The Role of a Specific PP2A holoenzyme, PP2A-B56α, and Scaffold Protein, Axin1, in Regulating the Potent Oncoprotein c-Myc

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List of Abbreviations

APC	Adenomatous Polyposis Coli
ARF	ADP Ribosylation Factor
B	Basic Redion
B55	PP2A Regulatory B Subunit/B Family/PR55
B56	PP2A Regulatory B Subunit/B' Family/PR56
B-Myc	Brain Myc
CDK9	Cyclin dependent kinase 9
CK1	Casein Kinase 1
c-Myc	Cellular Myc
Daxx	Death Domain-Associated Protein
DIX	Dishevelled Interaction Domain
DMEM	Dulbecco's Modified Eagle's Medium
Dox	Doxycycline
DP1	Transcription Factor Dp-1
DVL	Dishevelled
E4orf4	Early Region Open Reading Frame 4
ERK	Extracellular Receptor Kinase
FBS	Fetal Bovine Serum
FLAG-A	FLAG-tagged PP2A-A subunit
Frat	Frequently Rearranged in Activated T Cells
GSK3β	Glycogen Synthase Kinase 3β

HA-C	HA-tagged PP2A-C subunit
HAT	Histone acetyltransferase
HCC	Hepatocellular Cancer
HDAC	Histone deactylase
HIPK2	Homeodomain-Interacting Protein Kinase-2
His	Histidine
HLH	Helix Loop Helix
JNK	Jun N-terminal Kinase
LEF	Lymphoid Enhancer Binding Factor
L-Myc	Lung Myc
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
P	Phosphorylation
p107	Retinoblastoma-like 1
p53	Tumor Protein 53

PI3K	Phosphoinositide-3-Kinase
Pin1	Prolyl Isomerase 1
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
RNAi	RNA Interference
S62	Serine 62
Small T	SV40 Small T Antigen
SAPK	Stress Activated Pathway Kinase
SCC	Oesophageal Squamous Cell Carcinoma
SCF	Skp/Cullin/F-box
shRNA	Short Hairpin RNA
Siah1	Seven In Absentia Homolog 1
SIP	Tumor Protein p53 Inducible Nuclear Protein 1
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
WNT	Wingless
WT	Wildtype

β-gal	β-Galactosidase
βTRCP	F-box and WD repeat domain containing 11

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Chapter One :

Introduction

c-Myc Background

C-Myc is a powerful oncogenic transcription factor. Originally discovered through its homology to the avian expressed myelocytomatosis virus transforming gene, *v-myc* (Vennstrom et al. 1982), *c-myc* has been intensely studied for the past 20 years due to both the requirement of *c-myc* for normal development (Davis et al. 1993) and the high prevalence of elevated c-Myc expression in a wide array of human cancers (Nesbit et al. 1999). The potent oncogenic capacity of c-Myc has been demonstrated by increased proliferation, neoplastic transformation, and inhibition of differentiation in cultured cells with sustained overexpression of c-Myc (Evan et al. 1992). Moreover, expression of c-Myc in mice with inducible *c-myc* transgenes develop neoplastic pre-malignant and malignant phenotypes that often spontaneously regress when *c-myc* expression is shutoff (Felsher and Bishop 1999; Pelengaris et al. 1999). Part of a non-redundant family of proteins that includes N-Myc, L-Myc, S-Myc, and B-Myc, c-Myc or cellular-Myc is the ubiquitously expressed form of the Myc family that is believed to regulate the expression of ~15% of all genes (Patel et al. 2004). C-Myc has been characterized extensively as a transcription factor since it was shown to contain several domains common to other transcription factors that include a transactivation domain in the N-terminal region as well as basic region, leucine zipper and helix-loop-helix motifs in the C-terminal region that allow for sequence specific DNA binding and heterodimerization with Max, see

Figure 1.1 (Landschulz et al. 1988; Davis et al. 1990; Kato et al. 1990; Luscher and Eisenman 1990).

As a transcription factor, c-Myc has been shown to regulate the expression of numerous genes involved in cellular proliferation, growth, differentiation, angiogenesis and apoptosis (Cole 1986; Luscher and Eisenman 1990; Prendergast 1999). More specifically, several studies have demonstrated that c-Myc drives cellular proliferation and growth by increasing the expression of genes that positively regulate the cell cycle and ribosomal biogenesis and inhibiting the expression of genes that negatively regulate cell cycle progression (Zeller et al. 2003; White 2005). Furthermore, c-Myc expression inhibits differentiation (Coppola and Cole 1986; Miner and Wold 1991) and c-Myc plays an important role in promoting angiogenesis and vascularogenesis in tumors (Baudino et al. 2002). Moreover, c-Myc has been shown to be one of four critical factors for the self-renewal of stem cells (Coppola and Cole 1986; Takahashi and Yamanaka 2006). Altogether, these findings demonstrate potential mechanisms through which c-Myc can act as potent oncoprotein. However, c-Myc expression was also shown to induce apoptosis in “normal” cells (Pelengaris et al. 2002; Nilsson and Cleveland 2003), thereby confounding the previous findings supporting the oncogenic function of c-Myc. More recent studies have now shown that the induction of apoptosis by overexpression of c-Myc is likely a “normal” response to a high cellular oncogenic load. Apoptosis under these conditions of elevated c-Myc expression results from the activation of p53

through ARF's ability to prevent p53 degradation as well as c-Myc mediated release of cytochrome c through up-regulation of Fas (Kavurma and Khachigian 2003; Dai et al. 2006). Interestingly, these pathways for inducing apoptosis are often compromised early in tumorogenisis, thereby allowing the oncogenic functions of c-Myc to drive tumorogenisis. Altogether, these findings highlight the importance of maintaining "normal" c-Myc expression.

Figure 1.1: Diagram of the c-Myc transcription factor.

C-Myc has several domains common to transcription factors including a transactivation domain (TAD), nuclear localization signal (NLS), basic region (B), helix-loop-helix (HLH), and leucine zipper domain (LZip). There are two highly conserved regions within the TAD named myc box I (MB1) and myc box II (MB2) that contain two highly conserved phosphorylation sites, Threonine 58 (T58) and Serine 62 (S62). These phosphorylation sites are critical for the regulation of c-Myc protein stability.

Regulation of c-Myc expression occurs at all levels. Since elevated expression of c-Myc is observed in ~70% of human tumors, mechanisms that regulate c-Myc expression have been intensely studied. Expression of c-Myc is regulated at the transcriptional, translational and post-translational levels (Kelly et al. 1983; Jones and Cole 1987; Luscher and Eisenman 1990; Sears et al. 1999). Transcription of *c-myc* is very low in quiescent cells upon mitogenic stimulation, which results in the accumulation of *c-myc* mRNA peaking at two hours post-stimulation. *C-myc* mRNA levels then decline to ~50% peak level and are maintained at this level as cells continue to proliferate and grow. The increase in transcription of the *c-myc* gene in parallel with an increase in *c-myc* mRNA stability (Jones and Cole 1987) increase *c-myc* mRNA levels.

As seen with *c-myc* mRNA levels, c-Myc protein levels are maintained at very low levels in quiescent cells and upon mitogenic stimulation c-Myc protein

levels increase, peaking by four hours post-stimulation and then declining to ~30% of peak level (Sears 2004). Control of c-Myc expression at the post-translational level is managed in large part through protein stability and turnover of c-Myc protein via multi-ubiquitination and degradation by the 26S proteosome (Flinn et al. 1998; Gross-Mesilaty et al. 1998; Salghetti et al. 1999). Many proteins that are degraded by ubiquitination and the 26S proteosome are marked by phosphorylation (Hoyt 1997; Krek 1998; Karin and Ben-Neriah 2000). C-Myc is no exception as T58 phosphorylation of c-Myc signals the SCF^{Fbw7} ubiquitin ligase machinery to multi-ubiquitinate c-Myc marking it for degradation by the 26S proteosome (Welcker et al. 2004; Yada et al. 2004). Interestingly, T58 phosphorylation of c-Myc is dependent upon a set of hierarchical reversible phosphorylation events on T58 and S62 (Henriksson et al. 1993; Lutterbach and Hann 1994; Pulverer et al. 1994). The hierarchical phosphorylation events on T58 and S62 are discussed in more detail below.

C-Myc heterodimerizes with Max to bind specific DNA sequences and regulate transcription. C-Myc heterodimerizes with a small nuclear protein, Max and together c-Myc/Max bind DNA sequence specific sites (CACGTG) called E-boxes (Blackwood and Eisenman 1991; Blackwood et al. 1992a; Blackwood et al. 1992b; Kretzner et al. 1992). When bound to E-boxes, c-Myc/Max heterodimers regulate the transcription of numerous genes by recruiting proteins involved in chromatin remodeling, ubiquitination, and histone acetylation

reviewed in (Grandori et al. 2000; Patel et al. 2004). More specifically, c-Myc has been shown to recruit INI1, a subunit of the SWI/SNF chromatin remodeling complex associated with modifying chromatin to a transcriptionally active state (Cheng et al. 1999). Furthermore, c-Myc can recruit transformation/transcription-domain-associated protein (TRRAP) and its associated histone acetyltransferase (HAT) complexes as well as ATPase/helicases, which create a transcriptionally active environment (McMahon et al. 1998; McMahon et al. 2000; Wood et al. 2000; Frank et al. 2003). CDK9 can also be recruited to E-boxes by c-Myc and CDK9 has been shown to directly phosphorylate RNA polymerase II increasing elongation activity (Eberhardy and Farnham 2002). In contrast, c-Myc/Max heterodimers can also associate with Miz1 and suppress transcription (Staller et al. 2001; Herold et al. 2002; Wanzel et al. 2003; Wu et al. 2003). Interestingly, the c-Myc/Max/Miz1 complex does not appear to bind E-box elements, but rather binds to initiator (INR) elements where transcription begins. C-Myc/Max transcriptional activity can also be antagonized by the competitive association of Mad family proteins or Mnt with Max, sequestering Max away from c-Myc. Moreover, Mad/Max and MNT/Max heterodimers have been shown to bind E-box elements and recruit SIN3, a scaffold protein that in turn recruits histone deacetylases (HDACs) to create a transcriptionally inactive environment (Eisenman 2001). The ability of c-Myc to regulate transcription depends largely on the stochiometric levels of c-Myc, Max, Mnt and Mad family members as well as the phosphorylation status of Mnt (Hurlin and Huang 2006; Rottmann and

Luscher 2006). While Max is constitutively expressed, the expression of c-Myc, Mnt and most Mad family members are tightly regulated with respect to the proliferative status of the cell. In short c-Myc expression must overcome Mnt and Mad expression levels in order for c-Myc/Max heterodimers to form and regulate transcription (Walker et al. 2005; Hurlin and Huang 2006; Rottmann and Luscher 2006). Moreover, it has been shown that Mnt is phosphorylated upon mitogenic stimulation preventing its ability to interact with SIN3 and recruit HDACs, which also alleviates the suppressive affects of Mnt on c-Myc transcriptional activity (Popov et al. 2005). In summary, c-Myc transcriptional activity is regulated at many levels that include expression of *c-myc* by transcription and translational mechanisms, protein stability/turnover at the post-translational level, and the ability to interact with its binding partners Max and Miz1.

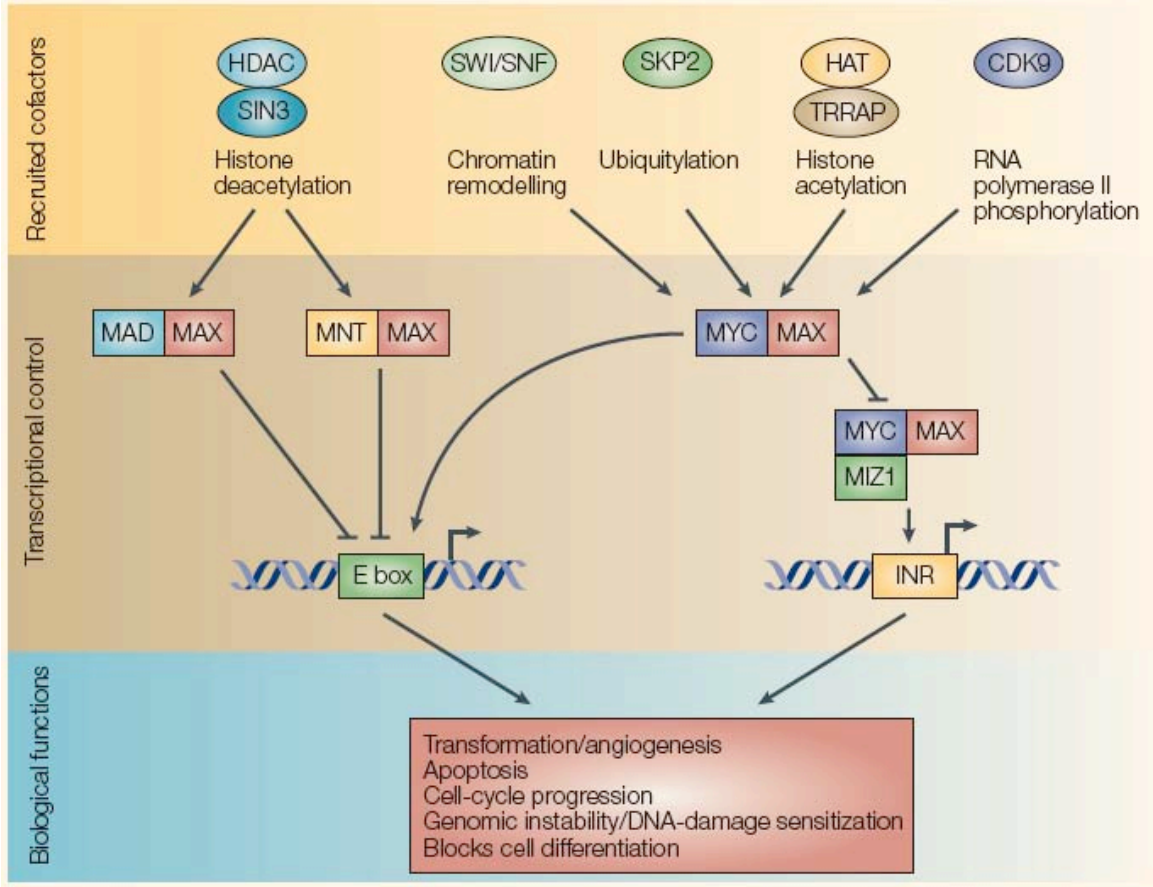

Figure 1.2: C-Myc/Max transcriptional network (Patel et al. 2004).

C-Myc heterodimerizes with Max and together they bind specific DNA elements called E-boxes within the promoter regions of numerous genes. Alternatively, c-Myc/Max heterodimers associate with Miz1 and bind INR elements masking the transcription initiation site found in the promoters of some genes thereby inhibiting their transcription. C-Myc transcriptional activity is also regulated by its relative expression to Mnt and Mad, which competitively bind Max preventing the formation of c-Myc/Max heterodimers and subsequent c-Myc transcriptional activity. C-Myc transcriptional activity generally results in the expression of genes that drive proliferation, growth, angiogenesis, and under “normal” cellular conditions can induce apoptosis. In contrast, c-Myc transcriptional regulation blocks differentiation and increases genomic instability and cellular transformation. C-Myc/Max positively regulate transcription by recruiting chromatin remodeling proteins and HATs that create a transcriptionally active chromatin/histone environment as well as recruit CDK9 to increase RNA polymerase II elongation. On the other hand, Mnt/Max and Mad/Max heterodimers oppose c-Myc/Max transcription by recruiting HDACs to create a transcriptionally inactive environment.

