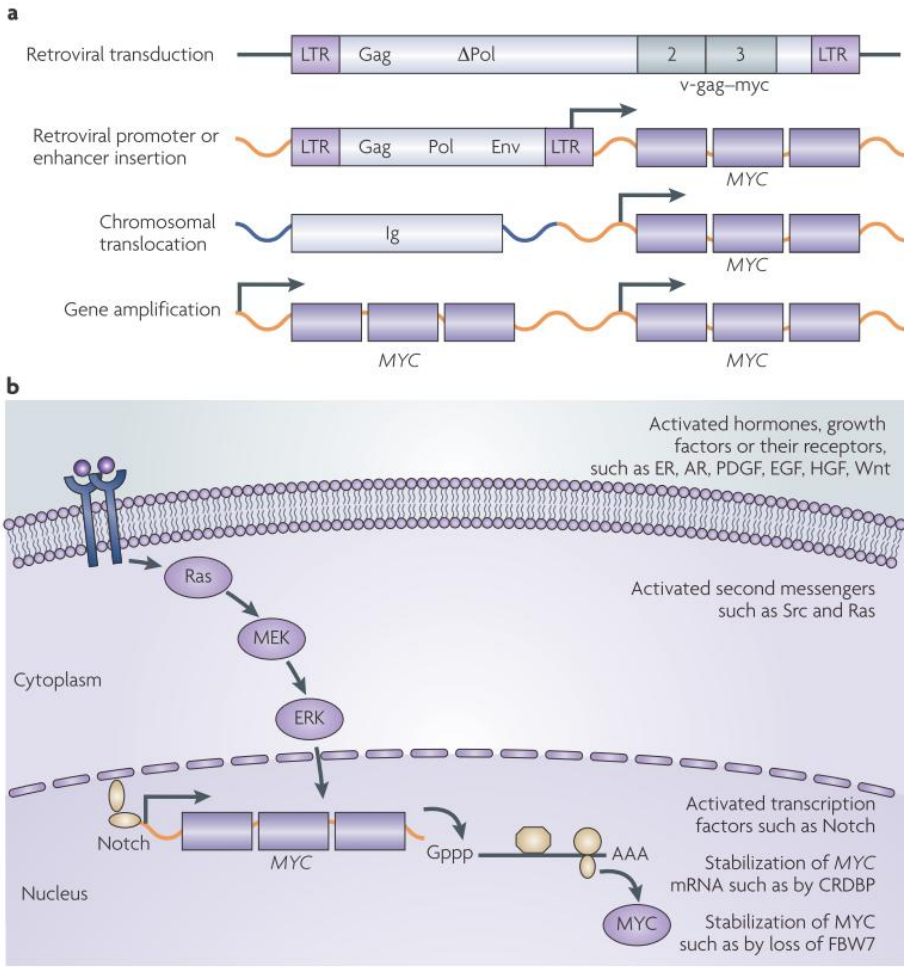

Figure 1.11: Mechanisms of c-Myc deregulation in cancers.
(a) Genetic mechanisms of c-Myc deregulation include retroviral transduction (avian myelocytomatosis), insertional mutagenesis (avian leukosis), Chromosomal translocation (Burkitt's lymphoma) and gene amplification. (b) Deregulation of upstream factors such as activated mitogenic signals, second messengers (RAS, ERK), increased mRNA stability or decreased degradation (loss of FBW7) are other mechanisms in which c-Myc is deregulated. (From Meyer and Penn, 2008, used with permission)

While elevated c-Myc expression is observed in many cancers, only ~20% of them have gene amplification or translocations of the c-Myc gene, indicating that other mechanisms, like increased protein stability, might also play a role in increasing c-Myc expression in cancers. We have previously shown that c-Myc is stabilized in breast cancer and this is associated with a switch in Axin1 expression from variant 1 to variant 2 (X. Zhang et al. 2012). Furthermore, we have also shown that c-Myc has increased protein stability in primary acute lymphoblastic leukemia bone marrow samples compared to normal bone marrow (Malempati et al. 2006). Our lab has also shown that c-Myc is stabilized in the pre B ALL cell line SupB15 and that this stabilization is probably due to a splicing out of exon 5 of Axin1 in this cell line. This deletion removes the Gsk3β binding site of Axin1 and thus decreases Axin1's activity towards c-Myc (Arnold et al. 2009). Taken together, it is clear that deregulation of c-Myc occurs through multiple mechanisms and forms a major driver of tumorigenesis in different cancers.

Chapter Two:

Role of Axin1 in regulating c-Myc oncoprotein in Leukemia

Abstract

Axin1 is a scaffold protein that was originally identified from the mouse fused locus. It is a multi domain protein that acts on the Wnt/β-Catenin, SAPK/JNK, p53, TGFβ and c-Myc pathways. Although Axin1 acts differently on various pathways, its overall function is generally accepted to be a tumor suppressor. Decreased expression of Axin1 has been found to correlate with poor differentiation, lymphatic invasion, lymph node metastasis and poor prognosis in non small cell lung cancer and esophageal carcinoma. Axin1 mutations are also found in HCC's in which activating mutations of β-Catenin are not present. Also, ectopic expression of Axin1 induces apoptosis in some HCC cell lines. We have previously shown that there is a switch in the expression of Axin1, from variant 1 to variant 2, in breast cancer, and this correlates with increased c-Myc protein stability. Work in our lab has shown that leukemia cell lines harbor a splice variant of Axin1 which is less effective in binding to c-Myc and is less efficient in regulating c-Myc protein levels. Based on these findings, I hypothesized that Axin1 plays an important role in regulating c-Myc in leukemia. In this study, I show that increasing Axin1 protein levels is able to decrease c-Myc protein levels in the U937 leukemia cell line, and Axin1 variant 2 is expressed preferentially to variant 1 in this leukemia cell line. In this study, I also sequenced Axin1 in 26 leukemia patient samples and have found many sequence variations in Axin1, but did not find any protein sequence changes, indicating that Axin1 mutations are relatively rare in leukemia.

Introduction

Axin1 is a scaffold protein containing multiple domains. It was originally identified as a protein product from the fused locus in mouse (Zeng et al. 1997). It was later renamed as Axin because it inhibits additional axis formation from Wnt overexpression. Axin1 has been well studied for its function in negatively regulating the Wnt/β-Catenin pathway (MacDonald et al. 2009). The Wnt/β-Catenin pathway plays an important role in determining dorsal ventral axis formation. Deletion of Axin1 causes deregulation of Wnt/β-Catenin signaling, and this leads to aberrant axis formation (Zeng et al. 1997; Farr et al. 2000). In addition, Axin1 has also been found to act on the TGFβ, SAPK/JNK pathway, p53 and c-Myc pathways (Wen Luo & Lin 2004; Y. Rui et al. 2004; Arnold et al. 2009).

Axin1 has a critical function in the Wnt/β-Catenin signaling pathway by acting to increase β-Catenin degradation. Axin1, along with APC, Gsk3β, and CK1 forms a degradation complex, which phosphorylates the transcription factor β-Catenin when Wnt/β-Catenin signaling is in the off state (MacDonald et al. 2009). This phoshporylated β-Catenin is then recognized by βTRCP ubiquitin ligase and degraded. However, in the presence of Wnt, the degradation complex of β-Catenin is suppressed by yet unknown mechanisms, leading to accumulation of β-Catenin stabilization and upregulation of its target genes.

Although Axin1 acts on multiple pathways and regulates different functions, its net effect is considered to be a tumor suppressor since it

suppresses proliferation and induces apoptosis (Salahshor & Woodgett 2005). Upregulation of Wnt signaling has been shown to be important in HCCs and Axin1 has been found to be mutated in HCCs that do not harbor activating β-Catenin mutations (Miyoshi et al. 1998; de La Coste et al. 1998). Axin1 mutations have been found in three HCC cell lines and all these cell lines have high levels of nuclear β-Catenin (Satoh et al. 2000). Ectopic expression of Axin1 has been induces apoptosis, indicating that deregulated Axin1 forms a critical factor in tumorigenesis of HCC. Decreased expression of Axin1 correlates with poor differentiation in non small cell lung cancer (Xu et al. 2011; L.-H. Yang et al. 2010). Also, Axin1 expression might play a more important role than β-Catenin mutations in determining prognosis of NSCLC (Han et al. 2009). In esophageal squamous cell carcinoma, Axin1 expression was found to be inversely correlated with depth of invasion, lymph node metastasis and lymphatic invasion, suggesting that Axin1 plays an important role in suppressing tumor progression (A. F.-Y. Li et al. 2009). Sequence variations of Axin1 have been found in multiple cancers, including colorectal cancer, medulloblastoma, adenoid cystic carcinoma as shown in Figure 2.1 (Salahshor & Woodgett 2005). These mutations occur in previously characterized binding regions of Axin1 through which it binds to its partner proteins, and could lead to disruption of binding and thereby alter function. Taken together, this suggests that Axin1 functions as a tumor suppressor and is mutated and downregulated in multiple cancers.

Table 1 Sequence variants of the Axin1 gene found in human cancer

Tissue type	Exon/nt or aa	Position (aa)	Domain(s) affected	Ref
CRC	1	S215L	APC–MEKK1?	94
CRC	1	R103M	APC	95
CRC	1	L101P	APC	95
CRC	3	R353H	GSK3	95
CRC	1	T122A	APC	95
CRC	4	E411D	GSK3	95
CRC	4	M418I	GSK3	95
CRC	4	R395C	GSK3	95
CRC	5	G584S	β Catenin	95
CRC	2	Y305X	MEKK1	96
CRC	1	K203M	APC	96
CRC	2	N307K	MEKK1	96
CRC	4	H394N	GSK3	96
CRC	10	P848L	Axin–DVL	96
CRC	10	E852G	Axin–DVL	96
CRC	1	S215L	APC–MEKK1?	94
MB	1–5 (aa 89–528)	Del	APC–GSK3–β catenin	21
MB	1–5 (aa 111–571)	Del	APC–GSK3–β catenin	21
MB	1–5 (aa 59–532)	Del	APC–GSK3–β catenin	21
MB	9–10	Del	Axin–DVL	21
MB	8–10	Del	Axin–DVL	21
MB	6–10	Del	PP2A–axin–DIX	21
MB	7–10	Del	PP2A–axin–DIX	21
MB	1	P255S	APC	21
MB	1	P255S	MEKK1	20
MB	1	S263C	MEKK1	20
HCC	1	Stop	APC–DVL–axin	2
HCC	4	Stop	GSK3–DVL–axin	2
HCC	4	5 bp del	β Catenin	2
HCC	5	13 bp del	β Catenin	2
HCC	3	P345L	MEKK1	97
HCC	1	L106R	APC	97
HCC	2 (1076 del 1 bp)	Frameshift	MEKK1?	97
HCC	5	Ins QVHH	β Catenin–PP2A?	97
HCC	5	G425S	β Catenin	97
HB	10	R842Q	Axin–DIX	97
HB	6	G651S	PP2A?	97
HB	2	T95M	APC	98
EM	4	A397V	GSK3	99
SCC	4	G419A	GSK3	100
OEA	4	L416X	GSK3	101
ACC	7	P661L	PP2A	102
ACC	5	G433E	β Catenin	102
ACC	10	E842K	Axin–DVL	102
ACC	10	F824K	Axin–DVL	102
ACC	6	S628Y	PP2A?	102

Mutations found in the axin1 coding sequence are scattered throughout the whole gene. There are some differences between the alterations found in different forms of tumours. Large deletions are more common in medulloblastomas, whereas missense mutations occur more frequently in hepatocellular carcinomas. Silent mutations and polymorphisms reported in axin1 are not included in this list. Protein and mRNA sequences are based on Swissprot and GenBank accession numbers 015169 and AF009674, respectively.
aa, amino acids; ACC, adenoid cystic carcinoma; APC, adenomatous polyposis coli; bp, base pair; CRC, colorectal cancer; del, deletion; DVL, Dishevelled; EM, endometrial cancer; GSK3, glycogen synthase kinase 3; HB, hepatoblastoma; HCC, hepatocellular cancer; MB, medulloblastomas; MEKK1, mitogen activated protein/extracellular regulated kinase kinase kinase 1; nt, nucleotide; OEA, ovarian endometrioid adenocarcinoma; PP2A, protein phosphatase 2A; SCC, oesophageal squamous cell carcinoma.

Figure 2.1: Axin1 mutations are prevalent in different types of cancers.
Mutations found in Axin1 sequence by different studies are listed along with the type of cancer, the exon in which the mutation is located and the binding partners that are possibly affected. (From Salshor and Woodget, 2004, used with permission)

We have previously shown that Axin1 acts to regulate c-Myc protein levels (Arnold et al. 2009). More specifically, Axin1 along with Gsk3β, PP2A, and Pin1 forms a destruction complex, which decreases c-Myc protein levels. Ectopically expressed Axin1 in the 293 cells is able to decreases c-Myc protein levels (Figure 2.2). Knockdown of Axin1 increases S62 phosphorylation, decreases T58 phosphorylation and increases c-Myc protein levels, and half-life indicating that Axin1 functions as an important regulator of c-Myc degradation.