Hierarchical phosphorylation of c-Myc on T58 and S62 regulate c-Myc protein stability. The regulation of c-Myc expression occurs at many levels through transcription, translation, and post-translational mechanisms, see above. Work from our lab and others have focused on the regulation of c-Myc expression at the post-translational level and identified a hierarchical set of reversible phosphorylation events that regulate c-Myc protein stability. These phosphorylation events center on two residues conserved across species, T58 and S62 (Luscher and Eisenman 1990). Both T58 and S62 residues fall within the conserved Myc Box I (MBI) region in the transactivation domain of c-Myc (Figure 1.1). Phosphorylation of these residues has opposing affects on c-Myc protein stability with S62 phosphorylation stabilizing and T58 phosphorylation destabilizing c-Myc protein stability (Sears et al. 2000). As previously mentioned, c-Myc protein levels are tightly regulated with respect to the proliferative status of the cell and the cell cycle, with low c-Myc protein levels observed in quiescent cells. However, following mitogenic stimulation and entry of cells into the cell cycle, c-Myc protein levels accumulate and peak by four hours post-stimulation in early G_1 and then quickly decline and are maintained at low levels by late G_1 (Sears 2004). This “bell-shaped” curve of c-Myc protein levels in the cell cycle is regulated in part by changes in c-Myc protein stability through the phosphorylation of T58 and S62.

Mitogenic stimulation of cells results in the activation of a small GTP-binding protein, Ras, which is a central component of the mitogen signal

transduction pathway (White et al. 1995). The activation of Ras results in the activation of several signaling pathways including Raf/MEK/ERK and PI3K/Akt. Raf/MEK/ERK activation has been primarily associated with mitogenic-induced cellular proliferation (Seger and Krebs 1995; Lavoie et al. 1996). PI3K/Akt activation has been primarily associated with increased cell mass and cell survival (Franke et al. 2003). Importantly, c-Myc can be phosphorylated on S62 by ERK kinases (Seth et al. 1992; Pulverer et al. 1994) as well as other MAP activated kinases and cyclin dependent kinases (Lutterbach and Hann 1994; Noguchi et al. 1999). Simultaneous activation of the PI3/Akt pathway by Ras can result in the phosphorylation and inactivation of GSK3β (Cross et al. 1995). These two Ras regulated pathways keep c-Myc singly phosphorylated on S62, which stabilizes c-Myc protein (Figure 1.3). Ras activity falls in late G_1 relieving the inhibition of GSK3β activity, allowing GSK3β to phosphorylate c-Myc on T58 (Lutterbach and Hann 1994; Pulverer et al. 1994). It is important to note that GSK3β is a processive kinase that prefers substrates already phosphorylated on a residue four amino acids downstream (Chu et al. 1996). Therefore, prior S62 phosphorylation of c-Myc is an important event in the eventual destabilization of c-Myc despite its initial stabilizing effect. Following T58 phosphorylation by GSK3β, the prolyl-isomerase, Pin1 associates with c-Myc and converts Proline 63 (P63) from cis- to trans-conformation (Yeh et al. 2004). This allows the conformation sensitive protein phosphatase 2A (PP2A) to bind and dephosphorylate S62 (Yeh et al. 2004; Arnold and Sears 2006) leaving c-Myc

singly T58 phosphorylated. Singly T58 phosphorylated c-Myc is unstable (Figure 1.3) since it is recognized by the SCF^{Fbw7} ubiquitin machinery, which multi-ubiquitinates c-Myc, marking it for degradation by the 26S proteosome (Welcker et al. 2004; Yada et al. 2004). Of note, it has also been shown that c-Myc can be ubiquitinated by Skp2 independent of T58 phosphorylation and degraded (Kim et al. 2003; von der Lehr et al. 2003; Herbst et al. 2004). This degradation mechanism appears to depend on elements within MBII rather than MBI (Figure 1.1). Altogether, c-Myc protein stability is tightly regulated by an exquisite set of hierarchical, reversible phosphorylation events occurring at the highly conserved T58 and S62 residues.

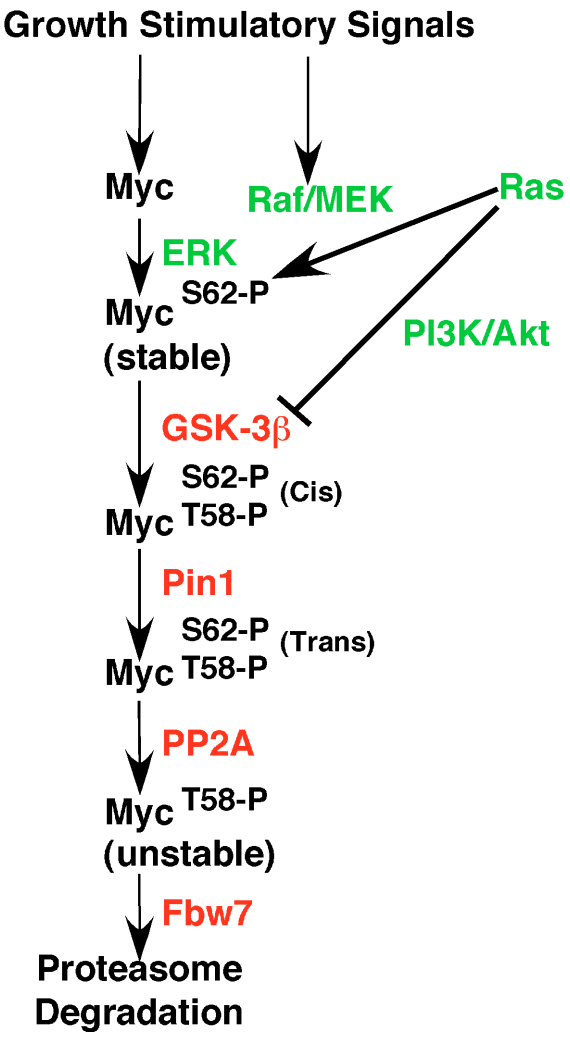

Figure 1.3: Schematic showing the mitogenic regulation of c-Myc protein stability.

In response to mitogen stimulation, Ras is activated resulting in the activation of the Raf/MEK/ERK and PI3/Akt pathways. ERK can phosphorylate c-Myc on S62 and simultaneously, PI3/Akt inhibits GSK3β phosphorylation of T58, keeping c-Myc in a stable form early in G_1. Late in G_1, Ras activity decreases and GSK3β becomes active, phosphorylating c-Myc on T58. This destabilizes c-Myc by allowing SCF^{Fbw7} to recognize and multi-ubiquitination c-Myc marking it for degradation via the 26S proteosome.

C-Myc expression is elevated in many types of human cancer. While elevated expression of c-Myc is observed in the majority of human cancers only ~20% of tumors show amplification or translocation of the *c-myc* gene, which could explain the elevated expression of c-Myc (Nesbit et al. 1999). This finding strongly suggest that deregulation of c-Myc expression at the post-translational level may be an important mechanism for elevated c-Myc expression in cancer. In support of this hypothesis, we have found that c-Myc is stabilized in a number of human breast (Xiaoli, data not shown) and leukemia (Malempati et al. 2006) cancer cell-lines as compared to "normal" control cell-lines. Moreover, primary acute lymphoblastic leukemia (ALL) bone marrow patient samples showed increased c-Myc protein stability compared to bone marrow samples from healthy patients (Malempati et al. 2006). Interestingly, none of these cell-lines or patient samples contained mutations within the MBI region of c-Myc that we have characterized to regulate c-Myc protein stability. Therefore, it is likely that proteins involved in regulating c-Myc phosphorylation at these sites are mis-regulated in cancers exhibiting elevated c-Myc stability.

Starting at the top of the signaling pathway outlined above in Figure 1.3, activating Ras mutations are often observed in human cancers (Barbacid 1987) and it has been shown that constitutively active point mutants of Ras are required in combination with c-Myc to stably transform some cells in culture, especially primary cells (Cole 1986). Conceptually, constitutively active Ras would stabilize

c-Myc protein by activating ERK to phosphorylate S62 as well as suppress GSK3β phosphorylation of T58. This would keep c-Myc in a stable form, allowing it to accumulate (Figure 1.3). However, elevated c-Myc expression in "normal" cells has been shown to induce apoptosis (Pelengaris et al. 2002; Nilsson and Cleveland 2003). Therefore, Ras activity confers another important function, which is survival signaling through the PI3/Akt pathway preventing the elevated expression of c-Myc from inducing apoptosis (Kauffmann-Zeh et al. 1997). Consequently, mutations that result in constitutively active Ras can cooperate with c-Myc to drive oncogenesis, especially if they occur early in tumorigenesis. However, c-Myc driven tumors can occur without activation of Ras, which suggest that mutations in other components of the c-Myc degradation pathway are also important.

Interestingly, common mutations or mis-regulation of other known components in the c-Myc degradation pathway have not been widely reported in human tumors. For example, mutations in GSK3β have not been reported; or in the case of Pin1, most observations find overexpression of Pin1 in human cancer (Lu et al. 2006). Overexpression of Pin1 should promote the turnover of c-Myc thereby inhibiting tumorigenesis. On the other hand, mutations and mis-expression of Fbw7 have been observed in human cancers, but generally are found in 10% or less of human tumors examined (Kwak et al. 2005; Koh et al. 2006; Yan et al. 2006). While global inhibition of PP2A has been shown to aid in the transformation of primary human cells (Chen et al. 2004), identification of

mutations relevant to the tumor suppressor activity of PP2A have not been well described and specific molecular targets for particular PP2A holoenzymes are limited. Therefore, further characterization of identified proteins as well as the identification of new proteins involved in c-Myc degradation is critical to furthering our understanding as to mechanisms by which c-Myc expression is deregulated at the post-transcriptional level in human cancer.

Identification of a specific PP2A holoenzyme, PP2A-B56α, and a new protein, Axin1, which regulate c-Myc protein stability and turnover. As mentioned above, our understanding of the mechanisms by which c-Myc expression becomes deregulated requires further analysis of already identified players as well as identification of new players involved in regulating c-Myc expression. To this end, my thesis focuses on further characterizing PP2A and the identification of a new player, Axin1, in the regulation of c-Myc expression at the post-translational level. More specifically, further characterization of PP2A centers on identifying the PP2A holoenzyme that promotes dephosphorylation of S62 in c-Myc. PP2A represents a large number of different heterotrimeric holoenzymes, which may include as many as 75 different members. Therefore, chapter two of my thesis focuses on identifying a specific PP2A holoenzyme that recognizes c-Myc and promotes the dephosphorylation of S62 in c-Myc. The third chapter of my thesis focuses on the identification and characterization of the scaffold protein, Axin1 that coordinates GSK3β, Pin1 and PP2A into a

degradation complex for c-Myc. This complex is analogous to the degradation complex coordinated by Axin1 for β-catenin. Altogether, these findings greatly expand our understanding of the regulation that controls c-Myc expression at the post-translational level. Furthermore, these findings offer important new insight into mechanisms by which c-Myc expression may become deregulated at the post-translational level, as mutations can be found in both PP2A and more frequently in Axin1 that likely affect their ability to properly regulate c-Myc expression.

PP2A Background

Protein phosphatase 2A (PP2A) is a serine/threonine protein phosphatase. Cells utilize a multitude of signaling pathways to govern virtually all aspects of cellular function in order to maintain “normal” cellular homeostasis. These signaling pathways are exquisitely regulated through a multitude of mechanisms. One mechanism of regulation is the post-translational modification of proteins by reversible phosphorylation. Two key groups of proteins are involved in the reversible phosphorylation process, kinases that add phosphates to target proteins and protein phosphatases that remove phosphates from target proteins. While a plethora of kinases have been identified and well characterized, only a handful of protein phosphatases have been identified and much less is known about their function. Protein phosphatases are grouped into two families based on their ability to either dephosphorylate tyrosine or serine/threonine residues. PP2A falls into the Serine/Threonine family of protein phosphatases, which includes PP1, PP2B (also called calcineurin), PP2C, PP3, PP4, PP5, PP6, PP7, PP8, and possibly others yet to be identified. To date PP2A has primarily been considered a tumor suppressor as global inhibition of PP2A contributes to the transformation of primary human cells (Shenolikar 1994; Schonthal 1998; Millward et al. 1999; Virshup 2000; Chen et al. 2004). However, there is growing evidence that contradicts this broad statement regarding PP2A activity as recent reports now demonstrate some oncogenic roles for PP2A (Abraham et al. 2000; Jaumot and Hancock 2001; Li et al. 2002; Yang et al. 2003). Much of the

ambiguity regarding PP2A tumor suppressor verses oncogenic activity stems from the lack of specific targets identified for specific PP2A holoenzymes (see below for holoenzyme structure). Consequently, significant contributions to our understanding of PP2A function result from the identification of specific PP2A holoenzyme/target protein interactions. As part of my thesis I describe the identification of a PP2A holoenzyme, PP2A-B56α that associates with c-Myc and negatively regulates both c-Myc protein levels and transcriptional activity.

PP2A is a heterotrimeric holoenzyme. The PP2A holoenzyme is comprised of two common components, the structural (A) and catalytic (C) subunits that create the dimeric form of PP2A, to which a third variable regulatory (B) subunit binds (Figure 1.4). Both the A and C subunits is found in two forms, α and β, and each are encoded by a different gene. In both cases of the A and C subunits, the α isoform is the predominantly expressed form. Furthermore, these different isoforms do not appear to be completely redundant as knockout of Cα is embryonic lethal (Gotz et al. 1998), demonstrating that Cβ can not fulfill the required functions of Cα. To date knockout of the A subunit has not been reported, yet a number of mutations have been identified in both Aα and Aβ isoforms in cancer (Wang et al. 1998; Ruediger et al. 2001a; Ruediger et al. 2001b) suggesting that both forms of the A subunit are critical for PP2A's tumor suppressor function. These findings demonstrate the biological requirements for both the A and C subunits, which form the structural catalytic core of PP2A.

The regulatory B subunits of PP2A fall into four distinct families, B, B', B'', and B'''. Importantly, a single regulatory B subunit is associated with a given PP2A holoenzyme and the regulatory B subunit dictates substrate specificity. The regulatory B subunits are also thought to regulate PP2A holoenzyme activity through regulation of subcellular localization and potentially by targeting PP2A holoenzymes to large complexes. For example, regulation of β-catenin protein levels by PP2A-B56α, this PP2A holoenzyme is found to associate with a degradation complex including the Axin1 scaffold protein (McCright et al. 1996b; Virshup 2000; Li et al. 2001). The requirement for particular B subunits is currently being explored and it has been shown that loss of B56α in zebra fish does have a distinct phenotype associated with axis formation defects (Li et al. 2001). Although, no reports of regulatory B subunit knockout mice have been published, we have been informed that there is currently a B56δ knockout mouse being made (Dr. Virshup, personal communication). Moreover, we are currently generating a B56α knockout mouse, which will help determine the relative importance of different PP2A holoenzymes in development and as tumor suppressors or oncogenes *in vivo*.

The physical structure of PP2A is largely mediated through the structural A subunit, which contains 15 non-identical HEAT (huntingtin-elongation-A subunit-TOR-like) repeats (Xu et al. 2006) that facilitate binding of the B and C subunits (Figure 1.4). The association of the B and C subunits is mediated through different regions of the A subunit with the B subunit binding HEAT

repeats 1-10 and the C subunit binding HEAT repeats 11-15 (Figure 1.4) (Kremmer et al. 1997). Furthermore, the association of different B or C subunits is mutually exclusive, such that distinct ABC heterotrimers of PP2A are formed (Kremmer et al. 1997). Post-translational modifications also play an important role in regulating PP2A holoenzyme formation (Figure 1.5) (Janssens and Goris 2001). Methylation of the C subunit by the leucine carboxyl methyltransferase (LCMT) regulates the ability of some regulatory B subunits to associate with the PP2A catalytic core to form a PP2A holoenzyme (Tolstykh et al. 2000). The methylation of the C subunit is reversible as the phosphatase methylesterase (PME-1) can remove the methyl modification and maintain the C in an inactive form (Longin et al. 2004). PME-1 can also keep the dimeric catalytic core form of PP2A in an inactive form until the protein phosphatase activating (PTPA) protein promotes the formation of a PP2A holoenzyme (Janssens and Goris 2001; Longin et al. 2004). Very recently it was shown that the incorporation of a regulatory B subunit into a PP2A holoenzyme increases the catalytic activity of the C subunit (epub REF Hombauer, H, 2007). Lastly, phosphorylation of the C subunit has been shown to negatively regulate the catalytic activity of the C-subunit (Brautigan 1995). Altogether the ability of PP2A to form distinct holoenzymes is regulated both intrinsically and post-translationally.

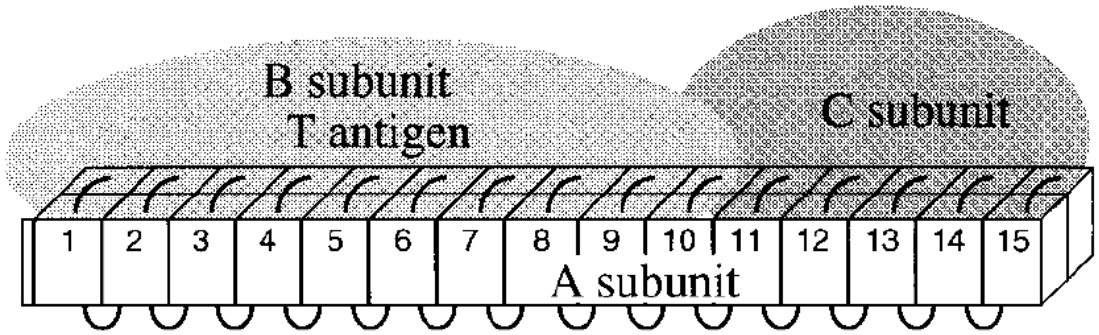

Figure 1.4: Structure of PP2A (Kremmer et al. 1997).

The A subunit of PP2A serves as the structural backbone which bring the catalytic C and regulatory B subunits together forming the PP2A holoenzyme. There are 15 non-identical HEAT (huntingtin-elongation-A subunit-TOR-like) repeats that mediate interaction with the B and C subunits. The B subunit interacts with repeats 1-10 and the C subunit interacts with repeats 11-15.

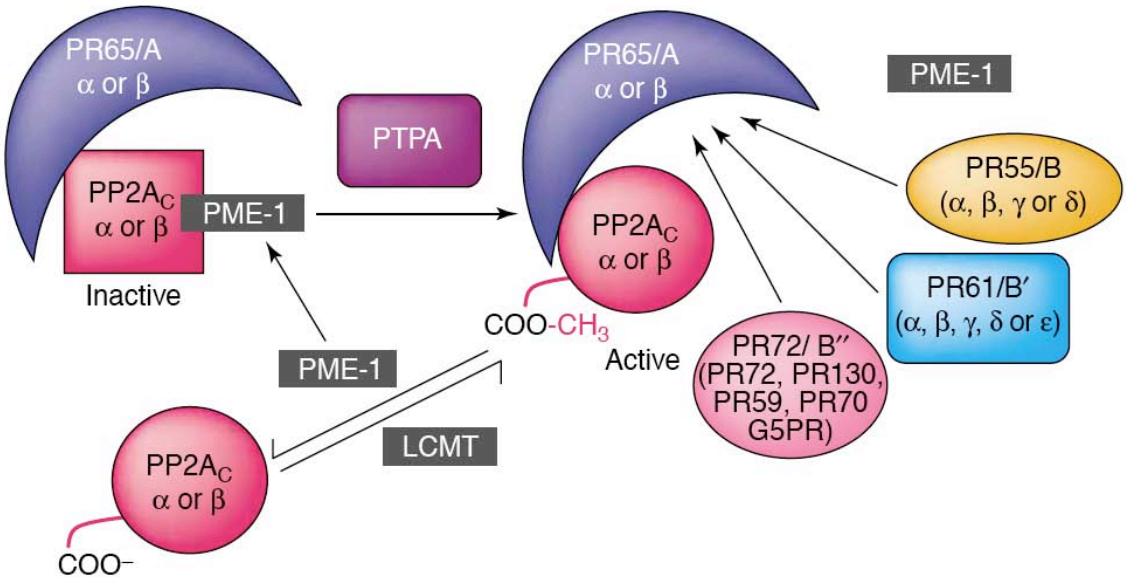

Figure 1.5: PP2A holoenzyme formation is regulated by post-translational modification (Van Hoof and Goris 2004).

The formation of an active PP2A holoenzyme is a regulated process involving several proteins. Methylation of the C subunit by Leucine carboxyl methyltransferase (LCMT) is reported to facilitate the association of some regulatory B subunits into the PP2A holoenzyme. This methylation event can be removed by the phosphatase methylesterase (PME-1). PME-1 can hold the catalytic core of PP2A in an inactive state until the protein phosphatase activating (PTPA) activates PP2A, allowing holoenzyme formation to occur. Not shown in the diagram is the phosphorylation of Y307 in C, which inactivates the C subunit.

PP2A can act as an oncogene and tumor suppressor depending upon PP2A holoenzyme composition. PP2A was originally described as a tumor suppressor, as it was shown that the inhibition of PP2A catalytic activity by okadaic acid resulted in increased tumor formation (Bialojan and Takai 1988). Subsequently it was discovered that oncogenic viruses expressed proteins which associate with PP2A (Pallas et al. 1990; Sontag et al. 1993; Mumby 1995). Specifically, simian virus 40 (SV40) small T antigen associates with the A subunit within the B subunit binding region and prevents the association of regulatory B

subunits with the A subunit (Yang et al. 1991). Moreover, the adenovirus encoded E4orf4 protein interacts with a subset of PP2A holoenzymes containing B or B' family members (Kleinberger and Shenk 1993; Shtrichman et al. 1999; Shtrichman et al. 2000), and redirects their activity toward processes involved in viral production (Kanopka et al. 1998; Shtrichman et al. 2000). Inhibition of normal PP2A function by these virally expressed proteins leads to increased cell transformation and tumorigenesis. In addition, overexpression of the A subunit of PP2A effectively inhibits normal PP2A function resulting in multinucleated cells (Wera et al. 1995). Altogether, these findings demonstrate that global inhibition of PP2A results in increased tumorigenesis and aberrant cellular division.

More recent experiments have begun to examine the role of specific regulatory B subunits in tumorigenesis. This started with the finding that inhibiting the B family member B55α caused defects in chromosome segregation (Mayer-Jaekel et al. 1993). Interestingly, screening of malignant melanoma cells revealed that the B' family member B56γ expression is elevated suggesting an oncogenic role for B56γ (Francia et al. 1999). However, more recent experiments suggest that this is a deletion mutant form of B56γ, ΔB56γ, from mouse melanoma cells that is overexpressed and increases both genomic instability and metastasis (Ito et al. 2000; Ito et al. 2003). More recently it was shown that RNAi knockdown of B56γ, similar to global inhibition of PP2A by SV40 small t antigen expression, is a key step in transformation of human primary cells (Chen et al. 2004). These findings suggest that full-length B56γ is a

critical tumor suppressor, while the mutant form of B56γ, ΔB56γ, acts in dominant negative manner and has an oncogenic effect. We have also shown that a PP2A resistant form of c-Myc, c-Myc^{T58A} can substitute for SV40 small T in transforming primary human cells (Yeh et al. 2004). Another regulatory B subunit that has been shown to have tumor suppressor function is B56α, which negatively regulates Wnt signaling by promoting β-catenin protein degradation (Seeling et al. 1999). B56α has also been shown to inactivate Bcl2, thereby inhibiting the survival activity of Bcl2 (Ruvolo et al. 2002). Moreover, B56α has been shown to associate with the oncogenic form of p63, ΔNp63, which results in increased β-catenin protein levels presumably by ΔNp63 acting as a "substrate trap" (Patturajan et al. 2002). Altogether, these experiments demonstrate a tumor suppressor role for specific PP2A holoenzymes.

In contrast to the tumor suppressor effects described above, there are now potential oncogenic roles characterized for PP2A. As described above, B56α negatively regulates Wnt signaling and reduces β-catenin protein levels. In contrast, another B' family member, B56ε has been shown to be required for Wnt signaling, which results in increased β-catenin protein levels (Yang et al. 2003). In a similar manner PP2A has been shown to have both positive and negative regulatory roles in MAP kinase signaling (Wasserman, DA 1996). Within the MAP kinase pathway it has been shown that B55α positively regulates Raf activity, which could have an oncogenic affect should B55α become constitutively active. Although global suppression of PP2A indicates that the predominant

function of PP2A is as a tumor suppressor, it cannot be ignored that certain PP2A holoenzymes may have oncogenic capabilities.

Given the opposing roles of PP2A to function as an oncogene or tumor suppressor, it is critical to further our understanding of PP2A with respect to which PP2A holoenzymes have oncogenic or tumor suppressor potential. To this end, chapter two is devoted to identifying and characterizing a particular PP2A holoenzyme, PP2A-B56α that negatively regulates c-Myc protein levels and transcriptional activity. As noted, B56α has already been shown to function as tumor suppressor in Wnt signaling by promoting the turnover of β-catenin and inactivating Bcl2 anti-apoptotic activity. This identifies PP2A-B56α as a PP2A holoenzyme with a strong tumor suppressor potential.

Axin1 Background

Axin1 is a scaffold protein that regulates several signaling pathways and is often mutated in a wide variety of cancers. Axin1, originally named fused, is a multi-domain scaffold protein that plays a critical role in axis formation during development as demonstrated by disruption of normal Axin1 expression resulting in aberrant axis formation (Zeng et al. 1997; Farr et al. 2000). Similar to the requirement of *c-myc*, homozygous disruption of full-length *fused/axin1* results in embryonic lethality at day E9.5 due to truncation of the forebrain, neural tube defects, and axis duplication in mice (Perry et al. 1995; Vasicek et al. 1997). Numerous studies of Axin1 have focused on its role in regulating β-catenin due to the critical role of Wnt/β-catenin signaling in axis formation (Marikawa 2006). However, more recent studies also show that Axin1 is involved in regulating, TGFβ, SAPK/JNK, (Figure 1.7) and p53 signaling (Zeng et al. 1997; Hart et al. 1998; Ikeda et al. 1998; Kishida et al. 1998; Kishida et al. 1999; Sakanaka and Williams 1999; Zhang et al. 1999; Ikeda et al. 2000; Neo et al. 2000; Furuhashi et al. 2001; Zhang et al. 2001; Levina et al. 2004; Rui et al. 2004; Liu et al. 2006; Li et al. 2007; Lin and Li 2007). Axin1 differentially regulates these pathways by associating and coordinating different complexes of proteins. To date Axin1 has been shown to function as a tumor suppressor in each of these pathways, as reviewed by (Salahshor and Woodgett 2005). Moreover, numerous *axin1* gene mutations have been identified from a number of different cancers that likely compromise the interaction of known Axin1 associating proteins such as DVL,

MEKK, GSK3β, PP2A, APC, β-catenin, and even Axin1 with itself (Figure 1.6) (Satoh et al. 2000; Webster et al. 2000; Dahmen et al. 2001; Moreno-Bueno et al. 2002; Shimizu et al. 2002; Taniguchi et al. 2002; Baeza et al. 2003; Jin et al. 2003; Miao et al. 2003; Daa et al. 2004; Zhou and Gao 2007). Molecular analysis of some Axin1 mutations in the GSK3β binding domain have been shown to disrupt GSK3β association with Axin1 and impair Axin1 function (Webster et al. 2000). Moreover, in some cases of hepatocellular cancers (HCCs) with mutations in *axin1*, re-expression of wild-type Axin1 induced apoptosis suggesting that Axin1 may be an important molecular target to treat HCC (Satoh et al. 2000). Altogether, these studies highlight the importance of maintaining wild-type expression of Axin1.

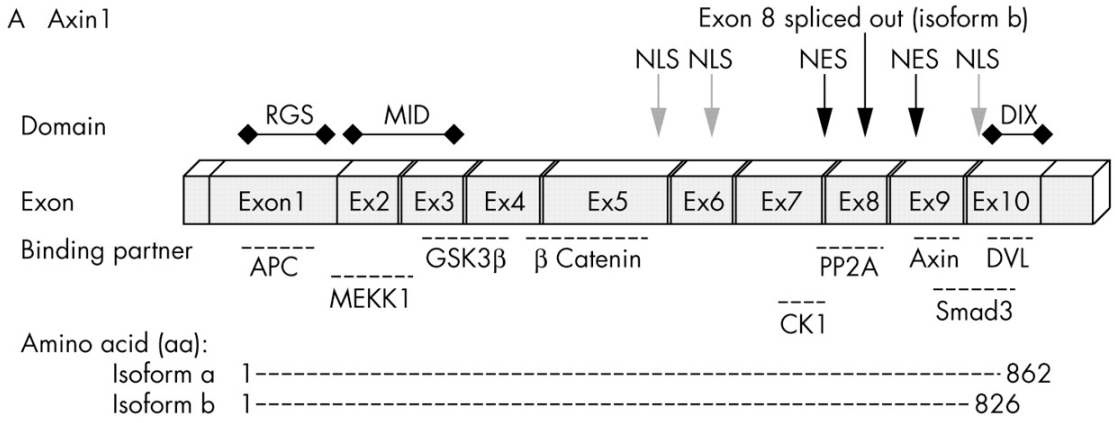

Figure 1.6: Axin1 is a multidomain scaffold protein (Salahshor and Woodgett 2005).

The scaffold protein Axin1 has 10 exons and multiple binding domains for proteins such as adenomatous polyposis coli (APC), mitogen activated protein/extracellular regulated kinase kinase kinase 1 (MEKK1), glycogen Synthase 3β (GSK3β), β-catenin, casein kinase 1 (CK1), protein phosphatase 2A (PP2A), disheveled (DVL), Axin, and Smad3. Axin1 also contains three nuclear localization signals (NLS) and two nuclear export signals (NES). There are two

naturally occurring splice variants of Axin1 with exon 8 being removed in the second version.

Axin1 regulates Wnt/β-catenin signaling. Perhaps the best characterized role for Axin1 function is in regulating Wnt signaling by promoting the degradation of β-catenin in the absence of Wnt stimulation. Axin1 promotes β-catenin degradation by coordinating a complex containing adenomatous polyposis coli (APC) and GSK3β to phosphorylate β-catenin (Hart et al. 1998; Ikeda et al. 1998; Sakanaka et al. 1998). Phosphorylated β-catenin is recognized and ubiquitinated by the $SCF^{\beta TcRP}$ ubiquitin machinery marking β-catenin for degradation by the 26S proteosome (Latres et al. 1999; Liu et al. 1999; Winston et al. 1999). However, upon Wnt stimulation Dishevelled (DVL) is activated, which associates with and inhibits Axin1 function towards β-catenin (Smalley et al. 1999) through two mechanisms. First, DVL prevents Axin1 dimerization, which has been shown to be required for Axin1 degradation of β-catenin (Sakanaka and Williams 1999; Zhang et al. 2000). Second, DVL recruits Frat1 and activated Akt into the Axin1-GSK3β complex, which results in increased GSK3β phosphorylation which is believed to inactivate GSK3β as well as increase β-catenin/Tcf/Lef dependent transcription (Li et al. 1999; Fukumoto et al. 2001). Importantly, this increase in β-catenin/TCF/Lef transcription is relevant with respect to c-Myc, as it has been shown that *c-myc* is a target gene for β-catenin/Tcf/Lef transcription (He et al. 1998). However, it is not consistently observed that an increase in nuclear β-catenin results in increased *c-myc* mRNA levels or increased c-Myc protein

expression and therefore regulation of c-Myc expression at post-translational level is a critical aspect to c-Myc expression (Wang et al. 2002). Moreover, a recent report demonstrated that loss of Axin1 function was not equivalent to the gain of β-catenin function, which suggests that Axin1 regulates other important non-Wnt related pathways in tumorogenisis (Zucman-Rossi et al. 2007).