Also, previously we have shown that in the pre B ALL leukemia cell line, SupB15, sequencing of Axin1 cDNA showed a heterozygous in-frame deletion of Axin1 exon 5 (Malempati et al. 2006; Arnold et al. 2009). Recent data from our lab suggests that this is a new splice variant of Axin1, which we have termed Axin1 Variant 3, and is expressed in many leukemia cell lines (Figure 2.3). We have sequence verified and confirmed that exon 5 is spliced out in these cell lines. We have found that Axin1, in which exon 5 is spliced out, shows decreased binding to c-Myc and no binding to Gsk3β (Figure 2.4). Consistently, the SupB15 cell line also showed increased c-Myc stability, and decreased c-Myc, Gsk3β interaction, likely due to a defective regulation of c-Myc by Axin1.

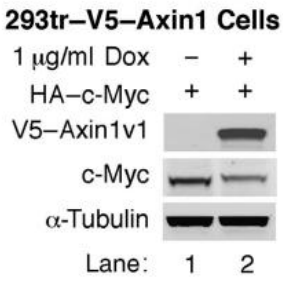

Figure 2.2 Ectopically expressed Axin1 is able to decrease c-Myc protein levels in 293 cells.
293tr cells which were stably transfected with V5-Axin1 cells were then transfected with HA-c-Myc, and treated with Doxycycline for 4hrs as indicated. The lysates were separated by western blot and proteins were detected by immunoblotting. (Data published in Arnold et al, 2009).

Although our new data demonstrates that leukemia cell lines express a splice variant of Axin1 which shows decreased binding to c-Myc, whether Axin1 plays a role in regulating c-Myc levels in leukemia is not known. Moreover, while Axin1 mutations have been found in many solid tumors (Salahshor & Woodgett 2005) whether mutations in Axin1 occur in leukemia patients is not known.

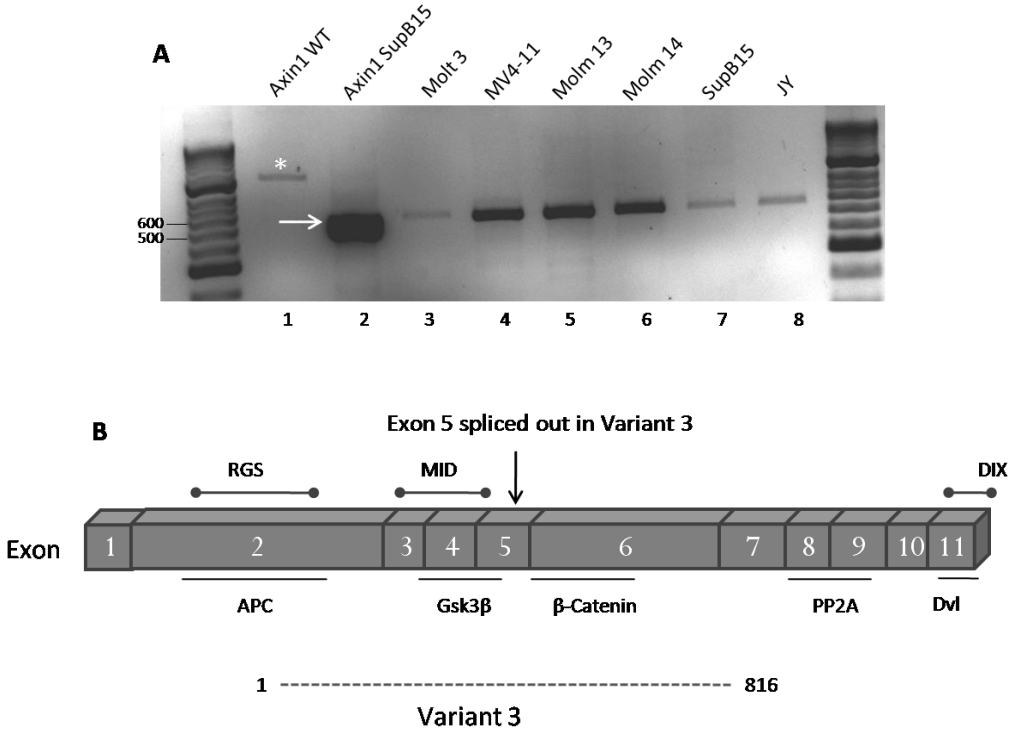

Figure 2.3: Leukemia cell lines express a novel splice variant of Axin1.
(A) cDNA from leukemia cell lines was used as a template for PCR amplification using a forward primer which is complementary to the junction of exon 4 and 6, and a reverse primer complementary to the 3' end of exon 6. Axin WT and Axin SupB15 (cloned from SupB15 cDNA) plasmids are used as template for positive and negative controls in lane 1 and 2 respectively. The sequence verified positive band (542 base pairs) is indicated by the arrow, and a non specific band is marked by *. (B) Schematic of Axin1 Variant 3 showing the exon that is spliced out. (Ellie Juarez, unpublished data).

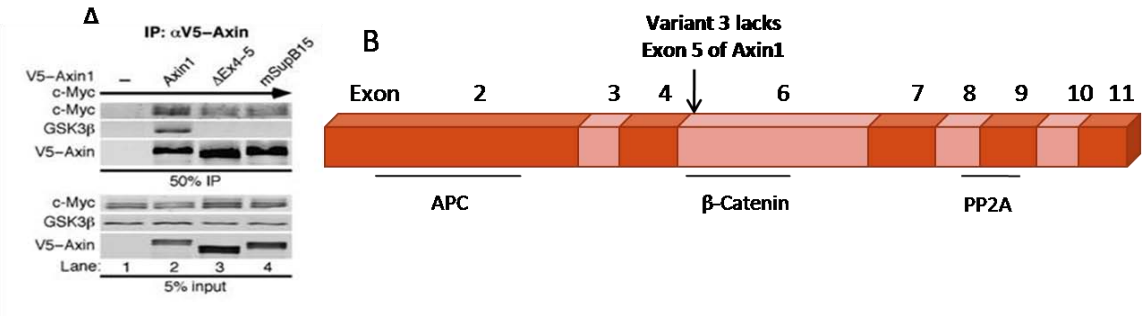

Figure 2.4: Leukemia cell lines express a variant of Axin1 which lacks exon 5 and affects Gsk3β and c-Myc binding to Axin1.
(A) 293 cells were co transfected with c-Myc and empty V5, V5 Axin1 WT, V5 Axin ΔExons 4-5 or V5 mSupB15 (Axin1 cDNA sequence cloned from SupB15 with missing exon 5). The input and immunoprecipitated proteins were separated and detected by western blot. (Data published in Arnold et al. 2009) (B) Schematic showing the location of the exon 5 which is spliced out in variant 3 of Axin1 expressed in leukemia cell lines.

To answer these questions, I used the U937 leukemia cell line as a leukemia model. I also obtained leukemia patient samples and sequenced *Axin1* in these samples to screen for Axin1 mutations.

Results

Axin1 is a multidomain scaffold protein which acts on multiple signaling pathways (Wen Luo & Lin 2004; Arnold et al. 2009). Altered Wnt/β-Catenin signaling has been implicated in hepatocellular carcinomas and colorectal cancers, and it has been shown that samples with wild type β-Catenin, Axin1 is either downregulated or mutated, suggesting that deregulation of Axin1 could be a mechanism of tumorigenesis (Miyoshi et al. 1998; de La Coste et al. 1998; Webster et al. 2000; W. Liu et al. 2000). Axin1 has been elucidated to mainly act as a tumor suppressor, and decreased expression of Axin1 has been correlated with poor differentiation, increased invasion, increased metastasis in many

cancers and also correlates with poor survival in non small cell lung cancer and esophageal cancer patients (Salahshor & Woodgett 2005).

We have previously shown that Axin1, by recruiting Gsk3β, PP2A, and Pin1, coordinates a degradation complex for c-Myc (Arnold et al. 2009). Ectopically overexpressed Axin1 is able to decrease c-Myc protein levels in 293 cells. Our lab has also shown that deregulation of this c-Myc degradation complex in one the breast cancer cell lines leads to increased c-Myc protein stability (X. Zhang et al. 2012). Also, we have found that there is a shift in the expression of Axin1, to predominantly variant 2 over variant 1, and this correlates with increased c-Myc protein levels. Since variant 2 binds less robustly to PP2A and c-Myc, this suggests that deregulation of the c-Myc degradation complex, coordinated by Axin1, plays a role in breast cancer. Moreover, we have also found that in the SupB15 pre B ALL cell line, in which c-Myc is stabilized, expresses a new splice variant of Axin1 that decreases its binding to Gsk3β and c-Myc (Malempati et al. 2006; Arnold et al. 2009). Taken together, these findings suggest that Axin1 coordination of c-Myc degradation complex plays an important role in regulating c-Myc. Whether Axin1 protein levels play a role in regulating c-Myc in leukemia is not known.

In order to begin to examine whether Axin1 plays a role in regulating c-Myc level in leukemia, I used the U937 leukemia cell line as a model. In order to alter Axin1 protein levels I used the small molecule, XAV939, which increases Axin1 levels by decreasing its degradation. Briefly, Axin1 is PARsylated by tankyrase (PARP5) (S.-M. A. Huang et al. 2009). This PARsylated Axin1 is then

linked to ubiquitin dependent degradation by the PARsylation dependent E3 ubiquitin ligase, RNF146 (Yue Zhang et al. 2011; Callow et al. 2011). XAV939 inhibits Tankyrase and decreases Axin1 PARsylation and thereby inhibits its degradation, leading to increased Axin1 levels. Tankyrase is also known to affect the telomere binding protein TRF1 (Smith et al. 1998). But the function of tankyrase is not well understood.

I incubated the U937 leukemia cell line with XAV939, at 1uM, for 72 hours. As a control, I used DMSO at the same volume as XAV939. The cells were then harvested after 72 hours and the proteins were visualized by western blot. As shown in Figure 2.5, I found that XAV939 is able to increase Axin1 levels as reported before. I also found that the increased Axin1 levels produces a corresponding decrease in c-Myc levels in the XAV939 treated cells, but not in the DMSO treated cells. This is consistent with my hypothesis that Axin1 levels might play a role in regulating c-Myc protein levels in leukemia.

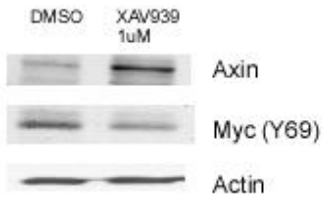

Figure 2.5: Treatment with XAV939 is able to increase Axin1 levels and decrease c-Myc protein levels in the U937 leukemia cell line.
U937 cells were incubated with either DMSO or XAV939 as indicated for 72 hrs. The cells were then lysed. Axin1 and c-Myc in the lysate were detected by immunoblotting. β-Actin was used as a loading control.

The U937 leukemia cell line can be induced to differentiate into the macrophage lineage by 12-O-tetradecanoylphorbol-13-acetate (TPA) and has been used as a model for differentiation studies (Stöckbauer et al. 1983). It has been shown that overexpressed c-Myc can prevent differentiation of U937 cell line induced by TPA (Larsson et al. 1988). Furthermore, phosphorylation at the Serine 62 site is required for c-Myc's ability to prevent differentiation (Hydbring et al. 2010). Based on these findings, I hypothesized that decreased c-Myc might be able to induce differentiation of the U937 leukemia cell line. In order to test this hypothesis, I incubated U937 cells with XAV939 at 1uM for 72 hours. But I did not find evidence of differentiation of U937 cells into the macrophage lineage based on morphology and the CD33 marker. This suggests that while c-Myc is able to prevent differentiation of U937 leukemia cells to macrophage lineage

decreased c-Myc is not sufficient for induction of differentiation into the macrophage lineage.

The *Axin1* gene is reported to encode two splice isoforms called variant 1 and variant 2, and we have found a 3rd variant as described above. Axin1 variant 2 differs from Axin1 variant 1 in that it lacks the region coded by exon 9. Lack of exon 9 removes part of the PP2A binding domain of Axin1 in variant 2 and we have shown that it has weak binding to PP2A. We have previously shown that the variant 2 of Axin1 is less effective in binding c-Myc (Arnold et al. 2009). Since variant 2 lacks the PP2A binding domain and shows less binding to c-Myc its might be less effective in coordinating a degradation complex for c-Myc. Moreover, we have also shown that this switch in the expression of Axin1 from variant 1 to variant 2 might play a role in the deregulation of c-Myc in breast cancer (X. Zhang et al. 2012). However, the relative level of expression of Axin1 variant 1 versus variant 2 in leukemia is not known.

To determine if Axin1 variant 2 is preferably expressed to Axin1 variant 1 in leukemia, I used the U937 cell line as the leukemia model. RNA was isolated form U937 cells cultured under normal conditions, using Trizol extraction protocol, and cDNA was prepared from the isolated RNA. In order to assess the relative levels of expression of variant 1 versus variant 2, I performed a semi-quantitative PCR using the cDNA prepared from the RNA as the template using forward primer on exon 8 (2229 forward) and reverse primer on exon 11 (2782

reverse). Axin1 variant 1, which contains the exon 9, will produce a 553 base pair band and Axin1 variant 2, which does not contain exon9, will produce a 446 base pair band. As show in figure 2.6, panel (A), we found that there is relatively more expression of Axin1 variant 2 compared to Axin1 variant 1. This contrasts to other non transformed cells we have examined in the lab which express variant 1 and variant 2 at similar levels (Figure 2.6, panel B).

Axin1 variant 2 does not have part of the PP2A binding domain, and we have previously shown that it is less effective in binding c-Myc. We have also shown that a switch in the expression of Axin1 from variant 1 to variant 2 might play an important role in deregulated c-Myc expression in breast cancer. Higher expression of variant 2 relative to variant 1, as indicated by my data, suggests that this might be a possible mechanism of deregulation of c-Myc in leukemia. With our recent discovery of variant 3, a similar analysis of its relative expression and importance in leukemia is warranted.