Axin1 regulation of TGFβ signaling could repress *c-myc* transcription. Transforming growth factor beta (TGFβ) has been shown to regulate multiple cellular processes including proliferation, differentiation, migration, and apoptosis (Massague 1998; Massague 2000). Depending upon cellular context, TGFβ signaling can positively or negatively regulate the previously mentioned cellular processes. Of particular interest with respect to c-Myc is the ability of TGFβ signaling to suppress cellular proliferation and growth by repressing *c-myc* expression at the mRNA level through TGFβ activation of Smad3, which recruits E2F4/5, DP1 and p107 to a Smad-E2F element in the *c-myc* promoter (Chen et al. 2002). It has been shown that Axin1 is involved in the activation of Smad3 by acting as an adapter protein between the TGFβ-type-1 receptor and Smad3 (Furuhashi et al. 2001). Axin1 has also been shown to negatively regulate the inhibitory Smad, Smad7 (Liu et al. 2006), which has been shown to inhibit TGFβ signaling by competitively preventing the association of Smad2 and Smad3 with TGFβ receptors (Hayashi et al. 1997; Nakao et al. 1997). These findings

strongly suggest that Axin1 can negatively regulate *c-myc* mRNA levels and presumably c-Myc protein levels through its role in regulating TGFβ signaling.

Axin1 induces apoptosis by activating SAP/JNK and p53 signaling pathways. The stress activated protein kinase/Jun N-terminal kinase (SAPK/JNK) signaling pathway is involved in both cellular stress response and normal cellular processes by regulating cellular proliferation, tissue morphogenesis, cell survival, and apoptosis (Ip and Davis 1998). Axin1 has been shown to induce apoptosis through SAPK/JNK signaling by associating with and activation of mitogen activated protein/extracellular regulated kinase kinase kinase (MEKK), which in turn activates SAPK/JNK (Zhang et al. 1999). Interestingly, GSK3β and MEKK competitively associate with Axin1 such that GSK3β association with Axin1 attenuates SAPK/JNK activation (Zhang et al. 2001). Moreover, the ability of Axin1 to induce SAPK/JNK mediated apoptosis depends upon both the extent to which JNK is activated as well as the ability to turnover β-catenin (Neo et al. 2000). Although it is not clear why β-catenin turnover is important for SAPK/JNK induced apoptosis, these findings suggest that Axin1 can coordinate different complexes to regulate multiple signaling pathways and through Axin1 these different signaling pathways can influence one another.

Axin1 has also been shown to induce apoptosis by coordinating p53 with a death domain-associated protein (Daxx) and homeodomain-interacting protein

kinase-2 (HIPK2), which results in p53 activation (Rui et al. 2004; Li et al. 2007; Lin and Li 2007). In these studies the predominant p53 binding domain within Axin1 resides within the MEKK binding domain of Axin1 (Rui et al. 2004). It is therefore likely that p53 and MEKK competitively associate with Axin1 to regulate apoptosis through different cellular signaling pathways depending upon cellular context. It has also been shown that elevated β-catenin protein levels induce p53 activity and in a negative feedback manner p53 decreases β-catenin protein levels by inducing the expression of Siah-1, which interacts with APC and promotes β-catenin degradation (Liu et al. 2001). However, p53/Siah-1 induced degradation of β-catenin does not depend upon the well characterized Axin1/GSK3β/APC phosphorylation and $SCF^{\beta TcRP}$ ubiquitination of β-catenin, but rather a novel ubiquitin ligase complex comprised of Siah/SIP/Skp1/Ebi (Matsuzawa and Reed 2001). Although Axin1 has not been shown to coordinate this later β-catenin ubiquitin ligase complex, it has been shown that p53 mediated degradation of β-catenin does require GSK3β activity and importantly, p53 shifts the cellular distribution of Axin1 to a more soluble fraction in which β-catenin protein levels were significantly reduced (Levina et al. 2004). These findings identify an importance role for Axin1 in both the induction of p53-mediated apoptosis as well as the p53 regulated negative feedback regulation of β-catenin. In summary, Axin1 can induce apoptosis through two signaling pathways, SAPK/JNK and p53, and both are likely coordinated with Axin1 mediated

turnover of β-catenin, which suggest that Axin1 can simultaneously regulate multiple pathways in “crosstalk” manner.

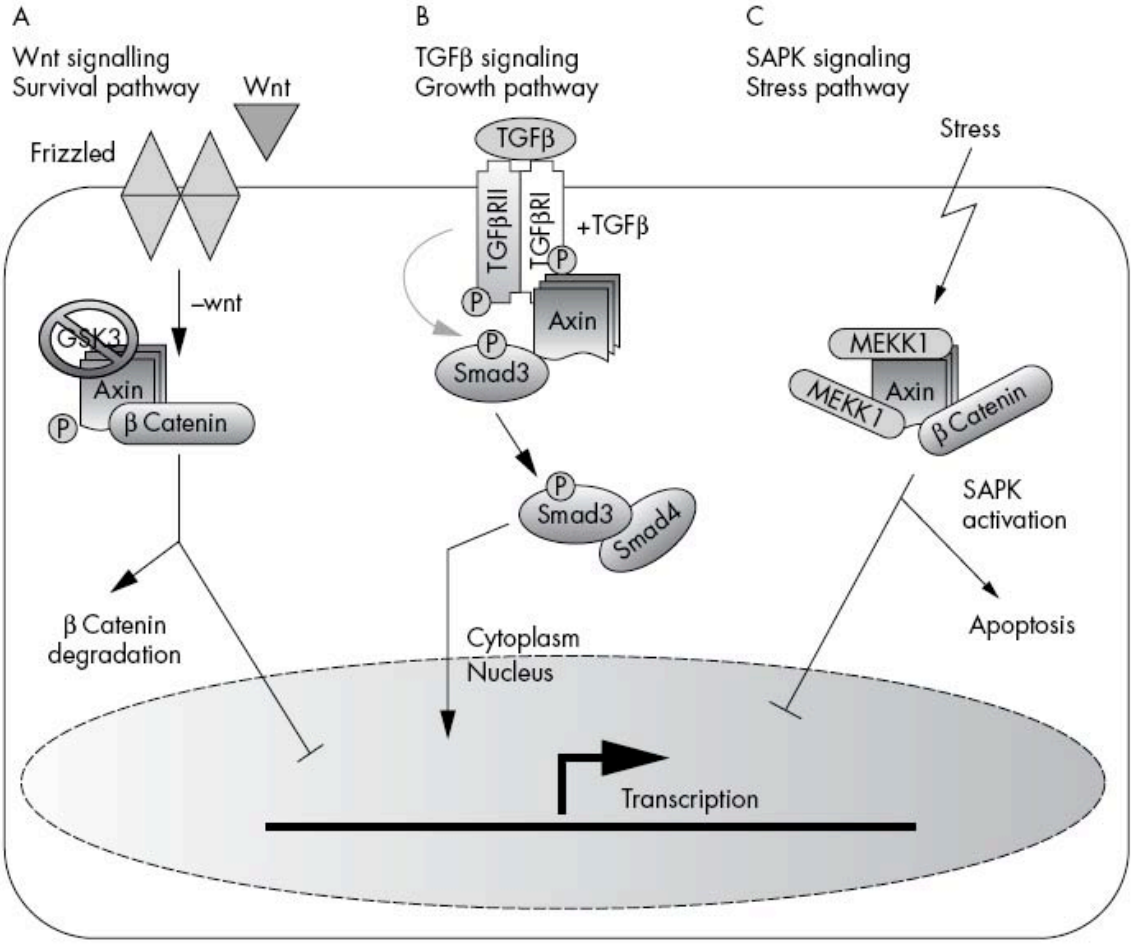

Figure 1.7: Axin1 is involved in regulating several signaling pathways (Salahshor and Woodgett 2005).

Axin1 coordinates several different complexes of proteins that are involved in Wnt, TGFβ, SAPK/JNK and not shown here, p53 signaling. It appears that most of these Axin1 mediated complexes regulate transcription factors that regulate the expression of a multitude of genes.

Chapter Two :

PP2A Regulatory Subunit B56α Associates with c-Myc and Negatively Regulates c-Myc Accumulation

PP2A Regulatory Subunit B56α Associates with c-Myc and Negatively Regulates c-Myc Accumulation

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Running Title: PP2A-B subunit, B56α, associates with c-Myc

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Abstract:

Protein phosphatase 2A (PP2A) plays a prominent role in controlling accumulation of the proto-oncoprotein, c-Myc. PP2A mediates its effects on c-Myc by dephosphorylating a conserved residue that normally stabilizes c-Myc, and in this way, PP2A enhances c-Myc ubiquitin-mediated degradation. Stringent regulation of c-Myc levels is essential for normal cell function as c-Myc overexpression can lead to cell transformation. Conversely, PP2A has tumor suppressor activity. Uncovering relevant PP2A holoenzymes for a particular target has been limited by the fact that cellular PP2A represents a large heterogeneous population of trimeric holoenzymes, composed of a conserved catalytic and structural subunit, with a variable regulatory subunit, which directs the holoenzyme to a specific target. We now report the identification of a specific PP2A regulatory subunit, B56α that selectively associates with the N-terminus of c-Myc. B56α directs intact PP2A holoenzymes to c-Myc, resulting in a dramatic reduction in c-Myc levels. Inhibition of PP2A-B56α holoenzymes, using shRNA to knockdown B56α, results in c-Myc overexpression, elevated levels of c-Myc Serine 62 phosphorylation, and increased c-Myc function. These results uncover a new protein involved in regulating c-Myc expression and reveal a critical interconnection between a potent oncoprotein, c-Myc, and a well-documented tumor suppressor, PP2A.

Introduction:

C-Myc is a transcription factor responsible for regulating a wide array of genes involved in cellular proliferation, growth, apoptosis, and differentiation. A number of experiments have demonstrated both the requirement for c-Myc and the importance of tightly regulating c-Myc protein levels for normal cellular function. For instance, lymphocytes and fibroblasts deleted for c-Myc cease to proliferate and exit the cell cycle (de Alboran et al. 2001; Trumpp et al. 2001). Furthermore, homozygous deletion of the *c-myc* gene results in embryonic lethality in mice (Davis et al. 1993). On the other hand, sustained overexpression of c-Myc in cultured cells blocks differentiation, induces neoplastic transformation, and can initiate apoptosis when survival factors are limiting (Evan et al. 1992). A wide array of naturally occurring tumors over-express c-Myc due in part to chromosomal translocations, amplification, and viral insertions at the *c-myc* locus (Cole 1986; Gregory and Hann 2000). Most notably, in mice with inducible c-*myc* transgenes, expression of c-Myc results in neoplastic pre-malignant and malignant phenotypes while withdrawal of c-Myc causes spontaneous regression of the neoplastic and malignant changes (Felsher and Bishop 1999; Pelengaris et al. 1999). All of these studies highlight the importance of understanding the mechanism as well as identifying the players involved in regulating c-Myc protein levels with respect to normal and neoplastic contexts.

PP2A Regulatory Subunit B56α Associates with c-Myc and Negatively Regulates c-Myc Accumulation

C-Myc expression is controlled at many levels including gene transcription, mRNA stability and post translational control of protein stability (Kelly et al. 1983; Jones and Cole 1987; Flinn et al. 1998). Post-translational regulation of c-Myc occurs through several Ras effecter pathways that control a series of sequential phosphorylation events on two highly conserved residues, Threonine 58 (T58) and Serine 62 (S62) (Sears et al. 1999; Sears et al. 2000; Yeh et al. 2004). These two phosphorylation sites exert opposing affects on c-Myc protein stability with S62 phosphorylation stabilizing c-Myc and T58 phosphorylation destabilizing c-Myc. Furthermore, T58 phosphorylation requires prior S62 phosphorylation (Lutterbach and Hann 1994; Sears et al. 2000). Upon exit from quiescence, during early G_1 phase, c-Myc is stabilized by phosphorylation on S62 that can be mediated by the Ras-activated Extracellular Regulated Kinase (ERK). Concurrent activation of PI3K by Ras can lead to inhibition of Glycogen Synthase Kinase-3 β (GSK3β), which is a negative regulator of c-Myc protein levels. In late G_1, when PI3K activity decreases, c-Myc can become phosphorylated on T58 by active GSK-3β. This dually phosphorylated form of c-Myc associates with the phosphorylation-directed prolyl isomerase 1 (Pin1), which can catalyze a *cis* to *trans* conformational change in the phospho-Ser62-Pro63 peptidyl bond of c-Myc . This form of c-Myc is then a target for PP2A, which dephosphorylates S62 resulting in an unstable, singly T58 phosphorylated form of c-Myc that is a substrate for ubiquitination by SCF^{Fbw7} and

degradation by the 26S proteosome (Welcker et al. 2004; Yada et al. 2004; Yeh et al. 2004).

PP2A is a heterotrimeric protein with two common components, a structural (A) subunit and catalytic (C) subunit forming the "catalytic core" to which a variable regulatory (B) subunit associates. To date 25 different B subunits have been identified that fall into four unrelated families, B, B', B'', and B'''. In total, it is estimated that there are 75-100 different PP2A holoenzymes responsible for 30-50% of the total cellular serine/threonine dephosphorylation activity depending on cell-type. PP2A has been shown to be involved in regulating proliferation, growth, differentiation, and apoptosis, (Janssens and Goris 2001). Similar to c-Myc, PP2A activity is required for normal cellular function as shown by a catalytic (Cα) subunit knock-out mouse model that results in death at embryonic day 5.5-6 (Gotz et al. 1998). However, unlike c-Myc, PP2A is generally regarded as a tumor suppressor. Global inhibition of PP2A activity results in increased cellular transformation (Schonthal 2001). Despite the importance of PP2A as a tumor suppressor, relatively few oncogenic PP2A targets have been identified. Moreover, specific PP2A holoenzymes that target these oncoproteins have not been well described.

We now report the identification of a specific PP2A holoenzyme containing the regulatory B subunit, B56α, that associates with c-Myc. We demonstrate that the PP2A- B56α holoenzyme interacts with the trans-activation domain of c-Myc containing the S62 residue that we previously reported to be dephosphorylated

by PP2A (Yeh et al. 2004). This interaction negatively regulates both c-Myc protein levels and activity. Given the potent oncogenic nature of c-Myc and the observed tumor suppressor function of PP2A, identification of the PP2A-B56α holoenzyme as a c-Myc regulatory protein adds to our understanding of two important cell regulatory pathways.

Results:

Increased PP2A activity down-regulates c-Myc protein levels dependent upon PP2A holoenzyme formation. We previously demonstrated that global inhibition of PP2A strongly stabilizes c-Myc protein, allowing it to accumulate to high levels (Yeh et al. 2004). These experiments relied on a variety of mechanisms for inhibiting PP2A activity, including chemical inhibition with okadaic acid, which inhibits the catalytic C subunit of PP2A (Fernandez et al. 2002), expression of SV40 small T antigen, which competitively inhibits regulatory B subunit binding to the structural A subunit (Pallas et al. 1990; Sontag et al. 1993; Mumby 1995; Chen et al. 2004), and RNAi knockdown of the catalytic C subunit (Yeh et al. 2004). To further characterize the role of PP2A in regulating c-Myc protein levels, we examined the effects of increasing cellular PP2A activity on c-Myc protein levels. I coexpressed c-Myc with increasing amounts of a stable and functional, N-terminal HA-tagged, PP2A catalytic (PP2A-HA-C) subunit (Wadzinski et al. 1992; Al-Murrani et al. 1999). As shown in Figures 2.1A and B, increasing amounts of PP2A-HA-C partially reduced c-Myc protein levels**.** Interestingly, the reduction was initially dose dependent, up to two-fold more transfected PP2A-HA-C subunit relative to c-Myc, but no further significant decrease in c-Myc protein levels was seen with higher concentrations of PP2A-HA-C (compares lanes 1-3 to lanes 4-5 and graph).

The limited capacity of PP2A-HA-C activity to reduce c-Myc protein levels suggests that some aspect of PP2A biology is limiting. Most likely this limiting

factor is related to PP2A holoenzyme formation, which involves association of the PP2A-A and C subunits with a variable regulatory B subunit that specifies substrate recognition. This hypothesis prompted us to determine whether formation of the heterotrimeric PP2A holoenzyme was required to negatively regulate c-Myc protein levels. To test this, I inhibited PP2A holoenzyme formation by expressing SV40 small T. I then coexpressed increasing amounts of PP2A-HA-C to determine whether the uncomplexed PP2A-HA-C subunit could negatively regulate c-Myc protein levels. Consistent with our results in murine fibroblasts (Yeh et al. 2004), SV40 small T expression increased c-Myc protein levels in human HEK-293 cells (Figure 2.1C, compare lanes 1 and 2). However, addition of PP2A-HA-C no longer reduced c-Myc protein expression, at levels previously shown to maximally reduce c-Myc (Figure 2.1C, lanes 3 and 4 compared to Figure 2.1A). It is important to note that SV40 small T expression reportedly does not affect PP2A-C subunit activity (Yang et al. 1991). These results strongly suggest that the PP2A-HA-C subunit must incorporate into a PP2A holoenzyme in order to negatively regulate c-Myc protein levels. Consequently, identifying the regulatory B subunit(s) that directs PP2A activity towards c-Myc is critically important to further understand the mechanism by which PP2A regulates c-Myc protein levels.

Adenovirus E4orf4 expression results in increased c-Myc protein levels. To limit the number of potential regulatory B subunits under investigation, I made

use of another viral protein, E4orf4, from adenovirus. Unlike SV40 small T, E4orf4 interacts with a subset of intact PP2A holoenzymes that contain either B55α (Kleinberger and Shenk 1993; Shtrichman et al. 1999) or B56 family members (Kanopka et al. 1998; Shtrichman et al. 2000). E4orf4 effectively inhibits these PP2A holoenzymes by redirecting their activity toward targets important for viral production (Kanopka et al. 1998; Shtrichman et al. 1999). As shown in Figure 2.1D, increasing amounts of E4orf4 resulted in a dose dependent increase in c-Myc protein levels, similar to that seen with increasing amounts of SV40 small T antigen (Yeh et al. 2004). Based on this result, we narrowed our focus to B55α and B56 family members to see if any had a role in regulating c-Myc protein levels.

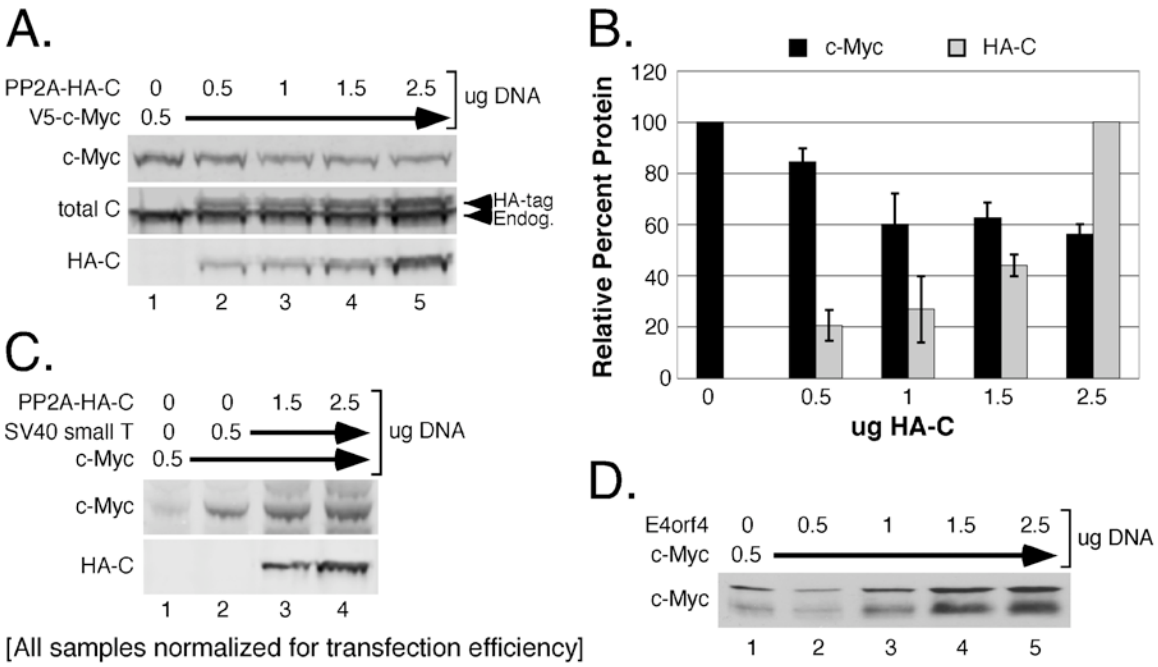

Figure 2.1: C-Myc protein levels are negatively regulated by PP2A holoenzyme activity.

(A) Increased cellular PP2A activity decreases c-Myc protein levels. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug pD40-His/V5-c-Myc, and increasing amounts from 0.5ug to 2.5ug of pD30-PP2A-HA-C, as indicated. Whole cell lysates were collected 36 hours post-transfection, normalized for transfection efficiency by β-galactosidase (β-gal)activity and visualized by western blot analysis with αV5 for c-Myc, αPP2A-Cα for total endogenous and ectopic PP2A-C, and αHA.11 for ectopic PP2A-HA-C. (B) Limited reduction of c-Myc protein levels by increased expression of PP2A-C subunit. C-Myc and PP2A-HA-C protein levels were quantified from Figure 2.1A and two repeat experiments using LI-COR software (see Methods: Western blotting and Quantitation). Average protein levels and error bars were calculated and graphed relative to the maximum level seen for each c-Myc or PP2A-HA-C. (C) PP2A holoenzyme formation is required to negatively regulate c-Myc protein levels. HEK-293 cells were cotransfected with 50ng CMV-β-gal, 0.5ug CMV-Myc, 0.5ug pCEP-SV40 small T-antigen, plus either 1.5ug or 2.5ug of pD30-PP2A-HA-C, as indicated. Whole cell lysates were prepared and normalized as in A. Immunoblots were probed for c-Myc with αN262 and for PP2A-HA-C with αHA.11. (D) Adenovirus E4orf4 expression causes an increase in c-Myc protein levels. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug CMV-Myc and increasing amounts from 0.5ug to 2.5ug of pCAN-E4orf4, as indicated. Lysates were prepared and normalized as in A and c-Myc protein was visualized by western blot analysis with αN262.

RNAi knockdown of the PP2A regulatory B56α subunit results in increased c-Myc protein levels. I used a RNA interference (RNAi) screen to individually knockdown expression of B55α and B56 family regulatory subunits to assess whether they are involved in negatively regulating c-Myc protein levels. I first examined B55α using small interfering RNA (siRNA). As shown in Figure 2.2A, knockdown of B55α did not cause a significant change in c-Myc protein levels as compared to scramble control siRNA (compare lanes 1 and 3). In contrast, knockdown of PP2A-C resulted in a substantial increase in c-Myc protein levels (lane 2), consistent with our previous results (Yeh et al. 2004). In both cases, PP2A-C and B55α were knocked-down by their siRNAs, 90% and 75%, respectively (Figure 2.2A, lanes 2 and 3, middle and bottom panels). We therefore concluded that B55α is not involved in negatively regulating c-Myc protein levels.

Next I examined knockdown of the B56 family of regulatory B subunits, which are encoded by five distinct genes: B56α, -β, -γ, -δ, and -ε (McCright and Virshup 1995; McCright et al. 1996a). B56α, -β, and -ε each express a single isoform whereas B56γ and -δexpress multiple isoforms due to alternative splicing, with four encoded by B56γ and three encoded by B56δ (McCright and Virshup 1995; Csortos et al. 1996; McCright et al. 1996a) (see Figure 2.2E for summary). I designed vector-expressed small hairpin RNAs (shRNAs) to knockdown the PP2A structural Aα (predominant adult form), B56α, -β, -γ, -δ, and -ε subunits. In the case of B56γ and δ, the constructed shRNAs are

designed to knockdown all splice variants (Figure 2.2E). I tested the specificity of these shRNAs by cotransfecting each shRNA construct with expression constructs for each of the targets: PP2A-A, B56α, -β, -γ1, -γ3, -δ1, or -ε. Each shRNA specifically reduced expression of its intended target by 70-90%, but did not affect expression of unintended targets (Figure 2.2B).

I cotransfected c-Myc with increasing amounts of each shRNA construct. As expected, increasing knockdown of the structural Aα subunit, which should result in near global inhibition of all PP2A holoenzymes, caused a robust linear increase in c-Myc protein levels (Figure 2.2C, top panel and 2D). In a similar manner, increasing knockdown of B56α resulted in a linear increase in c-Myc protein levels (Figure 2.2C, 2nd panel from top and 2D). In contrast, increasing knockdown of the remaining B56 subunits did not show a significant affect on c-Myc protein levels (Figure 2.2C, bottom four panels and 2D). Results from the E4orf4 experiment and the RNAi screen strongly suggest that B56α is likely the regulatory B subunit responsible for targeting PP2A activity towards c-Myc.

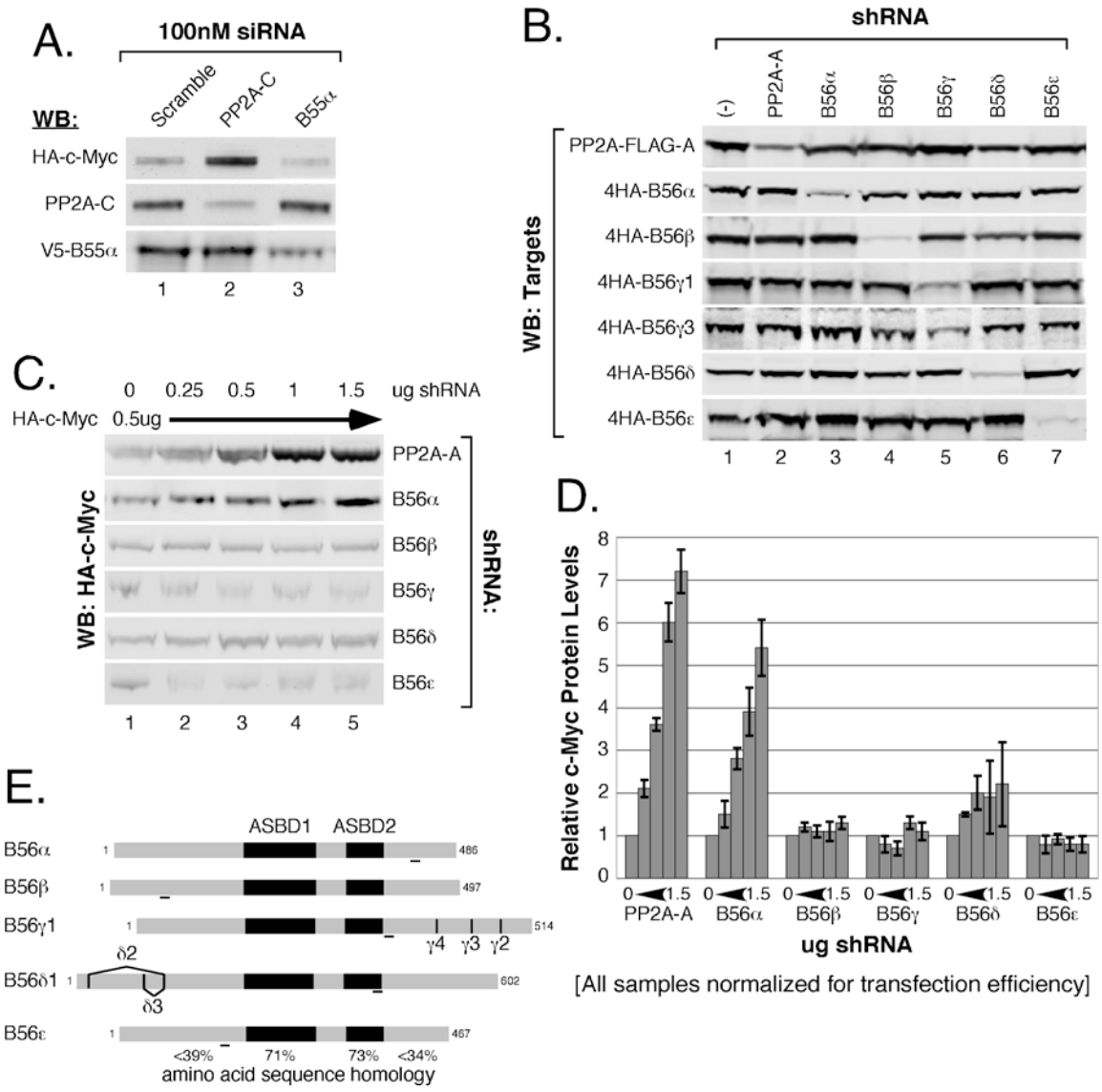

Figure 2.2: Increasing knockdown of B56α results in a robust linear increase in c-Myc protein levels.

(A) SiRNA knockdown of B55α does not affect c-Myc protein levels. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug CMV-HA-c-Myc, 0.5ug pD40-His/V5-B55α and siRNA (100nM final concentration) targeted to either PP2A-C, B55α, or scramble control. Whole cell lysates were collected 48 hours post-transfection, normalized for transfection efficiency by β-gal activity, and c-Myc, PP2A-C, and B55α proteins were visualized by western blot analysis with αHA.11, αPP2A-Cα , and αV5, respectively. (B) Vector expressed shRNAs targeted to PP2A-A and B56 family members specifically knockdown intended targets. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug of either

pD30-PP2A-FLAG-A or pCEP4HA-B56α, -β, -γ1, -γ3, -δ1 or -ε (Targets) and 2ug pSUPER-shRNA expression vector empty (-) or targeted to PP2A-A, B56α, -β, -γ1, -δ1 or -ε, as indicated. Cells were maintained in DMEM supplemented with 2% FBS and L-glutamine for 72 hours. Lysates were prepared and normalized as in A. Immunoblots were probed for PP2A-A with αM2-FLAG or for the indicated B56 family members with αHA.11. (C) Increasing knockdown of B56α results in increased c-Myc expression. HEK-293 cells were transfected with 50ng CMV-βgal, 0.5ug CMV-HA-c-Myc and increasing amounts from 0.25ug to 1.5ug of pSUPER-shRNA expression vector empty (-) or targeted to PP2A-A, B56α, -β, -γ1, -δ1 or -ε, as indicated. Cells were maintained and lysates prepared and normalized as in B. C-Myc was visualized with αHA.11 by western blot analysis. (D) Knockdown of PP2A-A or B56α results in a linear increase in c-Myc protein levels. C-Myc protein levels were quantified from Figure 2.2C and two repeat experiments, and average c-Myc expression and error bars were calculated (see Methods:Western blotting and Quantitation). Changes in c-Myc protein levels were then graphed as fold change relative to shRNA control (Figure 2.2C, lane 1). (E) Schematic of B56 family members. The B56 family of PP2A regulatory subunits share 71% and 73% amino acid sequence homology in the A subunit binding domains 1 and 2 (ASBD1 and 2), respectively, but significantly lower homology, less than 39% and 34%, in the N and C termini, respectively, that are believed to dictate substrate specificity (McCright et al. 1996b; Zhao et al. 1997; Li and Virshup 2002). Underlined regions indicate RNAi target sites used in the shRNA expression vectors for each B56 member. Splice variations in B56γ and B56δ are shown.