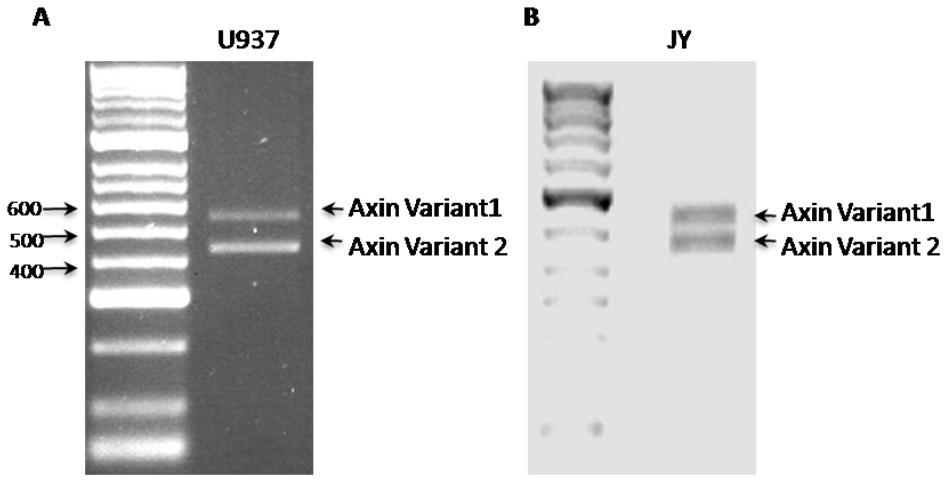

Figure 2.6: U937 leukemia cell line express relatively more Axin1 variant 2 than Axin1 variant1.

(A) cDNA was made from mRNA isolated from U937 cells cultured under normal conditions. The cDNA was used as a template in a PCR with primers spanning exon 8 and 10. The PCR products were then separated on an agarose gel. Variant 2 is visualized as the 446 base pair band and variant 1 is visualized as the 553 base pair band. The PCR was run for 20 cycles. (B) The non transformed JY cell expressed similar levels of Axin1 variant 1 and variant 2 (Hugh Arnold, unpublished data)

Axin1 functions on the Wnt/β-Catenin, TGFβ, SAPK/JNK, p53 and c-Myc pathways. However, its overall function is generally considered to be a tumor suppressor (Salahshor & Woodgett 2005). Over expression of Axin1 in A549 lung cancer cell line and BE1 cell lines has been shown to induce apoptosis by induction of the p53 or the JNK pathways (L.-H. Yang et al. 2010). It has been shown that Axin1 is homozygously deleted in the HCC lines including SNU475 and Alexander (Satoh et al. 2000). In another HCC cell line, SNU423, one of the allele harbors a deletion in exon3 and the other allele is lost. Furthermore,

reintroduction of Axin1 by adenovirus induces apoptosis in the Alexander cell line. This strongly suggests that Axin1 functions as a critical tumor suppressor in multiple cancers.

Decreased Axin1 expression has been shown to correlate with poor differentiation, TNM stage and increased lymph node metastasis in non small cell lung cancer (Xu et al. 2011; L.-H. Yang et al. 2010). Axin1, being a tumor suppressor, is frequently mutated in multiple cancers. Axin1 mutations have been found in prostate cancer and might contribute to the accumulation of nuclear β-Catenin (Yardy et al. 2009). In one study, Axin1 was found to be mutated in HCT-8, HCT-15 and DLD1 colon cancer cell lines (Webster et al. 2000). Furthermore, functional characterization of this mutant Axin1 showed altered binding to Gsk3β and it was less effective in suppressing Wnt/β-Catenin pathway. Another study found point mutations and deletions in as many as 12% of medulloblastoma samples (Baeza et al. 2003). Taken together, this indicates that Axin1 mutations play an important role in tumor progression and decreased Axin1 expression is correlated with poor differentiation and poor prognosis of many cancers. But whether Axin1 mutations occur in leukemia is not known. Since Axin1 mutations aid in the progression of different tumor types I hypothesized Axin1 mutations might play a role in leukemia.

In order to test this hypothesis, we obtained blood and bone marrow samples from 26 leukemia patients through Dr. Guang Fan MD, PhD and Dr. Harvey Fleming MD, PhD in OHSU. I isolated DNA from these leukemia samples and PCR amplified the 11 exons of *AXIN1* using 16 overlapping primer sets. The

primers also covered adjacent intronic regions in order to cover splice sites. I then sequenced the DNA using Sanger sequencing method at OHSU sequencing core (Clive Woffendin). The trace files of the sequences were then analyzed against NCBI sequence file NM_003502.3 using Mutation Surveyor software (Softgenetics). The results are tabulated in Table 2.1. I found many sequence variations in the *AXIN1* gene and many of these sequence variations were previously reported as Single Nucleotide Polymorphisms (SNPs) in NCBI SNP database. If a variation has been previously reported in the NCBI SNP database then its reference id is noted in the table. Some of the SNPs were found in as many as 18 samples out of the 26 samples sequenced. However, we did not observe any changes that would produce a change in the amino acid sequence.

Table 2.1: Sequence variations in *AXIN1* in Leukemia patients

Exon	Position	Reported SNP	Sequence reference>sample	Amino acid position	Amino acid subsitution	No of samples	Sample	Type of Leukemia
2	-19	dbSNP:758033	C>C/A	NA	NA	6	B68	B ALL
							T22	T ALL
							AP092707	AML
							AP122007	AML
							AP011708	AML
							AP032008	AML
2	762	dbSNP:1805105	T>C/C	254	D>D	5	B25	B ALL
							B40	B ALL
							T23	T ALL
							AP092707	AML
							AP01708	AML
2	762	dbSNP:1805105	T>T/C	254	D>D	13	B54	B ALL
							B61	B ALL
							B63	B ALL
							B68	B ALL
							T21	T ALL
							T22	T ALL
							T24	T ALL
							T34	T ALL
							T35	T ALL
							AP102707	AML
							AP120407	AML
							AP122007	AML
							AP032008	AML
3	1019+75	NO	G>G/A	NA	NA	3	AP122007	AML
							T20	T ALL
							T34	T ALL
4	1116+20	dbSNP:2301522	T>T/C	NA	NA	13	B61	B ALL
							B63	B ALL
							B68	B ALL
							T21	T ALL
							T22	T ALL
							T23	T ALL
							T34	T ALL
							T24	T ALL
							T34	T ALL
							T32	T ALL
							AP032008	AML
							AP120407	AML
							AP122007	AML
4	1116+20	dbSNP:2301522	T>C/C	NA	NA	6	B25	B ALL
							B40	B ALL
							B54	B ALL
							AP011708	AML
							AP102707	AML
							APO92707	AML
5	1254+17	dbSNP:62032881	G>G/A	NA	NA	4	AP011708	AML
							AP122007	AML
							APO92707	AML
							T22	T ALL
5	1254+73	NO	ins_C	NA	NA	3	T20	T ALL
							T34	T ALL
							B40	B ALL
6	1284	dbSNP:214250	G>G/A	428	S>S	8	AP011708	AML
							AP032008	AML
							APO92707	AML
							T24	T ALL
							T21	T ALL
							T20	T ALL
							B68	B ALL
							B25	B ALL
6	1284	dbSNP:214250	G>A/A	428	S>S	1	B40	B ALL
6	1677	NO	G>A/A	559	Q>Q	1	B54	B ALL
7	1827	dbSNP:214252	T>T/C	609	A>A	8	AP011708	AML
							AP032008	AML
							APO92707	AML
							B25	B ALL
							B68	B ALL
							T20	T ALL
							T21	T ALL
							T24	T ALL

7	1827	dbSNP:214252	T>C/C	609	A>A	1	B40	B ALL
9	2187-65	dbSNP:412243	A>A/G	NA	NA	4	AP011708	AML
							AP122007	AML
							T20	T ALL
							B40	B ALL
9	2187-65	dbSNP:412243	A>G/A	NA	NA	3	AP032008	AML
							APO92707	AML
							T22	T ALL
10	2354+25	dbSNP:387467	C>C/A	NA	NA	6	AP011708	AML
							AP032008	AML
							APO92707	AML
							B25	B ALL
							T21	T ALL
							T20	T ALL
10	2354+25	dbSNP:387467	C>A/A	NA	NA	1	B40	B ALL

The sequence variations found in 26 Leukemia patient samples are listed along with its location relative to exons, its reference ID if already reported, amino acid change, the sample ID, and the type of Leukemia.

The position of the sequence variation is derived from using the file NM_003502.3 from GenBank. The A of the ATG start codon is numbered as 1. The corresponding amino acid Methionine is counted as 1 for the amino acid position.

Variations in the intronic region are noted as the number of nucleotides from the nearest coding exon. For example 2187 – 65 means the variation is 65 nucleotides 5’ of exon 9 which starts at position 2187 in the sequence file NM_003502.3.

If the variation has already been reported then the corresponding reference ID from NCBI SNP database is listed.

The variations are indicated as Reference sequence > N when the sample is heterozygous at that particular location and as Reference sequence > N/N when the sample is heterozygous at that location.

Interestingly, I observed a SNP at 19 base pairs upstream of the ATG start codon (-19) in the 5' untranslated region of *AXIN1* mRNA. This -19 SNP has been previously reported in NCBI SNP database (dbSNP) and in our study we found this SNP in one B ALL, one T ALL and four AML samples. Since 5' regions of mRNA could have an effect on mRNA stability, I hypothesized that this -19 SNP might alter *AXIN1* mRNA levels. In order to test this hypothesis, I measured the expression levels of *AXIN1* mRNA in the AML samples. As shown in figure 2.7, while the average mRNA level was lower in samples containing the -19 SNP compared to the samples that do not contain -19 SNP, this was not statistically significant. But this might be due to our sample size being relatively small for comparative analysis.

Furthermore, I also analyzed the frequency of occurrence of the SNPs in leukemia by comparing it to the reported frequency in the NCBI SNP database. As shown in Table 2.2, I found that the frequency in which the SNPs occur in leukemia generally correlate with the reported frequency in dbSNP. Interestingly, I found a SNP at 1254+17 position which is 17 nucleotides 3' of exon 5 in 15% of our samples. It is interesting to note that since this SNP occurs near the intron exon junction it might have an effect on splicing of exon 5 in leukemia. This SNP could affect splicing even though this is not within the splice acceptor or the splice donor region since the spliceosome consists of large multiprotein complexes and changes in the DNA sequence might affect binding of splicing factor proteins. Furthermore, this is significant with regard to our recent data showing that leukemia cell lines express a variant of Axin1 in which exon 5 is

spliced out (Figure 2.3). But, frequency information for this SNP is not currently available in dbSNP. Moreover, I also found a SNP at 2185-65 which is 65 nucleotides 5' of exon 9, which is spliced out in Axin1 variant 2, in 26% of samples. This SNP might also affect the splicing of exon 9 and alter the relative expression of Axin1 variant 2 over variant 1. This is interesting with respect to my finding that the U937 leukemia cell line expresses Axin1 variant 2 preferentially over variant 1 and our previous report of switch in expression from Axin1 variant 1 to variant 2 in breast cancer (X. Zhang et al. 2012).

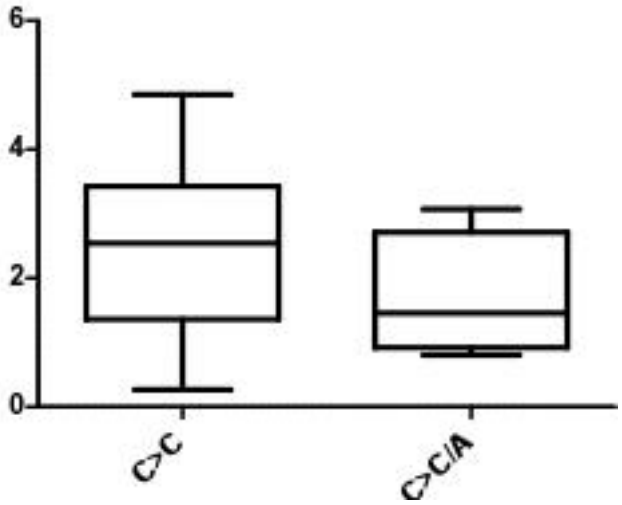

Figure 2.7: Axin1 expression in AML samples with (C>C/A) and without (C>C) the -19 SNP.

mRNA was isolated from equal number of AML cells and cDNA was made using ABI reverse transcription kit. Axin1 expression was measured using quantitative PCR and normalized with 18S expression and plotted. (Deanne Tibbitts, unpublished data)

Many previous sequencing studies of Axin1 have found point mutations, deletions, and loss of alleles in hepatocellular cancer, medulloblastoma, colorectal cancer, and prostate cancer (Salahshor & Woodgett 2005). However, I sequenced Axin1 in 26 leukemia samples and found many SNPs, but did not find any amino acid changes. This suggests that Axin1 protein sequence changes are relatively rare in leukemia. Further analysis of Axin1 mutations in leukemia would require a much larger panel in order to uncover any rare mutations of Axin1 in leukemia.

Table 2.2: Frequency of SNPs in Leukemia compared to frequency reported in NCBI SNP database

Exon	Position	Reported as SNP	Sequence Reference>Sample	No of Samples	Frequency in this study	Reported frequency in dbSNP
2	-19	dbSNP:758033	C>C/A	6	23	29.7
2	762	dbSNP:1805105	T>C/C	5	19.2	37.2
			T>T/C	13	50	46.9
4	1116+20	dbSNP:2301522	T>T/C	13	50	48.7
			T>C/C	6	23	36.3
5	1254+17	dbSNP:62032881	G>G/A	4	15.3	-
6	1284	dbSNP:214250	G>G/A	8	30.7	34.5
			G>A/A	1	3.8	5.3
7	1827	dbSNP:214252	T>T/C	8	30.7	35.7
			T>C/C	1	3.8	4.5
9	2187-65	dbSNP:412243	A>A/G	4	15.3	-
			A>G/G	3	11.5	-
10	2354+25	dbSNP:387467	C>C/A	6	23	31
			C>A/A	1	3.8	5.2

The frequency of reported SNP's that have been found in this study is compared to its frequency in the NCBI SNP database. The overall frequency for a particular SNP is used when available in the database. When the overall frequency is not available for a SNP, average frequency from studies done in European population is listed. (–) indicates that no frequency information is available in the database for that particular SNP.

Discussion

In this study, I found that increasing Axin1 levels produces a corresponding decrease c-Myc protein levels in the U937 cells, suggesting that Axin1 plays a role in regulation c-Myc protein levels in leukemia. I also found that Axin1 variant 2 is expressed relatively more than Axin1 variant 1 in the U937 leukemia cell line. I have also sequenced Axin1 from 26 leukemia samples and found that Axin1 mutations are relatively rare in leukemia.

Axin1 functions as a scaffold protein and coordinates the formation of the degradation complex for β-Catenin (MacDonald et al. 2009). Axin1 has been found to be the rate limiting member of the degradation complex and over expression of Axin1 leads to suppression of nuclear β-Catenin (E. Lee et al. 2003). We have previously shown that Axin1 also functions to coordinate a degradation complex for c-Myc consisting of Gsk3β, PP2A, and PIN1 (Arnold et al. 2009). Over expression of Axin1 is able to increase c-Myc ubiquitination and degradation in 293 cells, and knocking down Axin1 leads to increased c-Myc protein levels and stability. In this study, I have used the small molecule XAV939, to increase Axin1 protein levels in the U937 leukemia cell line and have shown that this produces a corresponding decrease in c-Myc protein levels. This is consistent with our previous finding that Axin1 forms a degradation complex for c-Myc. This suggests that Axin1 can regulate of c-Myc protein levels in leukemia. However, since XAV939 acts on Tankyrase, the increase in c-Myc protein levels might also be due to Tankyrase's effect on its other targets other than Axin1. Also, XAV939 might also affect other unknown proteins and the decrease in c-

Myc protein levels might be due to off-target effects of XAV939. Further studies which knockdown Axin1 in leukemia cell lines are warranted.

We have previously shown that variant 2 of Axin1 is less effective in binding c-Myc (Arnold et al. 2009). In this study, I found that Axin1 variant 2 is expressed more than Axin1 variant 1 in the U937 leukemia cells. This is consistent with our earlier finding that there is a switch in expression from Axin1 variant 1 to variant 2 in breast cancer cells compared to normal cells (X. Zhang et al. 2012). This switch is associated with increased c-Myc stability, decrease in T58 phosphorylation and increased S62 phosphorylation. In this regard, my finding that the U937 cells express more of variant 2 suggests that this might have a significant role in deregulation of c-Myc in leukemia. Future studies of regulation of the splicing factors that control splicing of exon 9 might provide valuable insights into the deregulation of c-Myc in leukemia.

In this study, I have sequenced Axin1 in leukemia samples and have found that Axin1 coding mutations are relatively rare in leukemia. Previous studies have found multiple coding mutations in Axin1 in hepatocellular cancer, colorectal cancers, medulloblastoma and prostate cancer (Salahshor & Woodgett 2005). However, my study of Axin1 mutations in leukemia did not find any amino acid changes. In this study, I found the -19 SNP in four out of 8 AML samples. However, whether this -19 SNP is associated with decreased expression of Axin1 in cancers as shown by previous studies remains to be seen. One of the previous studies functionally characterized a point mutation of Axin1 found in cancer and have shown that it decreases Axin1 association to Gsk3β (Webster et

al. 2000). However, functional characterization of other mutations found by previous studies is lacking. Moreover, these studies were conducted prior to our finding that Axin1 forms a degradation complex for c-Myc. In this regard, it is interesting that one of the previously found mutations fall in the binding site of c-Myc as described in the next chapter (Taniguchi et al. 2002). But, whether this mutation affects Axin1's function towards c-Myc remains to be seen.

In this study, I found a SNP at position 1254 + 17 which is present 17 nucleotides 3' from the end of exon 5. Interestingly, recent data from our lab suggests that leukemia cells express an isoform of Axin1 in which exon 5 is spliced out (Figure 2.3). Since this SNP occurs near the intron exon junction, and is found in 15% of the leukemia samples sequenced, it is possible that this SNP, by affecting binding of splicing factors, leads to exon 5 being spliced out in leukemia. However, since frequency of this SNP in the general population is not known, whether this SNP is present in increased frequency in leukemia samples remains to be seen. Future studies should include sequencing mRNA of the leukemia patient samples in which the 1254 + 17 SNP is present to check whether these samples express the new Axin1 splice variant (Variant 3) which lacks exon 5. Furthermore studies which sequence Axin1 in the leukemia cell lines that express this splice variant to check whether they contain the 1254 + 17 SNP are warranted. Moreover, we also found a SNP at position 2187 - 65 which occurs in 7 of the 26 samples sequenced. Since this SNP also occurs near the intron-exon junction of exon 9, it is possible that this SNP alters the relative expression of Axin1 variant 2 over variant 1. We have previously shown that

Axin1 variant 2 is less effective in binding to c-Myc and this form of Axin1 is enriched in breast cancer cell lines with stabilized c-Myc, suggesting that variant 2 might be less effective in regulating c-Myc. Thus, the SNP at 2187-65 could affect splicing of exon 9, increasing variant 2 levels and could lead to increased c-Myc stability and predispose to leukemogenesis. This might suggest that this SNP would be enriched in leukemia samples. Unfortunately, since the frequency of occurrence of this SNP in the general population is currently not known, we cannot determine whether SNP 2187-65 is enriched in leukemia samples. In this regard, it is interesting to note that the U937 leukemia cell line also expresses Axin1 variant 2 preferentially over variant 1 (Figure 2.6). It remains to be studied whether the U937 leukemia cell line carries this particular SNP, correlating with its high expression of variant 2. Future studies of Axin1 sequence in leukemia cell lines which express more Axin1 variant 2 could throw light on the significance of the 2187-65 SNP in leukemia.

In this study, I have found many sequence variations of Axin1 that might potentially alter Axin1 mRNA expression and splicing in leukemia. I also found that mutations of Axin1 were not present in the sequenced leukemia samples. This suggests that Axin1 mutations are relatively rare in leukemia. Further studies with a much larger sample size might bring to light rare mutations of Axin1 in leukemia.

In summary, my data is consistent with Axin1 regulating c-Myc protein levels in leukemia. I have also shown that the U937 leukemia cell line preferably expresses Axin1 variant 2 over variant 1. In addition, I have analyzed a panel of

leukemia patient samples for Axin1 mutations and show that Axin1 mutations are relatively rare in leukemia.

Chapter Three:

Role of Axin1 Exon7 in the regulation of c-Myc

Abstract

Axin1 is a scaffold protein that harbors multiple domains and is known to regulate multiple pathways, namely Wnt/β-Catenin, p53, SAPK/JNK, TGFβ and c-Myc pathways. Axin1 employs multiple discriminatory domains to function in different pathways. Previous studies have identified domains in Axin1 through which Axin1 binds to its partner proteins, namely the RGS domain for binding to APC in its N-terminal, the DIX domain to bind Dvl in its C-terminal, a Gsk3β binding domain, a β-Catenin binding domain and a PP2A binding domain. Axin1 regulates the Wnt/β-Catenin pathway by forming a degradation complex for β-Catenin through recruitment of APC and Gsk3β, and thereby aiding β-Catenin phosphorylation and marking it for degradation. We have previously shown that Axin1 association with Gsk3β, PP2A and Pin1 coordinates a degradation complex for c-Myc and the exon 7 region of Axin appears important for c-Myc binding. In this study, I report that the region coded by exon 7 of Axin1 harbors a domain that is conserved across species and that deletion of this conserved domain or the whole exon 7 from Axin1 disrupts Axin1 binding to c-Myc. Furthermore, I also show that deleting exon 7 from Axin1 disrupts Axin1's ability to negatively regulate c-Myc and its transactivation function while preserving its ability to suppress β-Catenin activity. My identification of the conserved domain for c-Myc binding and generation of a mutant Axin1 which is specifically deficient in its c-Myc regulatory function further expands our understanding of Axin1's activity towards c-Myc and will help in discriminating Axin1's effects on different pathways.

Introduction:

Axin is a multidomain scaffold protein that was originally identified from the mouse fused locus (Zeng et al. 1997). There are two genes in the Axin family Axin1 and (conductin) Axin2 which share about 44% sequence identity. Axin1 being a scaffold protein is able to interact with multiple proteins. Axin1 contains specific domains through which it interacts with its partner proteins and thereby regulates multiple different signaling pathways. Axin1 has been shown to act on Wnt/β-Catenin, SAPK/JNK, TGFβ, and p53 pathways (Wen Luo & Lin 2004). We have previously shown that Axin1 also coordinates the formation of a destruction complex for c-Myc which is distinct form the β-Catenin destruction complex. Axin1 utilizes separate discriminatory domains to interact with multiple proteins and is thus able to play distinct roles in different pathways. Axin1 has emerged as a major scaffold protein that regulates diverse cellular function like proliferation, cell fate determination and also acts as a tumor suppressor (Salahshor & Woodgett 2005).

Axin1 contains multiple domains; particularly well known are the Regulator of G protein Signaling (RGS) domain in its N-terminus (amino acids 89 to 216) and the DIX domain in its C-terminus (amino acids 757 to 820) (Figure 3.1). The RGS domain is a consensus domain that is conserved in the family of proteins that function as regulators of G protein signaling. Axin1's function on Wnt/β-Catenin signaling is facilitated by APC, and it has been found that APC interacts with Axin1 in the RGS domain (Kishida et al. 1998). Analysis of the crystal structure of APC binding to Axin1 has revealed that APC interacts with Axin1 in

an interface that is distinct from the G protein interface of classical RGS proteins (Spink et al. 2000). Furthermore, it has also been found that the critical amino acids required for binding to Gα are not conserved in Axin1 indicating that the RGS domain of Axin1 probably does not participate in G protein signaling. Also the amino acids in Axin1 that APC interacts with are not conserved in other RGS proteins probably, excluding APC binding to other RGS domain containing proteins (Wen Luo & Lin 2004).

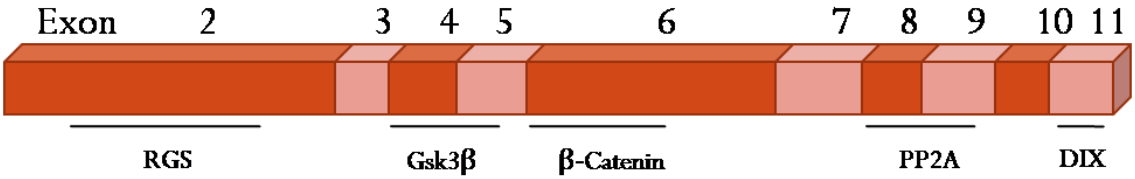

Figure 3.1: Schematic of various binding domain of Axin1 relative to its exons.

APC binds to Axin1 in its RGS domain and Dvl binds to its DIX domain. The Gsk3β and β-Catenin binding domains are nearby to each other presumably for allowing Gsk3β to phoshporylate β-Catenin.

The C-terminus of Axin1 contains the DIX domain between amino acids 757 to 820. The DIX domain is called so because it is present in Dishevelled (Dvl) and Axin1. The DIX domain of Dvl is present in its N-terminus (Sussman et al. 1994). The DIX domain of Axin1 and Dvl share amino acid sequence similarity of 37%. Axin1 is able to dimerize with itself forming homodimers due to interaction between its DIX domains (Hsu et al. 1999). Previous studies have suggested that this dimerisation of Axin1 might be important for its function (Y Zhang et al. 2000). Dvl is shown to upregulate the Wnt/β-Catenin pathways. Disheveled is also shown to interact with Axin1 through its DIX domain binding to the DIX domain of Axin1 and it has been reported that the DIX domain is sufficient for Axin1 interaction with Dvl (Kishida et al. 1999). Dishevelled might perform its function of upregulating Wnt/β-Catenin pathway by recruiting Axin1 to the Fzd/LRP6 membrane complex upon signaling by Wnt (Tamai et al. 2004; Mao et al. 2001; Davidson et al. 2005; Zeng et al. 1997). This could lead to

decreased Axin1 concentration in the cytoplasm and suppress β-Catenin degradation. The DIX domain of Axin1 is particularly interesting in that along with the DIX domain of Dvl it might lead to multiple molecules of Axin1 and Dvl being recruited in the membrane upon Wnt signaling, potentially forming a signalosome and thus decreasing Axin1 concentration in the cytoplasm (MacDonald et al. 2009).