Knock-down of B56αincreases c-Myc protein stability. PP2A activity has been shown to regulate transcription (Wadzinski et al. 1993; Altiok et al. 1997; Nowak et al. 2003), mRNA stability (Keen et al. 2005), and translation (Andjelkovic et al. 1996; Lechward et al. 1999). However, our previous data shows that PP2A activity affects c-Myc protein stability (Yeh et al. 2004). To determine whether PP2A-B56α might regulate *c-myc* transcription or mRNA stability, I examined endogenous *c-myc* mRNA levels by RT-PCR upon shRNA knockdown of B56α in 293 cells. *C-myc* mRNA levels did not change when B56α was knocked down compared to control (Figure 2.3A, top panel). However, in

the same experiment endogenous c-Myc protein levels did increase 4.1-fold with B56α knock-down (Figure 2.3A, second panel from bottom), consistent with my results from Figure 2.2C and D indicating a post-transcriptional affect. I next assessed whether knock-down of B56α affects c-Myc protein stability. As shown in Figure 2.3B, knock-down of B56α significantly decreases the rate of c-Myc protein degradation following inhibition of further protein synthesis with cyclohexamide (compare middle and top panels). The quantitated increase in c-Myc half-life with B56α inhibition, from 25 minutes to greater than 2 hours (Figure 2.3B, graph), is consistent with our previous data showing a 5-6 fold increase in c-Myc stability upon global inhibition of PP2A activity (Yeh et al. 2004). These results demonstrate that the increase in c-Myc protein levels upon knockdown of B56α is due to increased c-Myc protein stability rather than increased levels or stability of *c-myc* mRNA.

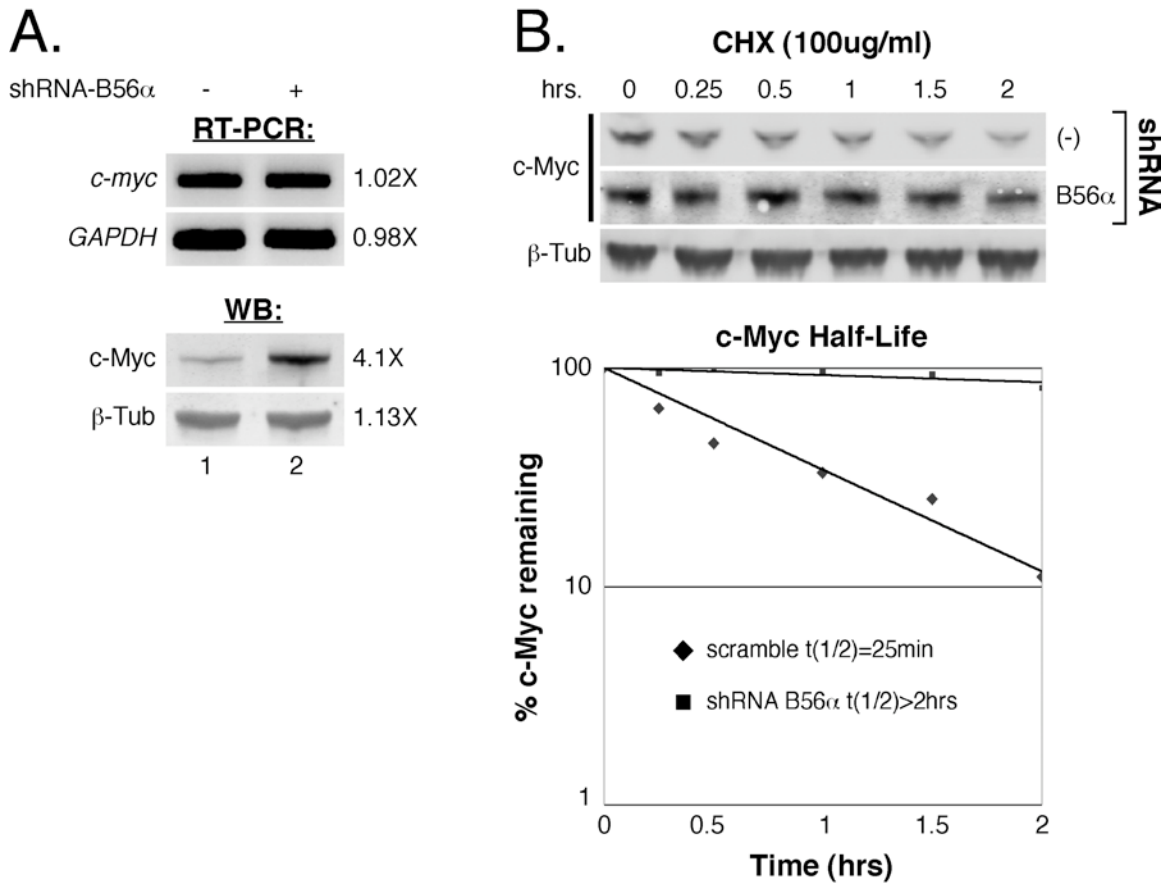

Figure 2.3: PP2A-B56α regulates c-Myc protein stability.

(A) Knockdown of B56α does not affect endogenous c-*myc* mRNA levels. HEK-293 cells were cotransfected with 50ng CMV-βgal and 3ug pSUPERshRNA-empty (-) or pSUPERshRNA-B56α under 10% FBS serum conditions for 24hrs and then starved in 0.2% FBS serum for 48hrs. Cells exhibiting transfection efficiencies by β-gal assay within 5% of each other were used for RT-PCR analysis of endogenous c-*myc* and *GAPDH* mRNA levels. Endogenous c-Myc and β-Tubulin protein levels were also examined in the same experiment by western blotting with αN262 and αβ-Tubulin, respectively. (B) C-Myc protein stability is increased upon B56α knock-down. 100mM dishes of HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug pD40-His/V5-c-Myc and 4ug pSUPER-empty or p-SUPER-B56α under 10% FBS serum conditions for 24hrs. Each transfection was split into six 60mM dishes with 10% FBS serum and then starved in 0.2% FBS serum for 48hrs. Cells were treated with 100ug/mL cyclohexamide and cell lysates were prepared at the indicated time points after treatment. C-Myc and β-Tubulin were visualized from each time point sample with αV5 and αβ-Tubulin by western analysis. C-Myc protein levels were quantified relative to β-Tubulin levels and graphed as percent c-Myc protein remaining after cyclohexamide treatment. Protein half-life was calculated using Excel (Microsoft) graphing function.

B56α associates with c-Myc. Although previous experiments presented here and elsewhere (Yeh et al. 2004) have demonstrated that manipulating PP2A function can affect c-Myc protein levels, no one has experimentally demonstrated an association between c-Myc and PP2A. We therefore initially characterized the general interaction between PP2A and c-Myc. Cells were cotransfected with an expression vector for c-Myc and either PP2A-HA-C or empty control. As shown in Figure 2.4A, c-Myc coimmunoprecipitated with anti-HA antibody only in the presence of the PP2A-HA-C subunit (compare lanes 1 and 2, upper panel). I also coimmunoprecipitated PP2A-HA-C with c-Myc. HIS_6-tagged c-Myc, c-Myc$^{\Delta TAD}$ (deleted for the trans-activation domain) or control Ni-NTA columns were generated and incubated with cellular lysates containing PP2A-HA-C. As shown in Figure 2.4B, the PP2A-HA-C subunit was pulled out by the c-Myc column (lane 2, top panel), but not by either control or c-Myc$^{\Delta TAD}$ columns (lanes 1 and 3, respectively). This not only confirms that PP2A and c-Myc associate, but also shows that the interaction occurs through the N-terminal trans-activation domain of c-Myc. Importantly, the trans-activation domain of c-Myc contains the highly conserved S62 residue that we have previously shown to be dephosphorylated by PP2A (Yeh et al. 2004).

Given that c-Myc associates with PP2A, we asked whether the interaction is mediated by one or more specific regulatory B subunit(s). We focused on the B56 family of regulatory subunits as suggested by the E4orf4 and RNAi

experiments. I coexpressed c-Myc with B56α, -β, -γ1, -γ3, -δ1, -ε, or -empty control and then immunoprecipitated the different B56 subunits. As shown in Figure 2.4C, c-Myc clearly coimmunoprecipitated with B56α (lane 2, upper panels), but not with B56β, -γ1, -γ3, -δ1 or -ε (Figure 2.4C, lanes 4, 6, 8, 10, and 12). Furthermore, endogenous PP2A C subunit coimmunoprecipitated with each of the B56 subunits, suggesting that PP2A holoenzymes are pulled down in each case (Figure 2.4C, even lanes, 2nd panel from top). I also immunoprecipitated endogenous c-Myc from 293 cells and showed association with endogenous B56α (Figure 2.4D, lane 3). Moreover, endogenous PP2A-C subunit also coimmunoprecipitated with endogenous c-Myc (lane 3, middle panel). Studies have shown that association between the regulatory B and catalytic C subunits is mediated through the structural A subunit (Ruediger et al. 1992; Ruediger et al. 1994; Kremmer et al. 1997; Strack et al. 2002). Therefore, it is likely that intact trimeric PP2A-B56α holoenzymes associate with c-Myc through the B56α regulatory subunit.

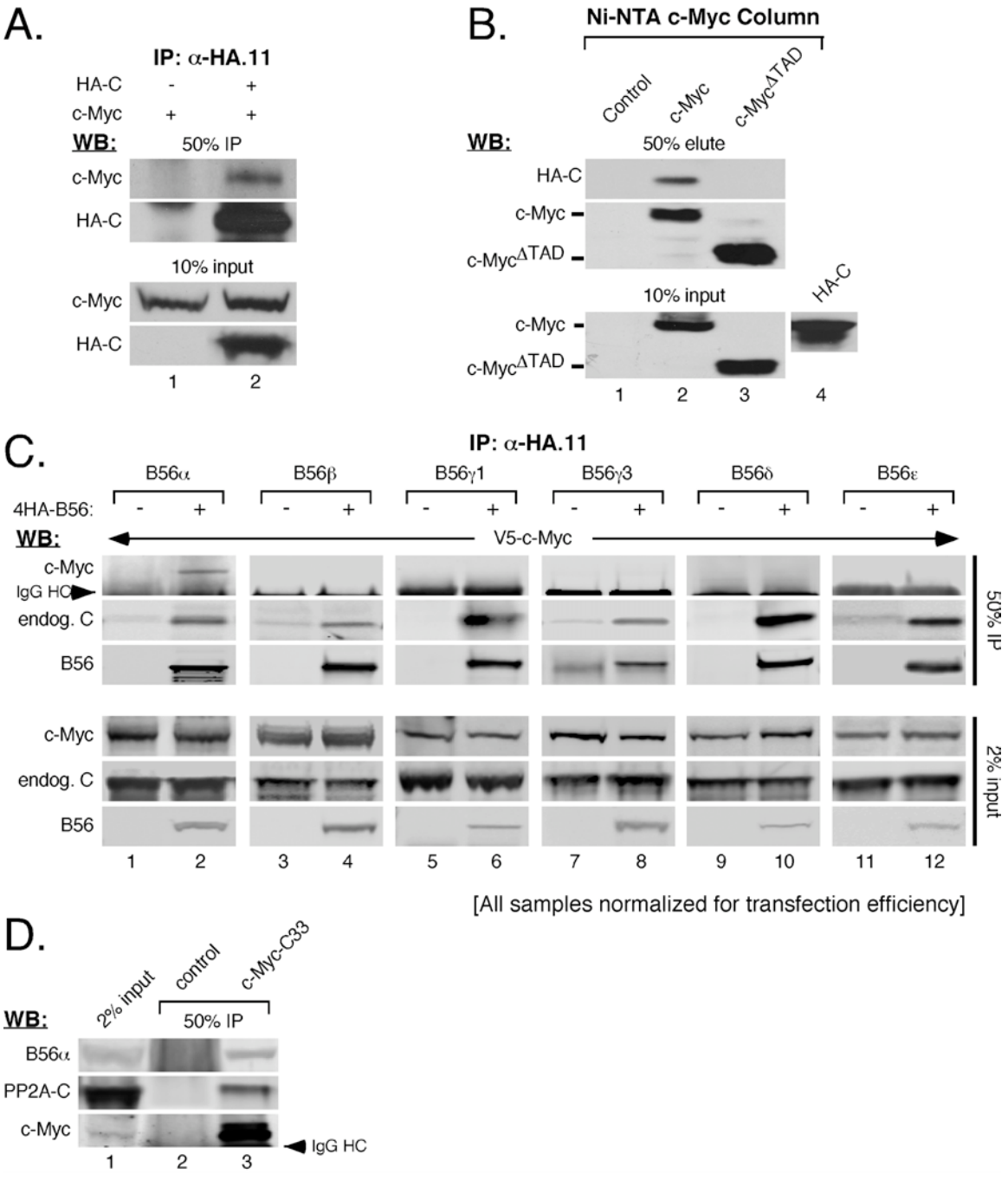

Figure 2.4: c-Myc specifically associates with B56α.

A) C-Myc complexes with PP2A-HA-C subunit. HEK-293 cells were cotransfected with 50ng CMV-βgal, 3ug CMV-Myc and 3ug of either pD30-PP2A-HA-C (+) or CMV-empty (-), as indicated. Cleared lysate volumes were normalized by β-gal activity and subjected to immunoprecipitation (IP) with αHA.11. 10% input volumes and 50% of IP samples were analyzed by western blotting for c-Myc with αN262 and PP2A-HA-C with αHA.11. (B) PP2A-HA-C

associates with the transactivation domain of c-Myc. HEK-293 cells cotransfected with 50ng CMV-βgal and 3ug either pD40-His/V5-control, -c-Myc, or –c-Myc$^{\Delta TAD}$ (input shown in lanes 1-3) were used to generate Ni-NTA-control, -c-Myc, or -c-Myc$^{\Delta TAD}$ columns (see Methods: Ni-NTA columns). . Ni-NT columns were incubated with cleared lysates from HEK-293 cell transfected with pD30-PP2A-HA-C (input shown in lane 4). Column bound proteins were eluted and 50% elute and 10% input samples were subjected to western blot analysis with αV5 for c-Myc and αHA.11 for PP2A-HA-C. (C) PP2A-B56α interacts with c-Myc. HEK-293 cells were cotransfected with 50ng CMV-βgal, 3ug pD40-His/V5-c-Myc and 3ug either CMV-empty (-) or pCEP4HA-B56α, -B56β, -B56γ1, -B56γ3, -B56δ1 or -B56ε, as indicated. Anti-HA.11 immunoprecipitations were carried out on cleared lysates adjusted for β-gal activity . 2% input and 50% IP samples were analyzed by western blotting with αV5 for c-Myc, αrabHA for B56 family members, and αPP2A-Cα for endogenous PP2A-C. (D) Endogenous PP2A-B56α holoenzymes associate with endogenous c-Myc. Endogenous c-Myc was immunoprecipitated from HEK-293cells with agarose-conjugated C-33 antibody. Control immunoprecipitation was done using agarose A+G beads. 2% input and 50% IP samples were subjected to western blot analysis with αPP2A-B'α for endogenous B56α , αN262 for endogenous c-Myc, and αPP2A-Cα for endogenous PP2A-C subunit.