β-Catenin is the final effector molecule of Wnt signaling. It is normally present in the membrane anchored to E-Cadherin and bridges E-Cadherin to α-Catenin which in turn links to the cortical Actin cytoskeleton thereby linking the cadherin complexes into the cellular cytoskeleton (Gumbiner & McCrea 1993; Schneider et al. 1993). Free β-Catenin in the cytoplasm is normally degraded by the Axin1 degradation complex which aids in phosphorylating β-Catenin by Gsk3β. The phoshporylated β-Catenin is then recognized by βTRCP ubiquitin ligase and is degraded (Bellamy et al. 1995). Thus β-Catenin performs the dual role of a transcription factor and also a cytosketal anchor protein. Axin1's function of degrading β-Catenin forms the critical point of regulating Wnt signaling. It has been found that Axin1 increases Gsk3β activity on β-Catenin many fold and knock down of Axin1 up regulates Wnt signaling (C. Liu et al. 2002; Ikeda et al. 1998). Axin1 binds to the armadillo repeats 2-7 of β-Catenin. β-Catenin has been shown to bind to Axin1 fragment 437 to 506 (Ikeda et al. 1998). Of particular note is that this β-Catenin binding domain is adjacent to Axin1's Gsk3β binding domain. This indicates that Axin, Gsk3β and β-Catenin might form a ternary

complex thereby greatly increasing Gsk3β phosphorylation of β-Catenin (Wen Luo & Lin 2004).

We have previously shown that Axin1 coordinates the formation of a degradation complex for c-Myc (Arnold et al. 2009). Induction of Axin1 expression with Doxycycline (Dox) in 293 cells stably expressing Dox inducible Axin1 showed decreased ectopically expressed c-Myc driven by the CMV promoter. This suggests that Axin1 could induce the degradation of c-Myc protein. We have also found that, cells in which Axin1 is knocked down by shRNA showed increased ectopic c-Myc protein levels indicating that Axin1 is critical in the degradation of the c-Myc oncoprotein. Consistent with this data, we found that upon knockdown of Axin1, S62 phosphorylation of c-Myc is increased with concomitant decrease in T58 phosphorylation. More importantly, we find that Axin1 is able to co-immunoprecipitate c-Myc along with Gsk3β, B56α, PP2AC, and Pin1. Furthermore knockdown of Axin1 is able to decrease c-Myc's association with Gsk3β and PP2AC. Taken together our data indicates that Axin1 along with Gsk3β, PP2A and Pin1 forms a complex which phosphorylates c-Myc at T58 and dephosphorylates it at S62 marking it for degradation (see Figure 1.8).

Further analysis of c-Myc binding to Axin1 by truncation studies showed that exon7 of Axin1 is important for its binding to c-Myc. More specifically, while c-Myc co-immunoprecipitated with full length Axin1, it did not precipitate with Axin1 exons 2 through 6. But upon adding exon 7 to the construct (exons 2

through 7), c-Myc was able to bind Axin1 (Figure 3.2). c-Myc is also able to bind Axin1 exons 2-8 and exons 2-9. This suggests that exon 7 of Axin1 is important for c-Myc binding to Axin1. But whether exon 7, in the context of full length Axin1, affects c-Myc binding is not known. Also whether exon 7 of Axin1 is critical for regulating the c-Myc protein is not known. Since c-Myc does not bind to Axin1 exons 2-6 but binds to exons 2-7, I hypothesized that exon 7 is required for c-Myc binding to Axin1 and deletion of exon 7 from Axin1 will affect Axin1's ability to bind c-Myc and to regulate c-Myc.

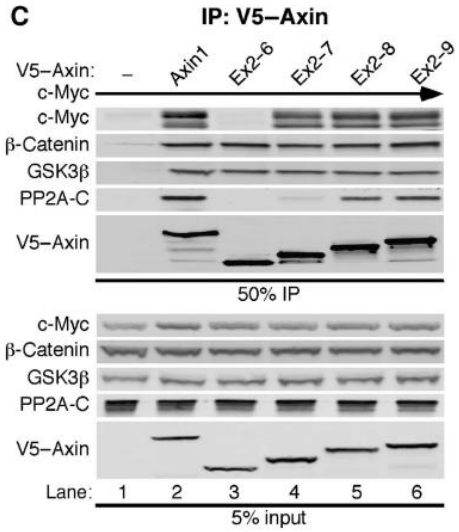

Figure 3.2: c-Myc binds to full length Axin1 and to Axin1 exons 2-7, but does not bind to Axin1 Exons 2-6.
293 cells were co-transfected with c-Myc and either empty V5 Plasmid or V5 tagged Axin1 WT, Axin1 Exons 2-6, Exon 2-7, Exon 2-8, Exon 2-9 expressing plasmids. The proteins were immunoprecipitated with V5 antibody. The input and immunoprecipitated proteins were then separated and detected by Western blot. (Data published in Arnold et al, 2009)

In order to test the hypothesis, that exon 7 of Axin1 is required for Axin1's effect on c-Myc, I synthesized a construct of Axin1 which lacks exon 7. In addition, I also synthesized a construct in which a domain that is conserved across species in Axin1 exon7 is deleted. In this study, I use these Axin1 mutants to study how deletion of either the conserved domain or the exon 7 affects Axin1's function in regulating c-Myc.

Results:

Axin1 is a scaffold protein that binds to multiple partner proteins through its conserved domains and acts on Wnt/β-Catenin, JNK, TGFβ and the p53 pathways (Wen Luo & Lin 2004). The Wnt pathway is conserved across species from worms to mammals. Axin1, which forms the rate limiting member of the Wnt/β-Catenin pathways (E. Lee et al. 2003), is conserved in human, rat, mice, chicken, xenopus, and drosophila (Zeng et al. 1997; Ikeda et al. 1998; Hedgepeth et al. 1999; Hamada et al. 1999). It has been shown that the function of Axin1 in the Wnt/β-Catenin signaling is conserved between drosophila and humans (L. Li et al. 1999; Hart et al. 1998; Nakamura et al. 1998; Zeng et al. 1997; Behrens et al. 1998). This suggests that the Axin1 gene is highly evolutionarily conserved and its function at least in the Wnt signaling pathway is conserved between drosophila and humans.

Since we have previously shown that a truncated form of Axin1 that is lacking exon 7 prohibits c-Myc binding, I hypothesized that exon 7 will contain a conserved domain that helps in binding of Axin1 to c-Myc.

In order to test this hypothesis, I collected the amino acid sequences of Axin1 from human, mouse, rat, chimpanzee, dog, cow, chicken and zebra fish from NCBI RefSeq. I then aligned the amino acid sequence coded by exon 7 of human Axin1 with the corresponding amino acids of other species using ClustalW multiple sequence alignment (Thompson et al. 1994). I found that there is a 26 amino acid sequence (641 to 666) out of the 56 amino acids encoded by exon 7 that is highly conserved across species with just a few amino acid

changes in dog, chicken and zebra fish (Figure 3.3). My finding that there is a domain that is highly conserved across species in exon 7 of Axin1 suggests that this domain performs an important function which is conserved across species. In order to gain additional information about the conserved domain of Axin1 exon 7 I used the secondary structure prediction server HHpred (Söding J et al., 2005).

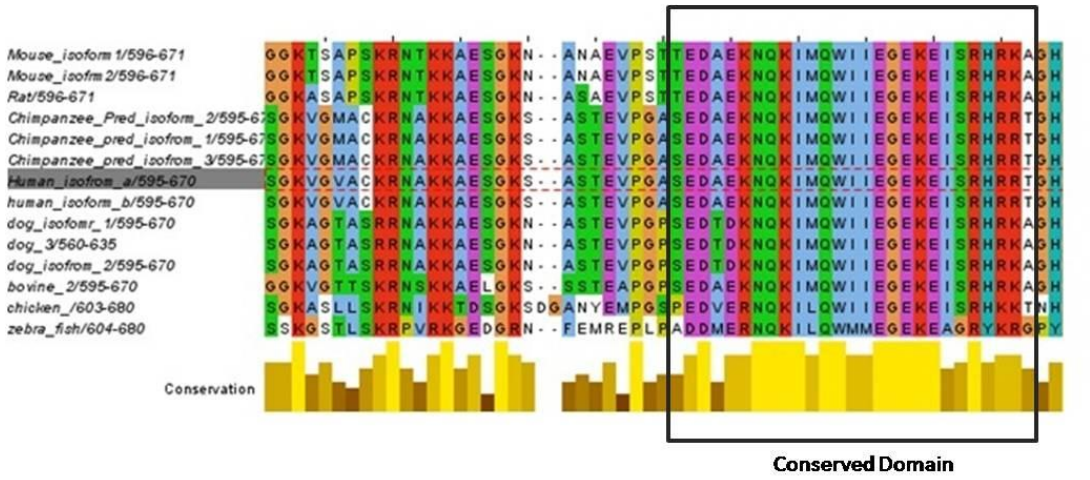

Figure 3.3: Exon7 of Axin1 contains a domain that is conserved across multiple species.

The amino acid sequence encoded by Exon7 of human Axin1V1 of multiple species was collected from NCBI RefSeq and the sequences were aligned by ClustalW. The human Axin1 variant 1 is shaded in grey. The conservation of particular amino acids at each position across different species is shown as yellow bars below the alignment. The coloring of amino acids is done according to ClustalW coloring scheme.

Interestingly, I found that the sequence encoded by exon 7 is predicted to form a helix loop helix structure with a high degree of confidence by HHpred (Figure 3.4). Moreover, I also found that the same sequence within exon 7 that is highly conserved across species, is the one predicted to form the helix loop helix structure. This suggests that the structure formed by the conserved sequence is also conserved across species. Since we have previously shown that exon 7 of Axin1 is important for Axin1 binding to c-Myc and I now find that it encodes a domain that is conserved across species, we hypothesized that this conserved domain is important for Axin1 binding to c-Myc.

Figure 3.4: Conserved domain of Exon7 of Axin1 is predicted to form a helix loop helix structure.

Secondary structure prediction of Axin1 exon 7 amino acid sequence using HHpred showing a Helix loop Helix structure corresponding to the highly conserved region in Exon7. The prediction is shown as either C (Coil) or H (Helix) for each amino acid and the confidence for each prediction is shown as blue bars on the top.

In order to test this hypothesis, I synthesized a mutant form of Axin1 in which the conserved domain in exon7 is deleted (Axin1 Ex7 ΔCD; deletion from aa 641 to 666, Figure 3.5). I also synthesized another mutant form of Axin1 in which the sequence coded by all of exon 7 is deleted. (Axin1 Ex7 ΔWE; deletion from aa 612 to 669, Figure 3.5). To test whether deletion of the exon 7 or the conserved domain decreases binding of Axin1 to c-Myc, I performed a co-immunoprecipitation experiment. Specifically, 293 cells were co-transfected with c-Myc and either empty vector or V5-tagged Axin1 wild type (WT), Axin1 exons 2-6, Axin1 Ex7 ΔCD, or Axin1 Ex7 ΔWE. I then used anti-V5 antibody to immunoprecipitate Axin1. I found that while Axin1 WT binds to c-Myc robustly, Axin1 Ex7 ΔCD and Axin1 Ex7 ΔWE showed weaker binding to c-Myc (Figure 3.6). I also found that while Axin1 Ex7 ΔCD showed less binding to c-Myc Axin1 Ex7 ΔWE was more deficient in binding to c-Myc. The reason for this is not

known. Even though I find pull down of c-Myc with Axin1 Exon 2-6 similar to Axin1 Ex7 ΔWE (lane 3 to lane 5) this cannot be compared because the c-Myc input is much greater in the Axin1 Ex7 ΔWE in contrast to Axin1Exon 2-6. This effect is due to dominant interfering effects of overexpressed Axin1, which has been observed in other scaffold proteins and is consistent with our previous findings (E. Lee et al. 2003; Arnold et al. 2009), and is probably due to the formation of incomplete complexes in which all the partner proteins are not present and thus results in an non functional complex. My finding that deletion of the conserved domain and also the exon 7 from Axin1 decreases Axin1 binding to c-Myc supports my hypothesis that c-Myc binds to Axin1 in the exon 7 region, probably through the conserved domain.

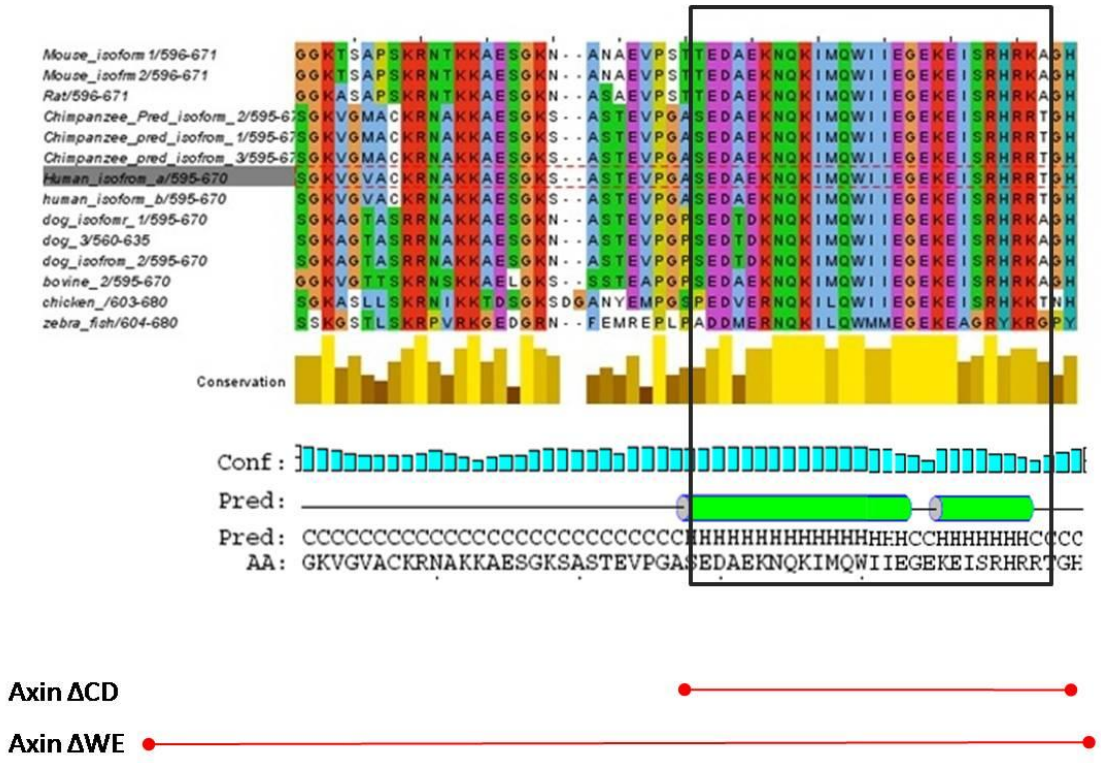

Figure 3.5: Schematic, marking the location of the deletion in Axin1 Ex7 ΔCD and Axin1 Ex7 ΔWE with respect to the sequence conservation.
Axin1 Ex7 ΔCD and Axin1 Ex7 ΔWE have the region corresponding to the red line deleted from its sequence.

In order to further analyze the effect of deletion of exon 7 on Axin1's activity on c-Myc, I used the SNU-475 hepatocellular carcinoma cell line as the model system. SNU-475 cells are known to harbor a homozygous genomic deletion of the exon 2 region of Axin1, and they have been found to not express Axin1 transcript (Satoh et al. 2000). To test whether deletion of exon 7 of Axin1 would affect the activity of Axin1 on c-Myc, I infected SNU-475 cell line with either Axin1 WT or Axin1 Ex7 ΔWE. After 24 hours the cells were harvested and the proteins were visualized by western blot. Interestingly, I found that while

Axin1 WT is able to suppress c-Myc at low concentrations, Axin1 Ex7 ΔWE mutant requires much higher expression to affect c-Myc protein level (Figure 3.7, compare lane 4 to lane 6). This suggests that deletion of exon 7 from Axin1 significantly reduces Axin1's activity on c-Myc. This is consistent with my previous finding that Axin1 Ex7 ΔWE is defective in binding to c-Myc.

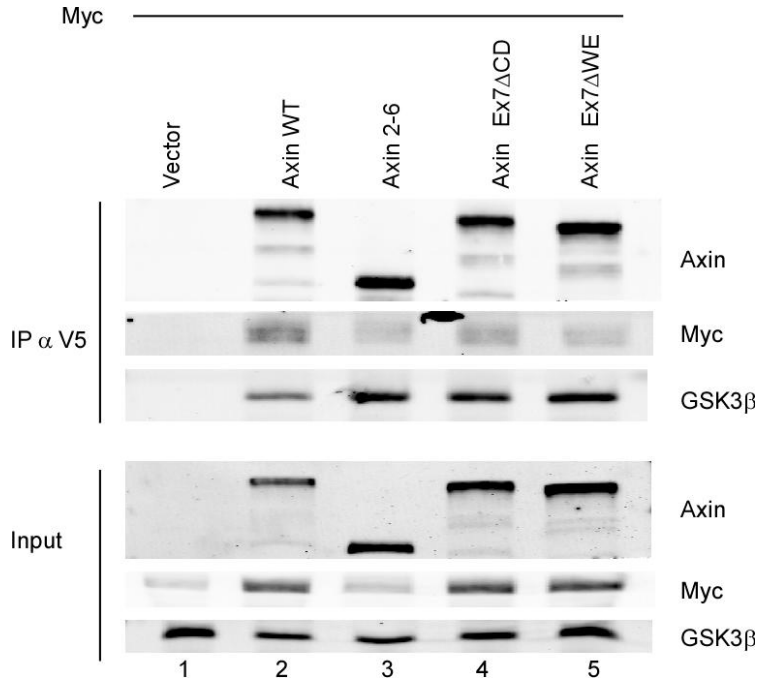

Figure 3.6: Deletion of the Conserved domain (Axin Ex7 ΔCD) or Whole Exon 7 (Axin Ex7 ΔWE) decreases c-Myc binding to Axin1.
293 cells were co-transfected with c-Myc and either empty V5 plasmid, or V5 tagged Axin1 WT, Axin1 2-6, Axin1 Ex7 ΔCD, and Axin1 Ex7 ΔWE. Axin1 was immunoprecipitated with V5 antibody. The input and immunoprecipitated proteins were then separated by western blot and then probed with the indicated antibodies.

Since I found that Axin1 Ex7 ΔWE is less effective in binding c-Myc and is less effective in decreasing c-Myc protein levels I hypothesized that Axin1 Ex7 ΔWE will be less effective in suppressing c-Myc's transcriptional function. To test this hypothesis I transfected SNU-475 cells with an E2F2 promoter construct that drives Luciferase gene as a reporter for c-Myc transcriptional activity (R Sears et al. 1997). I found that while Axin1 WT is able to suppress c-Myc transcriptional activity, Axin1 Ex7 ΔWE was less effective in decreasing it. This suggests that

Axin1 Ex7 ΔWE is less effective than Axin1 WT in negatively regulating the c-Myc oncoprotein.

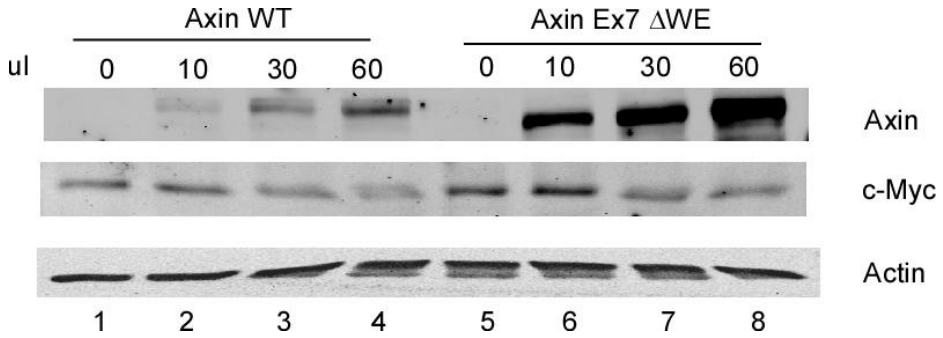

Figure 3.7: Axin1 Ex7 ΔWE is less effective in decreasing c-Myc protein levels when compared to Axin1 WT in the SNU-475 cells.

SNU-475 cells were transduced with Axin1 WT and Axin1 Ex7 ΔWE Lentivirus as indicated. 24 hours later cells were harvested and the proteins separated and visualized by western blot by probing with the indicated antibodies. β Actin was used as a loading control.

In order to address the possibility that Axin1 Ex7 ΔWE might be less functional overall and not specifically against Myc, I tested its ability to suppress the TOP flash Luciferase reporter (TCF Optimal Promoter driving Firefly Luciferase) as a measure of Axin1's activity on the Wnt/β-Catenin pathway. As shown in figure 3.9 I found that Axin1 Ex7 ΔWE was as effective as Axin1 WT in suppressing TOP flash Luciferase activity. This indicates that deletion of exon 7 does not non-specifically affect Axin1's activity on its other targets.

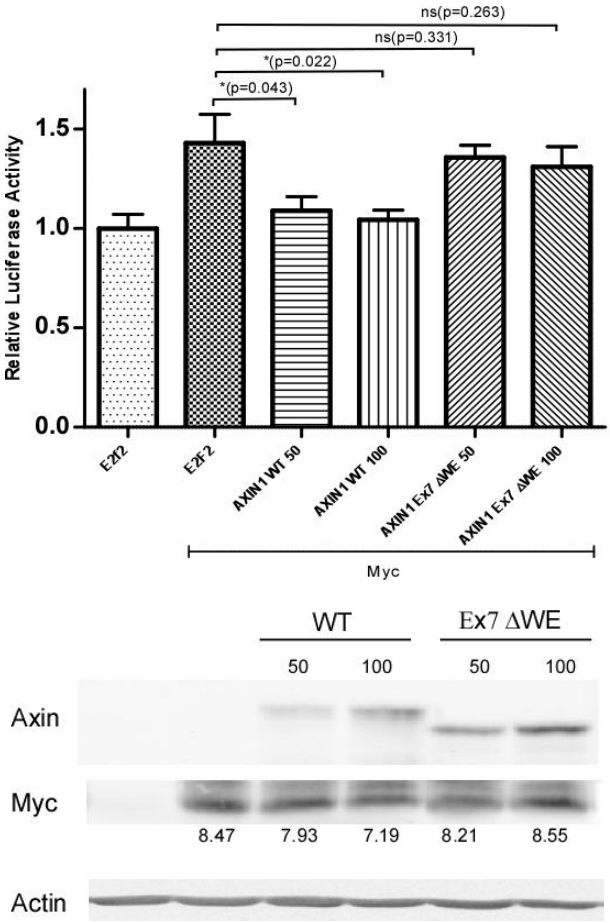

Figure 3.8: Axin1 Ex7 ΔWE is less effective in decreasing c-Myc transcriptional activity when compared to Axin1 WT in the SNU-475 cells.

SNU-475 cells were co-transfected with E2F2 Luciferase construct, CMV-β-gal, c-Myc and either Axin1 WT or Axin1 Ex7 ΔWE as indicated. Luciferase activity was measured and normalized with β-Gal activity for transfection efficiency. The cell lysates were also analyzed by Western blot by probing with indicated antibodies. β-Actin was used as a loading control. c-Myc is quantified using LI-COR Odyssey infrared imaging software and the values are shown below. The average results of two independent experiments performed in duplicate are shown.

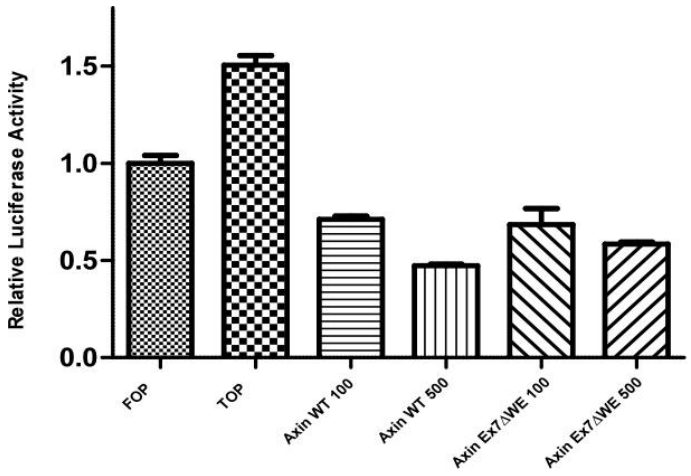

Figure 3.9: Axin1 Ex7 ΔWE has a similar effect to Axin1 WT on decreasing β-Catenin transcriptional activity.

SNU-475 cells were transfected with either TOP FLASH or FOP Flash Luciferase (Control plasmid with mutated TCF sites) construct along with either Axin1 WT or Axin1 Ex7 ΔWE as indicated. The Luciferase activity was measured and normalized with β-Gal for transfection efficiency.

Discussion:

In this study, I found that exon 7 of Axin1 encodes a domain that is highly conserved across species. I also found that either deletion of the conserved domain or the whole exon 7 from Axin1 reduces Axin1 binding to c-Myc. Furthermore, I found that deletion of exon 7 from Axin1 decreases its ability to regulate c-Myc protein levels and also decreases Axin1's activity specifically on c-Myc transactivation but not on β-Catenin.

Axin1 is a scaffold protein containing multiple domains through which it interacts with multiple partner proteins and regulates different pathways including Wnt/β-Catenin, JNK, p53, TGFβ and c-Myc (Wen Luo & Lin 2004; Arnold et al. 2009). The Wnt/β-Catenin pathway is shown to regulate development, cell fate determination, differentiation, and the development of cancer (Akiyama 2000; Wodarz & Nusse 1998; Bienz & Clevers 2000; MacDonald et al. 2009). The Wnt signaling pathway is conserved from worms to mammals. It has been shown that Axin1's function on the Wnt/β-Catenin pathway is conserved from flies to humans (L. Li et al. 1999; Hart et al. 1998; Nakamura et al. 1998; Zeng et al. 1997). c-Myc is a transcription factor which has been extensively studied and is known to regulate cell growth, proliferation, apoptosis and differentiation (Meyer & Penn 2008). c-Myc is conserved from flies to humans and recent genomic studies suggest that it regulates almost 15% of the genes. We have previously shown that Axin1 acts as a scaffold for the formation of a destruction complex for c-Myc (Arnold et al. 2009). In this study I found that the sequence encoded by exon 7 of Axin1 harbors a domain that is conserved across species and deletion of either

the conserved domain or exon 7 reduces Axin1 binding to c-Myc and also decreases its activity towards Myc. This is consistent with the previous reports that Axin1's multiple other domains, namely RGS, DIX, β-Catenin, and Gsk3β binding are also conserved across species (Kishida et al. 1999; Hsu et al. 1999; Y Zhang et al. 2000; Ikeda et al. 1998; Nakamura et al. 1998). In this study I have found that the domain that is important for c-Myc binding to Axin1 is conserved across species, suggesting that Axin1's function on c-Myc might also be evolutionarily conserved. Further studies in other model systems might elucidate this activity.