PP2A-B56α association with the transactivation domain of c-Myc is enhanced when Serine 62 dephosphorylation is inhibited. To determine whether B56α interacts with c-Myc near the Serine 62 phosphorylation site, I immunoprecipitated c-Myc^{WT} and c-Myc$^{\Delta TAD}$ from 293 cells cotransfected with B56α. As shown in Figure 2.5A, both B56α and endogenous PP2A-C subunit coimmunoprecipitated with c-Myc^{WT} (top 2 panels, lane 5), but not with c-Myc$^{\Delta TAD}$ compared to control (lanes 4 and 6). These results demonstrate that B56α targets PP2A holoenzymes to the transactivation domain of c-Myc.

The transactivation domain of c-Myc contains the T58 and S62 residues that coordinately regulate c-Myc degradation, and PP2A dephosphorylates S62.

Therefore, I tested whether the phosphorylation status of T58 or S62 could affect the interaction between PP2A-B56α and c-Myc. B56α was immunoprecipitated from 293 cells co-expressing c-Myc^{WT} or c-Myc point mutants: T58A, S62A, T58E, or S62D. Immunoprecipitates were washed under stringent conditions to challenge the interaction. As shown in Figure 2.5B, c-Myc^{WT} and all four point-mutants coimmunoprecipitated with B56α to varying degrees (top panel, even lanes). Since the S62A mutant lacks phosphorylation at both T58 and S62 (Sears et al. 2000), this result suggests that phosphorylation at T58 and/or S62 is not essential for PP2A-B56α association with c-Myc. On the other hand, both c-Myc^{T58A} and the S62 phosphorylation mimic, c-Myc^{S62D}, showed a stronger association with B56α (Figure 2.5B, top panel, lanes 4 and 10, respectively). Previous studies have demonstrated that c-Myc^{T58A} shows substantially higher S62 phosphorylation levels than c-Myc^{WT} and *in vitro* assays show that PP2A fails to dephosphorylate c-Myc^{T58A} (Lutterbach and Hann 1994; Sears et al. 2000; Yeh et al. 2004). Thus, the inability of PP2A to dephosphorylate S62, as occurs with c-Myc^{T58A} and the phosphorylation mimic c-Myc^{S62D} mutants, appears to create a “substrate trap” where relatively more P-S62 c-Myc remains bound to PP2A-B56α holoenzymes.

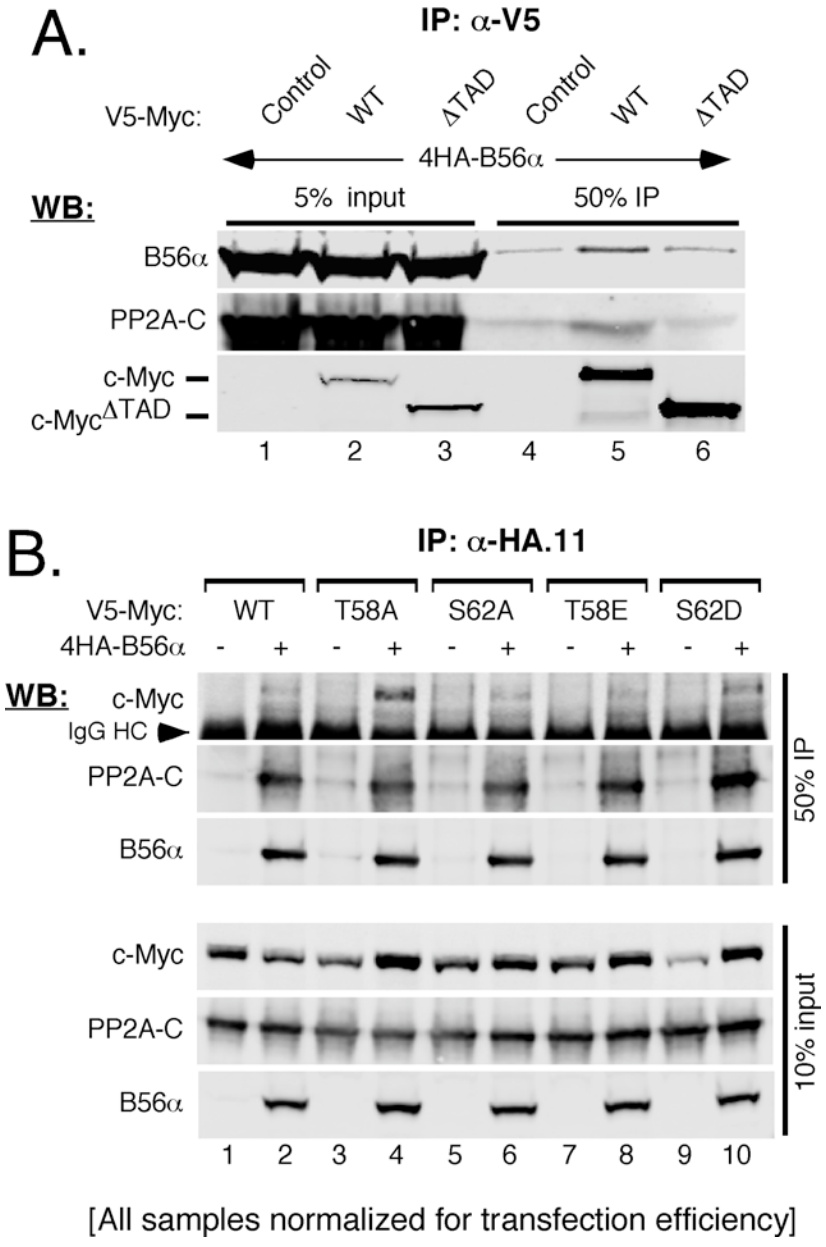

Figure 2.5: PP2A-B56α holoenzyme associates with the transactivation domain of c-Myc.

(A) PP2A-B56α requires the transactivation domain of c-Myc for association. HEK-293 cells were cotransfected with 50ng CMV-βgal, 3ug pCEP4HA-B56α, and 3ug of either pD40-His/V5-contol, -c-Myc^{WT}, or -c-Myc$^{\Delta TAD}$, as indicated. Cleared lysates were collected 36 hours post-transfection, normalized for transfection efficiency by β-gal activity and immunoprecipitations were performed with αV5. 5% Input and 50% IP samples were subjected to western blot analysis with αV5 for c-Myc, αrabHA for B56α, and αPP2A-Cα for endogenous PP2A-C. (B) PP2A-B56α shows a stronger association with c-Myc point mutants T58A and

S62D. HEK-293 cells were cotransfected with 50ng CMV-βgal, 3ug of either CMV-control (-) or pCEP-4HAB56α (+), and 3ug pD40-His/V5-c-Myc^{WT} or pD40-His/V5-c-Myc point mutants: T58A, S62A, T58E, and S62D, as indicated. Lysates were prepared and normalized as in A and subjected to immunoprecipitation with αHA.11. Immunoprecipitates were washed with a stringent buffer (see Coimmunoprecipitation, Methods). 10% Input and 50% IP samples were analyzed by western analysis with αV5 for c-Myc, αrabHA for B56α, and αPP2A-Cα for endogenous PP2A-C.

Combined expression of the catalytic and B56α subunits dramatically reduces c-Myc protein levels. In Figure 2.1, I demonstrated that increasing PP2A activity by expressing PP2A-HA-C had limited activity toward reducing c-Myc protein levels. Now that I have identified a specific PP2A holoenzyme, PP2A-B56α, that associates with c-Myc, I assessed the effects of increasing expression of this holoenzyme on c-Myc protein levels. As shown in Figure 2.6, expression of either B56α or PP2A-HA-C alone showed only a slight, but consistent reduction in c-Myc protein levels compared to control (top panel, lanes 1-3 and graph). Expression of the PP2A-A subunit alone generally resulted in increased c-Myc protein levels (lane 4). Other reports have also observed aberrant affects from overexpressing PP2A-A which could explain my results (Wera et al. 1995). However, the combination of B56α with PP2A-HA-C dramatically reduced c-Myc protein levels to about 20% compared to control (compare lanes 1 and 5 and graph). Coexpression of all three B56α, HA-tagged C, and PP2A-A subunits together also significantly reduced c-Myc protein levels to 45% (data not shown). Taken together, these results demonstrate that

increasing PP2A-B56α holoenzyme levels can substantially reduce c-Myc protein expression.

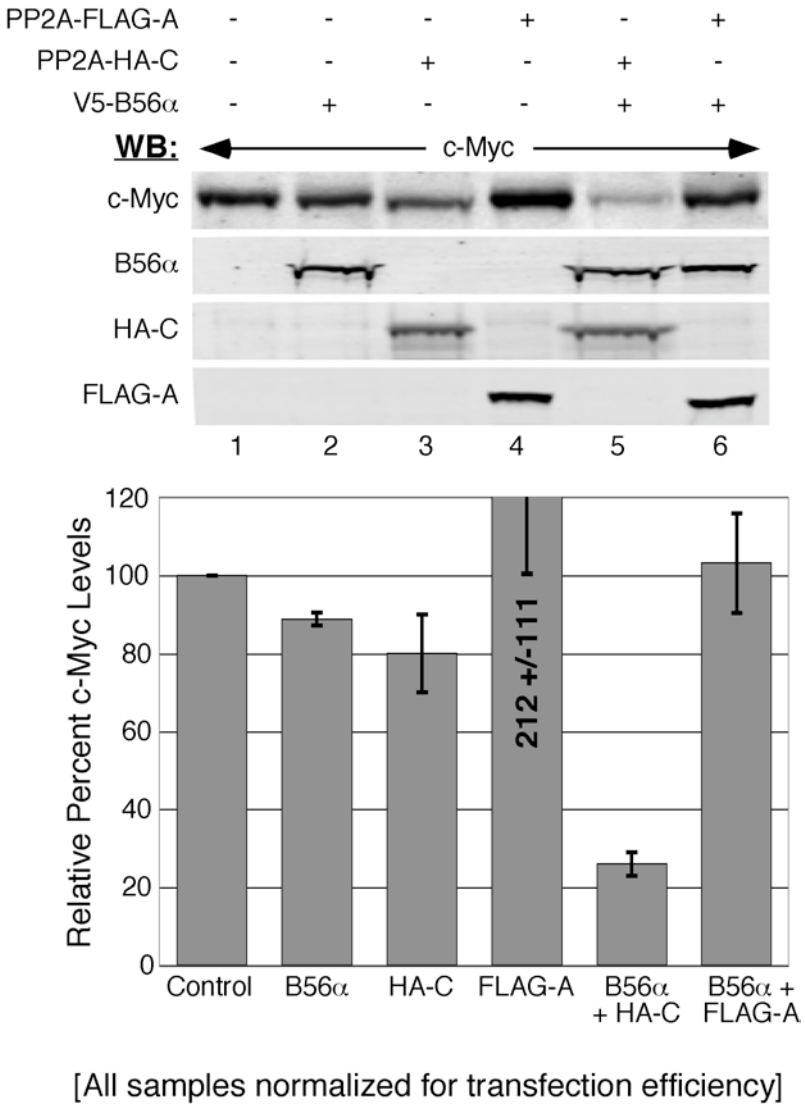

Figure 2.6: Increased PP2A-B56α activity reduces c-Myc protein levels.

HEK-293 cells were cotransfected with 50ng CMV-βgal, 1ug CMV-Myc and 1ug of pD30-PP2A-FLAG-A, pD30-PP2A-HA-C, or pD40-His/V5-B56α, as indicated. Whole cell lysates were collected 48hrs post-transfection, normalized for transfection efficiency by β-gal activity and samples were subjected to western blot analysis with αN262 for c-Myc, αM2-FLAG for PP2A-A, αHA.11 for PP2A-C, and αV5 for B56α. C-Myc protein levels were quantified from three separate experiments and average protein levels with error bars were graphed relative to control protein levels (see Methods, Western blotting and Quantitation).

Reduced expression of B56α increases the level of Serine 62 phosphorylated c-Myc. We have previously observed that global inhibition of PP2A activity by expression of SV40 small T causes an increase in S62 phosphorylated c-Myc (unpublished data). I investigated whether knock-down of B56α alone could affect the phosphorylation status of c-Myc at S62. Consistent with our previous findings, knockdown of B56α resulted in higher total c-Myc protein levels compared to control (Figure 2.7A, bottom panel, compare lanes 1 and 2). Using phospho-specific antibodies, I also observed a significant increase in both S62 and T58 phosphorylated c-Myc (top and middle panels). In contrast, knockdown of the other B56 family members did not significantly affect total c-Myc levels, nor S62 and T58 phosphorylation levels (Figure 2.7A, lanes 3-6). The concurrent increase of c-Myc T58 phosphorylation with B56α knockdown is not surprising since the degradation of c-Myc is blocked prior to dephosphorylation on S62 by PP2A, but after GSK3β phosphorylates c-Myc on T58, and thus doubly phosphorylated c-Myc accumulates. By simultaneously probing for total and phospho-c-Myc I was able to quantitate the relative increases in S62 and T58 phosphorylation with respect to total c-Myc. As shown in Figure 2.7B, I observed a significant enrichment in both S62 and T58 phosphorylated c-Myc species relative to total c-Myc with an average increase of 1.8- and 1.4-fold, respectively, upon B56α knockdown. A near doubling in the population of c-Myc that is S62 phosphorylated could significantly affect c-Myc function.

Knockdown of PP2A-B56α enhances c-Myc-dependent transcriptional activity. C-Myc is a transcription factor that positively regulates the transcription of a number of target genes critical for cellular proliferation, growth, and differentiation (Dang et al. 1999). Consequently, we asked whether the increase in c-Myc expression observed upon knockdown of B56α has functional significance. To asses c-Myc-driven transcription, I used two luciferase reporter plasmids that contain the E2F2 promoter, which either have consensus E-box c-Myc binding sites (E2F2-Luc) or have point mutations in these binding sites that prevent c-Myc binding (E2F2(-E-box)-Luc) (Sears et al. 1997). As shown in Figure 2.7C, knockdown of B56α again resulted in significantly increased c-Myc protein levels (lane 2) compared to knockdown of the other B56 family members and shRNA control (lanes 1 and 3-6). Importantly, this increased accumulation of c-Myc was accompanied by a statistically significant increase in E2F2-Luc reporter activity over that seen with c-Myc when B56α is not knocked down (Figure 2.7C, graph, E2F2-Luc, B56α compared to (-)). In contrast, the negative control E2F2(-E-box)-Luc reporter plasmid that does not support c-Myc binding, did not show a statistically significant change in activity when B56α was knocked down (Figure 2.7C, E2F2(-E-box)-Luc, B56α compared to (-)). The results demonstrate that the B56α effect on E2F2 promoter activity is c-Myc-dependent. In contrast, knockdown of the remaining B56 family members showed only minor changes in the activity of either E2F2-Luc or E2F2(-E-box)-Luc (Figure 2.7C,

B56β,-γ,-δ, and -ε). I also examined the effect of knocking down B56α on endogenous c-Myc driven transcription of the *E2F2* gene. Figure 2.7D shows a 1.9 fold increase in E2F2 mRNA levels when B56α (lane 2) is knocked down compared to control (lane 1). As a positive control knockdown of PP2A-A (lane 3) resulted in a 2.1 fold increase in E2F2 mRNA levels compared to control (lane 1). Interestingly, the ~2 fold increase in c-Myc-dependent E2F2 promoter activation with B56α knockdown is consistent with increases seen in c-Myc-driven transcription when c-Myc is stabilized by inhibition of PP2A activity with SV40 small T (Yeh et al. 2004). Based on these results, we conclude that PP2A-B56α negatively regulates c-Myc protein levels and this level of control is important for regulating c-Myc activity. In the absence of this control, aberrant expression of functional c-Myc can occur.