In this study, I also found that the conserved domain is predicted to form a helix loop helix structure. This suggests that the structure is conserved across species and further emphasizes the importance of this conserved domain. Helix loop helix structures are mostly found in DNA binding domains of proteins. But, it has to be remembered that secondary structure predictions are not always accurate and adjacent domains in the protein also play a role in protein folding (Nayeem et al. 2006; Dor & Zhou 2007). In this study I found that deletion of the conserved domain of Axin1 reduces c-Myc binding to Axin1 and also that deletion of the whole exon 7 further decreases Axin1's ability to bind c-Myc. The reason for this is currently not known. But it is possible that amino acids encoded by exon 7 that are adjacent to the conserved domain might also help in c-Myc binding to Axin1. Indeed multiple other amino acids encoded by exon 7 are also conserved as shown in figure 3.3.

Axin1 is a tumor suppressor and Axin1 mutations have been found to play a role in the tumorigenesis of different cancers like colon, prostate, hepatocellular carcinoma and medulloblastoma (Salahshor & Woodgett 2005), as discussed in chapter two. In this study I show that the exon 7 region of Axin1 is important for Axin1's activity on c-Myc. Interestingly one previous study which examined Axin1 mutational status in hepatoblastoma has reported mutations of Axin1 in the exon 7 region (Taniguchi et al. 2002). This study found Axin1 mutation at 631 position which changes amino acid from Glycine to Serine and is 5' position to the conserved domain in exon 7. This study was published prior to our report that Axin1 exon 7 is important for c-Myc binding. Since in this study I found that deleting exon 7 affects Axin1 binding to c-Myc it is possible that mutations in exon 7 might affect Axin1's function on c-Myc and contribute to tumorigenesis in these cancers. Functional studies with this Axin1 mutation are warranted.

In this study, I found that Axin1 Ex7 ΔWE is still able to suppress β-Catenin transcriptional activity suggesting that this mutant's inability to suppress c-Myc transactivation activity is not due to misfolded protein but is specifically defective in regulating c-Myc. This is particularly interesting in the context of a recent study which has found a small molecule, SKL2001, that prevents Axin1 binding to β-Catenin (Gwak et al. 2012). Further studies using Axin1 Ex7 ΔWE and SKL2001 will be instrumental in differentiating Axin1's role in the Wnt/β-Catenin pathway and the c-Myc degradation pathway.

In summary, I show that Axin1 exon 7 encodes a domain that is conserved across species and deletion of either the conserved domain or the whole exon 7

is able to inhibit Axin1 binding to c-Myc. Furthermore I show that deletion of exon 7 from Axin1 suppresses its function specifically toward c-Myc while preserving its activity on β-Catenin.

Chapter Four:

Summary and Discussion

Axin1's role in regulating c-Myc in Leukemia

Axin1 appears to regulate c-Myc protein levels in Leukemia.

c-Myc is a transcription factor that was originally identified as the cellular homologue of the transforming v-myc gene of avian myelocytomatosis virus MC29 (Vennstrom et al. 1982). c-Myc is over expressed in many cancers. c-Myc regulates multiple cellular functions like cell growth, proliferation, cell cycle regulation, differentiation and apoptosis (Meyer & Penn 2008) and it has been shown to be one of the necessary transcription factors for maintenance of stem cell pluripotency (Takahashi & Yamanaka 2006). c-Myc overexpression in mouse models induce cancers which are dependent on c-Myc for their survival. Our lab has previously established that the protein levels of c-Myc are in part regulated at the post translational level by phosphorylation at the conserved residues Serine 62 and Threonine 58 (Rosalie Sears et al. 2000). More specifically, newly synthesized c-Myc is phoshporylated by ERK kinases which are under the regulation of mitogenic signaling (Lutterbach & Hann 1994; R Sears et al. 1999) (Figure 1.7). This S62 phoshporylated c-Myc is a stabilized form of Myc and is then recognized by Gsk3β, which requires priming phosphorylation at this site (Chu et al. 1996). This dual phoshporylated c-Myc is then dephoshporylated at S62 by PP2A after isomerization of Proline 63 from *cis* to *trans* by Pin1 (Yeh et al. 2004). Our lab has shown that this step wise phosphorylation and dephosphorylation of c-Myc is coordinated on the scaffold protein Axin1 (Arnold et al. 2009). Axin1 is a scaffold protein and has previously been shown to act on the Wnt/β-Catenin, JNK, TGFβ and p53 pathways (Wen Luo & Lin 2004). Axin1

is expressed at low levels and has been shown to be the rate limiting member in the β-Catenin degradation complex (E. Lee et al. 2003). Decreased overall Axin1 protein levels and lower Axin1 levels in the cytoplasm form a major part in suppressed β-Catenin degradation in the presence of Wnt signaling (E. Lee et al. 2003; Mao et al. 2001; Tolwinski 2009). Since c-Myc protein levels are tightly regulated and the c-Myc degradation complex is coordinated by Axin1, it is possible that Axin1 also forms the rate limiting member of the c-Myc degradation complex. Indeed, as shown in figure 2.4 by increasing Axin1 protein levels in the U937 leukemia cell line, I have shown that c-Myc protein levels were able to be decreased. This indicates that increasing Axin1 protein levels is able to regulate c-Myc protein levels even in the oncogenic context.

Moreover, previous studies of c-Myc in Burkitt's lymphoma have shown that c-Myc is stabilized even in the absence of translocations or mutations at its phosphorylation site. Also, other studies have reported that PP2A inhibitors, SET and CIP2A might be over expressed in AML and primary leukemia samples. Taken together, these studies indicate that deregulation of c-Myc degradation pathway might form a major mechanism in which c-Myc might be upregulated in leukemia. Decreased expression of Axin1 has been shown to occur in non small cell lung cancer, esophageal cancer and prostate cancer and its expression is inversely correlated with metastasis, invasion, poor differentiation and poor prognosis (Nakajima et al. 2003; de Castro et al. 2000; Baeza et al. 2003; Yardy et al. 2009). Axin1 expression might also be downregulated in leukemia and this could contribute to tumorigenesis. In this context, it is interesting that Axin1

expression in thymic stromal cells has been linked to reduced thymopoiesis and increased thymic adiposity (H. Yang et al. 2009). Furthermore studies found that overexpression of Axin1 in the A549 lung cancer cell line and the C6 astrocytoma cell line induces apoptosis (Han et al. 2009; L.-Y. Zhang et al. 2009). Also our lab has shown that, decreased overall Axin1 expression and a switch in the expression of Axin1 from variant 1 to variant 2 occurs in breast cancer and might lead to deregulated c-Myc levels in breast cancer (X. Zhang et al. 2012). In this study, I have shown that the U937 cells show a preferable expression of Axin1 variant 2 over variant 1. This altered expression of Axin1 in the U937 leukemia cell line could also contribute to increased c-Myc in leukemia and aid in tumorigenesis. Taken together, this indicates that Axin1 forms a critical suppressor of tumorigenesis, and regulating Axin1 protein levels, using small molecules, could suppress tumor progression by inducing apoptosis, and might be a valuable therapeutic strategy in leukemia.

Axin1 mutations are relatively rare in leukemia

Axin1 protein has been extensively studies for its function in regulating the Wnt/ β-Catenin pathway. Axin1, along with APC and Gsk3β forms a complex that phosphorylates β-Catenin (MacDonald et al. 2009). This phoshporylated β-Catenin is then recognized by βTRCP ubiquitin ligase and degraded (Bellamy et al. 1995). This mechanism helps to keep β-Catenin protein levels in the cytoplasm at low levels. In the presence of Wnt, Axin1 protein levels are

decreased in the cytoplasm either by degradation or by recruitment to the membrane and β-Catenin is stabilized leading to its nuclear translocation and upregulation of its target genes (E. Lee et al. 2003; Mao et al. 2001; Tolwinski 2009; Tamai et al. 2004). The Wnt/β-Catenin pathway has been extensively studied for its regulation of colonic villous epithelium. Interestingly, over expressed Wnt pathway is implicated in the development of colon cancer (Miyoshi et al. 1998; de La Coste et al. 1998; Webster et al. 2000; W. Liu et al. 2000). Indeed, APC mutations lead to predisposition for colon cancer (Kinzler & Vogelstein 1996). Furthermore, not surprisingly, many studies have found mutations in Axin1 in colorectal cancers, including primary patient samples and in HCT-8, HCT-15 and DLD-1 colon cancer cell lines (Webster et al. 2000). Axin1 mutations are also found to occur frequently in hepatocellular cancers. Studies have found mutations in approximately 10% of hepatocellular carcinoma samples and hepatoblastoma samples (Taniguchi et al. 2002). Axin1 mutations also occur in three HCC cell lines SNU 423, SNU 475, and Alexander (Satoh et al. 2000). Axin1's role in liver development and/or whether Axin1 plays a role in maintaining normal liver homeostasis is not known. One recent study has found that Axin1 mutations are not found in low grade and high grade dysplastic nodules in the liver, but occur in hepatocellular carcinomas, indicating that Axin1 mutations occur relatively late in liver tumorigenesis (Park et al. 2005). In this study, I have sequenced Axin1 in 26 leukemia samples, but did not find any protein sequence changes in the Axin1 gene. Thus, even though Axin1 expression seems to be linked to thymopoiesis (H. Yang et al. 2009), this result suggests that Axin1

mutations are relatively rare in leukemia. It is possible that Axin1 mutations are selected for during tumorigenesis in particular cell types and not in others. Mutations in Axin1 might offer survival benefit in the context of oncogenic pathways that are deregulated in colonic cancers and hepato cellular cancers. In this regard, it is interesting that Axin1 mutations are found only in the late stages of HCC, indicating that Axin1 mutations are selected for only after previously acquired mutations in other still unknown pathways (Park et al. 2005). Moreover, Axin1 studies in esophageal and pancreatic cancers have not found any mutations in Axin1 (S.-C. Huang et al. 2011; A. F.-Y. Li et al. 2009; Kudo et al. 2007). However, decreased Axin1 expression has been correlated with poor prognosis, invasion and metastasis in esophageal cancers (A. F.-Y. Li et al. 2009; Kudo et al. 2007). This indicates that different mechanisms of Axin1 inactivation might play a role in different cancers.

Moreover our finding that leukemia cell lines express a new variant of Axin1 (variant 3), which lacks exon 5, is interesting since it affects Axin1's Gsk3β and c-Myc binding. This variant of Axin1, due to its loss of a part of Gsk3β binding domain, might affect Axin1's function towards c-Myc and also possibly towards β-Catenin. In this regard it is interesting that in this study, I have found a SNP at 1254+17, which might affect splicing of exon 5. This SNP might affect splicing of exon 5, thereby making it less effective in regulating c-Myc and possibly β-Catenin and thus supporting leukemogenesis. However, it has been previously shown that Gsk3β decreases Axin1's activity on the SAPK/JNK pathway. Axin1 variant 3, since it does not bind to Gsk3β, could also have

increased activity on the SAPK/JNK signaling and could promote apoptosis. However, whether this new splice variant of Axin1 is expressed in other cells and what might be the functional consequences of splicing out of exon 5, remains to be studied.

Axin1 Exon 7 role in regulating c-Myc protein levels.

In this study, I used a combination of sequence analysis and co-immunoprecipitation methods to identify and narrow down the c-Myc binding region of Axin1. I found that deletion of exon 7 of Axin1 decreases Axin1 binding to c-Myc and also decreases its regulatory activity towards c-Myc while maintaining its activity against β-Catenin.

Our lab has previously shown that Axin1 forms a critical coordinator of the c-Myc degradation pathway (Arnold et al. 2009). c-Myc degradation is regulated by two conserved residues S62 and T58, whose phosphorylation and dephosphorylation in a sequential and interdependent fashion serves to orchestrate an elegant mechanism for fine tuning c-Myc's protein stability (R. C. Sears 2004). c-Myc is a potent oncogene which is overexpressed in many cancers and tight regulation of its protein levels would be critical for maintaining cellular homeostasis. More specifically, c-Myc is phoshporylated at S62 by ERK kinases upon mitogenic stimulation and stabilized. Later, when growth signals subside the pS62 is recognized by Gsk3β, which phosphorylates Threonine at position 58. The S62 is then dephosphorylated by PP2A which then leads to c-

Myc being recognized by the FBW7 ubiquitin ligase (R Sears et al. 1999; Rosalie Sears et al. 2000; Yeh et al. 2004; Yada et al. 2004; Arnold et al. 2009). It is important to note that, the overall stability of c-Myc in such a sequential phosphorylation and dephosphorylation pathway is dependent upon the relative rates of binding of c-Myc to individual partner proteins at each of these individual steps. Thus, it is conceivable that the rate at which each of these proteins come together will have a major effect on c-Myc's stability. In this context, it is interesting to note that Axin1, which is a scaffold protein, regulates the coordination of the degradation complex by binding to c-Myc, Gsk3β, PP2A, and Pin1. Axin1, by bringing together all these proteins in a single multi domain complex can greatly aid in the degradation of c-Myc. Indeed, we have previously shown that knock down of Axin1 in 293 cells increases c-Myc protein levels that is expressed from a heterologous promoter (Arnold et al. 2009).

Axin1 is a multi domain protein of 862 amino acids that acts as a scaffold protein in coordinating the formation of multi protein complexes which presumably acts as the central node of regulating different pathways (Wen Luo & Lin 2004). Axin1's many domains have been studied by deletion studies and many of the protein interactions have been mapped to approximate regions in the Axin1 sequence. Many of the studies have also used these fragments for functional evaluation and have yielded mixed results (Julius et al. 2000). This study is unique in that it employs full length Axin1 with only the domain in question deleted so as to prevent any untoward effects of deletion of other domains in its function. This is particularly important with respect to studying

scaffold proteins that are expressed at rate limiting levels since fragments of Axin1 can sequester proteins from other pathways leading to untoward effects which might not be physiologically significant.

Furthermore, it has been shown that Axin1 lacking its C-terminal dimerization domain is deficient in its function, suggesting that dimerization is important in Axin1's function to bring together multi protein complexes (Y Zhang et al. 2000). Thus, expressing Axin1 mutants in cells expressing Axin1 wild type could lead to dimerization of ectopic Axin1 with endogenous Axin1 and might cause untoward effects. In this study I have used the SNU-475 cell line, which is Axin1 null, (Satoh et al. 2000) in order to prevent artifacts due to dimerization of mutant Axin1 proteins with endogenous Axin1.

Axin1 has been shown to coordinate the degradation complex for c-Myc (Arnold et al. 2009). Axin1's overall function is established to be a tumor suppressor (Salahshor & Woodgett 2005). The c-Myc oncogene is over expressed in many cancers (Meyer & Penn 2008) and part of Axin1's tumor suppressor activity may be through downregulating c-Myc. In this study, I have shown that Axin1 missing exon 7 is less effective in regulating c-Myc protein levels. Further studies analyzing the relative ability of Axin1 WT and Axin1 Ex7 ΔWE in suppressing the transformation ability of c-Myc, could shine light on Axin1's tumor suppressor activity through c-Myc, especially given that this mutant does not affect Axin1 regulation of β-Catenin. In this regard, generation of an Axin1 Ex7 ΔWE knock-in mouse, at the endogenous locus, might provide *in-vivo* evidence of Axin1's tumor suppressor activity through c-Myc.

In this study, I found that Axin1 Ex7 ΔWE is deficient in binding to c-Myc, but it has not completely lost binding to c-Myc (Figure 3.6). This phenomenon has been observed previously in other Axin1 mapping studies. Specifically, in a study by Fagotto et al. 1999, they observed that APC would still bind to Axin1 fragment 331-956 which lacks the RGS domain of Axin1 (Fagotto et al. 1999). Similarly, β-Catenin, while binding to fragments containing the β-Catenin binding site (aa 600 – 622), would still bind to Axin1 fragment 12-531 and 194 – 531. This residual pull down in co-immunoprecipitation is suggested to be due to indirect binding through other Axin1 interacting proteins, for example APC and β-Catenin. Similarly, in this study some amount of c-Myc pull down with Axin1 Ex7 ΔWE in co-immunoprecipitation is probably due to c-Myc indirectly binding through its association with Gsk3β or PP2A.

The scaffold protein Axin1 acts on multiple pathways and performs its scaffold function by binding to multiple proteins simultaneously. How Axin1's function on different pathways interplay is not known. It is interesting to note that while Gsk3β is a common partner of Axin1 in the β-Catenin and the c-Myc degradation complex, we have not found APC in the c-Myc degradation complex coordinated by Axin1 (Ikeda et al. 1998; Arnold et al. 2009). Similarly Axin1's activity on p53 involves binding to DAXX, HIPK2 and p53. Axin1 binds to p53 through HIPK2 and also Axin1 has a direct binding domain for p53, presumably preventing Axin1 from binding to APC, β-Catenin or Gsk3β (Q. Li et al. 2007; Y. Rui et al. 2004). Whether Axin1 regulates multiple pathways simultaneously or forms distinct complexes to regulate separate pathways remains to be studied. In

this regard, it is very interesting that the critical scaffolding regions of Axin1 were found to be natively unfolded, and it has been suggested that binding to specific proteins might aid folding of Axin1 in a manner that is specific to a particular pathway (Noutsou et al. 2011). Clearly, the question of how Axin1 is able to coordinate multi protein complexes and act on multiple different pathways is intriguing, and studies which include specific deletions like the Axin1 Ex7 ΔWE mutant described here, as well as specific binding inhibitors like SKL2001 (Gwak et al. 2012) in the Axin1 null background, would shed light on the complex function of Axin1.

Chapter Five:

Materials and Methods

Cell lines and media:

HEK293, SNU-475, U937, MCF10A cells were obtained from ATCC (American Type Culture Collection). All cells were grown in a 5% CO2 incubator at 37°C. HEK293 cells were grown in DMEM supplemented with 10% FBS 2.5mM L-Glutamine and 1x penicillin/streptomycin. SNU-475 cells, U937 and SupB15 cells were grown in RPMI supplemented with 10% FBS, 2.5mM L-Glutamine and 1x penicillin/streptomycin. MCF10A cells where grown in 45% DMEM, 45% F-12 Hams, 5% FBS, 2.5mM L-Glutamine, 20ng/ml EGF, 10ug/ml Insulin, 500ng/ml cholera toxin.

Western blotting:

Total cell lysates were collected in 1.5x SDS sample buffer. The proteins were separated by SDS page and then transferred to Immobilon-FL membranes. Membranes were blocked by 50% Aquablock, 50% PBST (PBS with 1% Tween 20) and probed with indicated primary antibodies. The primary antibodies were diluted in 1:1 PBST and Aquablock (East Coast Bio). The primary antibodies were then detected by near infrared Alexafluor 680 or IRDye800 used at 1:10,000 dilutions in 1:1 PBST and Aquablock. The immunoblots were then scanned using a LI-COR Odyssey infrared imager. The antibody signals were then quantified with a LI-COR Odyssey infrared imager software which allows for linear measurement over four orders of magnitude.

Cell proliferation assay:

MCF10A cells were plated at 150,000 cells/ml, (4mls/plate) in 6 cm plates with or without Doxycycline. Cells were harvested at the said time points by trypsinisation and resuspended in media. Cell numbers were counted by diluting 1:1 resuspended cells with Trypan blue and loading 10ul of the diluted sample into the hemocytometer.

Co-Immunoprecipitation:

Cells were washed with 1x cold PBS and aspirated dry. Then cells were scraped and collected in PBS and spin down briefly. Cells were then resuspended in 10x pellet volume of Co-IP Buffer (20mM Tris, pH 7.5, 12.5% glycerol, 0.2% NP-40, 200mM NaCl, 1mM EDTA, 1mM EGTA and 1mM DTT plus protease and phosphatase inhibitors). The pellet was pipetted up and down 5x to break down cell clumps and were incubated in ice for 20 minutes. The lysate was cleared by centrifugation at 14K rpm for 10 minutes at 4°C. The supernatant was used in subsequent steps. The volume between samples was adjusted according to B gal activity to normalize for transfection efficiency. 25 ul of sample is then removed as input. Anti V5 was added at 1:500 dilution and rotated at room temperature for one hour. 12ul of protein A beads (IPA300, Repligen) beads were added and rotated for 2 hrs. The beads were then washed 3X with 10 volumes of Co-IP buffer with one minute incubation between washes. The beads were then resuspended in 1.5x sample buffer and analyzed by western blot.

Antibodies:

The antibodies used were as follows: c-Myc antibody, Y69 was purchased from Abcam (ab32072, used at 1:1000 dilution in 1:1 PBST, Aquablock). Axin1 antibody was purchased from R&D systems (AF3287, used at 1:1000 dilution in 1:1 PBST, Aquablock). GSK3β antibodies were purchased from Cell Signaling Technology (5676, used at 1:5000 dilution in 1:1 PBST, Aquablock). β-Actin was used as a loading control for western blots and the antibody was purchased from Santa Cruz biotechnology (SC 130301, Used at 1:10000 dilution in 1:1 PBST, Aquablock).

Luciferase assay:

Cells were collected in 160ul/well of 1x Luciferase Buffer (Promega) with the protease and phosphatase inhibitors incubated in ice for 20 minutes. 30ul of the sample was removed and 7.5ul of 5x sample buffer was added and boiled for 10 minutes for Western blot analysis. The rest of the sample was cleared by centrifugation at 14K for 10 minutes. 60 ul of the supernatant was used for analysis of B gal activity and 60 ul for Luciferase activity which was measured by Promega Luciferase assay kit.

DNA isolation and sequencing

Genomic DNA was isolated from leukemia patient samples using the Blood and Tissue Kit from Quiagen according to the protocol. Then the eleven axin1 exons were PCR amplified using the genomic DNA as the template and primers which include adjacent intronic regions of each exon. PCR was

performed using 12.5 ul of MangoMix, 0.5 ul Forward primer 0.5 ul Reverse primer in a total volume of 25ul. The steps of PCR used are 1) 95°C for 30 sec. 2)55 to 60°C depending upon the primer pair. 3)72°C extension for 35 cycles. These steps was followed by a final extension at 72C for 10 min. 5 ul of the sample was then run on an agarose gel to verify amplification of a single band of DNA and were then purified using EXOsap-it according to the protocol. The samples were then sequenced according to traditional Sanger sequencing technique by Clive Woffendin in the OCTRI sequencing core. The trace files were then analyzed against NM_003502.3 using Mutation analyzer software (Softgenetics)

Plasmids and cloning

Construction of expression plasmids CMV-empty, CMV-βgal, CMV-Myc, pD40-c-Myc, prc CMV Myc, pD40 Axin1v1 and reporter plasmids, E2F2-Luc, and E2F2(-E-box)-Luc have previously been described (R Sears et al. 1997). Construction of the Axin1 deletion mutants, Axin1 Ex7 ΔCD and Axin1 Ex7 ΔWE were done using Stitching PCR method. The mutants were synthesized in two pieces, first piece containing the sequence before the deletion and the second piece containing the sequence after the deletion. The pieces where PCR amplified from pD40 Axin1 WT using the primers listed in Table 5.1. The PCR products were then run in an agarose gel and the correct size band is cut and purified using Qiagen Kit according to manufacturer's protocol. To generate the

full length deletion mutant, the two pieces were used as a template in a PCR with forward primer from set 1 and reverse primer from set 2 as shown in Figure 5.1.

	Axin1 ΔCD	
	Forward	Reverse
Set 1	ATGAATATCC AAGAGCAGGG TTTCC	GAAGACCCGT GGCCGGTGGC ACCTGGCACC TCGGTG
Set 2	GAGGTGCCAG GTGCCACCGG CCACGGGTCT TCG	TCAGTCCACC TTCTCCACTT TGC

	Axin1 ΔWE	
	Forward	Reverse
Set 1	ATGAATATCC AAGAGCAGGG TTTCC	CTCGTCCCCG AAGACCTGTG GGCGAGGCCA TCAC
Set 2	GGCCTCGCCC ACAGGTCTTC GGGGACGAGG AAG	TCAGTCCACC TTCTCCACTT TGC

Table 5.1: Primers used for synthesizing the Axin Ex7 ΔCD and Axin Ex7 ΔWE mutants

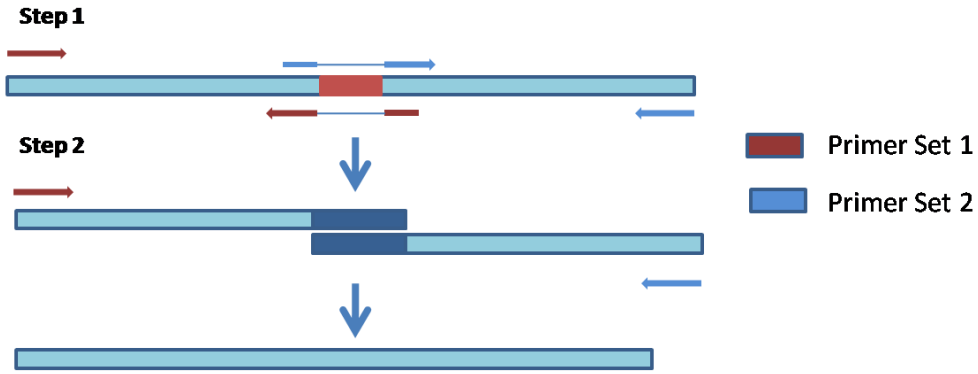

Figure 5.1: Stitching PCR technique.
During the first step two different PCR reactions using primer set 1 and primer set 2 are run. The resulting two PCR products are then used as a template in the second step with forward primer from set 1 and reverse primer from set 2.

Appendix

Contribution to figures

Figure 1.9: Colin Daniel designed the model.

Figrue 1.10: Hugh Arnold did the Co-IP and the semi quantitative ChIP.

Figure 2.3: Ellie Juarez isolated cDNA and did the PCR.

Figure 2.4: Deanne Tibbitts cloned the V5 Axin SupB15 splice variant.

Figure 2.6 (B): Hugh Arnold did the semi-quantitative PCR for the JY cell line.

Figure 2.7: Deanne Tibbitts isolated cDNA and did the quantitative PCR.

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