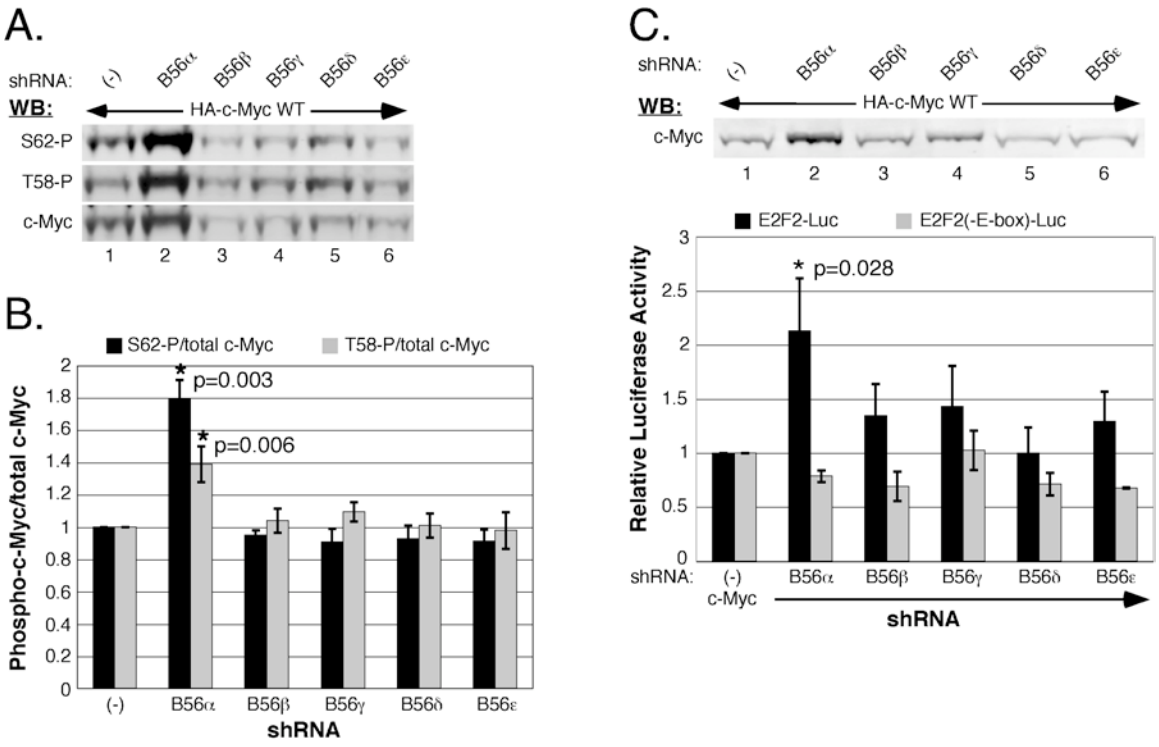

Figure 2.7: Knockdown of PP2A-B56α increase S62 and T58 phosphorylated c-Myc as well as c-Myc-driven transcription.

(A) C-Myc S62 and T58 phosphorylation increases upon PP2A-B56α knockdown. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug CMV-Myc-HA-WT, and 1.5ug of pSUPER-shRNA expression vector empty (-) or targeted to B56α B56β, B56γ, B56δ, or B56ε, as indicated. Whole cell lysates were collected 72hrs post-transfection. Samples were normalized for β-gal activity and analyzed by western blots simultaneously probed with αHA.11 for total c-Myc and either αT58phospho for T58 phosphorylation or αS62phospho for S62 phosphorylation. Anti-HA.11 was detected with secondary anti-mouse Alexa Fluor 680 and αT58phospho or αS62phospho were detected with secondary anti-rabbit IRDye800. Westerns shown are representative of S62 phosphorylation, T58 phosphorylation and total c-Myc protein levels from three separate experiments. (B) S62 and T58 phosphorylated c-Myc is enriched upon B56αknockdown. S62 or T58 phosphorylation levels along with total c-Myc levels, from simultaneously probed westerns, were quantitated using LI-COR software (see Materials and Methods, Western Blotting and Quantitation). Ratios of S62 phosphorylation/total c-Myc and T58 phosphorylation/total c-Myc were calculated. Average ratios with error bars from the experiment shown in A, and 2 repeat experiments were graphed relative to control (-) levels and statistical significant differences are indicated (*) (C) B56α knockdown selectively increases c-Myc-driven transcription. HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug CMV-HA-c-Myc, and 10ng of either E2F2-Luc or E2F2(-E-

box)-Luc, along with 1.5ug pSUPER-shRNA expression vector empty (-) or targeted to B56α B56β, B56γ, B56δ, or B56ε, as indicated. Whole cell lysates were collected 72hrs post-transfection. C-Myc protein levels are shown by western analysis with αHA.11. Luciferase activity was measured and corrected for transfection efficiency based on β-gal activity. Average corrected luciferase activity from three separate experiments was graphed with error bars and statistically significant differences are indicated (*). (D) Knockdown of B56α increases endogenous *E2F2* mRNA levels. HEK-293 cells were cotransfected with 50ng CMV-βgal and 3ug pSUPER-empty, pSUPER-B56α or pSUPER-PP2A-A under 10% FBS serum conditions for 24hrs and then starved in 0.2% FBS serum for 48hrs. Cells exhibiting transfection efficiencies by β-gal assay within 5% of each other were used for RT-PCR analysis of endogenous *E2F2*, c-*myc*, and *GAPDH* mRNA levels.

Discussion:

Our previous work demonstrated a prominent role for PP2A in regulating c-Myc protein levels and activity (Yeh et al. 2004). Given the significant number of regulatory roles assigned to PP2A, identifying the specific PP2A holoenzyme involved in regulating c-Myc gives us valuable insight into the control of c-Myc oncogenicity as well as further understanding of how PP2A can function as a tumor suppressor. In this report, we present data identifying a specific PP2A holoenzyme containing the regulatory B subunit, B56α, that interacts with the transactivation domain of c-Myc containing the S62 residue we previously demonstrated to be dephosphorylated by PP2A. Furthermore, we show that the PP2A-B56α holoenzyme negatively regulates c-Myc protein stability and function, demonstrating the importance of this interaction with respect to c-Myc biology.

PP2A function toward c-Myc is regulated by trimeric holoenzyme formation. It has previously been shown that ectopic expression of the PP2A-HA-C subunit in HEK-293 cells increases cellular PP2A activity (Al-Murrani et al. 1999). However, whether PP2A-HA-C requires trimeric PP2A holoenzyme formation to function has not been fully elucidated. Some studies have demonstrated that free PP2A-C and the dimeric catalytic core, PP2A-A/C, can dephosphorylate known PP2A targets *in vitro* (Yang et al. 1991; Turowski et al. 1997). However, other reports show that the regulatory B subunit is required for

PP2A substrate-specificity and greatly increases PP2A activity towards known targets (Agostinis et al. 1992; Mayer-Jaekel et al. 1994; Turowski et al. 1999; Voorhoeve et al. 1999; Yan et al. 2000; Chen et al. 2005). Our findings show that in order to affect c-Myc accumulation, ectopically expressed PP2A-HA-C must incorporate into PP2A holoenzymes. Moreover, our results suggest that holoenzyme formation directed toward c-Myc is limited, presumably due to low levels or limited accessibility of the specific regulatory B subunit, B56α. Accordingly, we show that manipulation of B56α expression levels can dramatically affect c-Myc accumulation.

PP2A holoenzyme formation has been strongly implicated in cellular tumor suppressor activity. For example, there are a number of PP2A-A subunit mutations reported in a variety of cancers that disrupt PP2A holoenzyme formation (Wang et al. 1998; Ruediger et al. 2001a; Ruediger et al. 2001b). Additionally, inhibition of PP2A holoenzyme assembly by expression of SV40 small T has been shown to be a critical step in the experimental conversion of primary human cells to a transformed tumorigenic state (Pallas et al. 1990; Rundell and Parakati 2001; Yu et al. 2001; Hahn et al. 2002). Interestingly, the requirement for PP2A inhibition in this assay can be circumvented by expression of a stable c-Myc mutant that is resistant to PP2A mediated dephosphorylation and degradation (Yeh et al. 2004). These findings highlight the importance of PP2A holoenzyme formation for tumor suppressor activity and reveal the critical nature of PP2A regulation of c-Myc for normal cellular function.

B56α targets c-Myc and is a key regulator of PP2A tumor suppressor activity. The B56 family of PP2A regulatory B subunits is diverse, with each member displaying distinct tissue expression, developmental expression and subcellular localization patterns (McCright and Virshup 1995; Martens et al. 2004). In general, the B56 family members have been regarded as tumor suppressors (Van Hoof and Goris 2004). To date, a great deal of attention has focused on the B56γ isoforms. A N-terminal deletion mutant of B56γ1, found in a mouse melanoma cell-line, has been shown to result in increased metastasis by deregulation of paxillin (Ito et al. 2000), and inhibition of the gamma-irradiation cell cycle check point, contributing to genomic instability (Ito et al. 2000; Ito et al. 2003). B56γ3 is reported to regulate Chk2 and p300 protein levels (Dozier et al. 2004; Chen et al. 2005) as well as to be involved in cell cycle control by regulating Mdm2 and p53, through its association with cyclin G (Okamoto et al. 1996; Okamoto et al. 2002).

Although the B56γ subunits are clearly tumor suppressors, knockdown of the B56γ subunits only recapitulates the requirement for SV40 small T in cellular transformation of primary human cells after a significantly longer period of time (Chen et al. 2004). This result suggests that other PP2A holoenzymes targeted by SV40 small T play an essential in the transformation of human cells. In this regard, our results showing that c-Myc is a target of PP2A-B56α, identifies

another such PP2A holoenzyme targeted by SV40 small T with tumor suppressor function.

The B56α regulatory subunit is reported to be the most ubiquitously expressed isoform of the B56 family (McCright and Virshup 1995; Martens et al. 2004), making it an ideal regulatory B subunit to target PP2A activity towards c-Myc, since c-Myc is also ubiquitously expressed. Although there are no known mutations in B56α in human cancers, two mutations identified in the PP2A-A subunit from human tumors have been shown to specifically disrupt the binding of B56α to PP2A-A, selectively inhibiting PP2A-B56α holoenzyme formation (Ruediger et al. 2001b). In addition, several other important cellular regulatory proteins have been shown to be targeted by B56α. The anti-apoptotic protein Bcl2 has been shown to be inactivated by B56α (Ruvolo et al. 2002), and B56α plays a crucial role in negatively regulating protein levels of the Wnt-signaling transcription factor, β-catenin (Seeling et al. 1999; Li et al. 2001). Discovery that c-Myc is a B56α target, places B56α at a critical junction point for regulating multiple potent oncoproteins.

Interplay between the oncogenic activity of c-Myc and the tumor suppressor function of PP2A-B56α. Despite its critical role in regulating multiple oncoproteins, B56α transcript levels are found to be relatively low in most tissues, and data presented here suggests that B56α protein is limiting toward c-Myc (Martens et al. 2004). Presumably, under normal or quiescent

cellular conditions, when c-Myc and β-catenin proteins levels are low and Bcl2 is not activated, these low levels of B56α are sufficient. However, aberrant deregulation of any one of these oncoproteins could sequester the majority of B56α and thereby cause deregulation of the other oncoproteins regulated by PP2A-B56α. In fact, ΔNp63α, an oncogenic form of the p53 family member, p63, that is overexpressed in squamous cell carcinomas, has been reported to associate with B56α, and this results in the accumulation of β-catenin (Patturajan et al. 2002). Our finding that c-Myc^{T58A} has a stronger association with PP2A-B56α than wild-type c-Myc, could act in a similar manner as ΔNp63, explaining in part the increased oncogenicity of c-Myc^{T58A} verses c-Myc^{WT} (Henriksson et al. 1993; Pulverer et al. 1994; Sears et al. 2000). Furthermore, c-Myc^{T58A} shows reduced apoptosis compared to c-Myc^{WT}, which could be facilitated by the efficient sequestering of PP2A-B56α by c-Myc^{T58A}, thus preventing B56α-mediated inactivation of the anti-apoptotic Bcl2 (Chang et al. 2000; Hemann et al. 2005).

Acknowledgements

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Chapter Three :

Axin1 Coordinates a c-Myc Degradation Complex

Abstract:

Both c-Myc and Axin1 have been shown to be critical for normal development as homozygous disruption/deletion of either *c-myc* or *axin1* result in embryonic lethality. Interestingly, c-Myc and Axin1 oppose each other in tumorogenisis where c-Myc is a potent oncoprotein and Axin1 is a critical tumor suppressor. We report here that this opposition between c-Myc and Axin1 occurs in part through a direct mechanism in which Axin1 coordinates a degradation complex for c-Myc. This complex includes GSK3β, PP2A-B56α and Pin1, all of which have been shown to play critical roles in negatively regulating c-Myc protein levels by negatively regulating c-Myc protein stability. Not surprisingly, I found that shRNA knockdown of Axin1 increases c-Myc protein stability and consequently c-Myc protein levels. Furthermore, expression of Axin1 acutely reduces c-Myc protein levels prior to any affects seen through Axin1 regulation of β-catenin/Tcf/Lef transcription, which has previously been shown to affect *c-myc* mRNA levels. Importantly, expression of two naturally occurring variants of Axin1 are potent suppressors of c-Myc dependent transcription, demonstrating that Axin1 can negatively regulate c-Myc oncogenic function. Our identification of an Axin1 mediated degradation complex for c-Myc greatly expands our understanding of an important tumor suppressor, Axin1, and the regulation of a potent oncoprotein, c-Myc.

Introduction:

C-Myc is a transcription factor that regulates the expressions of numerous genes involved in the regulation of cellular proliferation, growth, apoptosis, and differentiation. Importantly, c-Myc is required for development as demonstrated by embryonic lethality in mice with homozygous deletion of *c-myc* (Davis et al. 1993). Conversely, c-Myc is a potent oncoprotein as demonstrated in mice with inducible *c-myc* transgenes, which develop neoplastic pre-malignant and malignant phenotypes that often spontaneously regress upon withdrawal of c-Myc expression (Felsher and Bishop 1999; Pelengaris et al. 1999). These findings demonstrate the necessity for proper regulation of c-Myc expression, which occurs at multiple levels through transcription, translation, and post-translational mechanisms. Interestingly, 70% of human tumors exhibit overexpression of c-Myc protein with only 20% of these showing amplification or translocation of the *c-myc* gene, which could account for transcriptional overexpression of c-Myc (Nesbit et al. 1999). These findings suggest that translational and post-translational regulatory mechanisms are very important for maintaining proper c-Myc expression.

Work by our lab and others have characterized a highly conserved, sequential series of reversible phosphorylation events that regulate c-Myc protein stability. Normally, c-Myc protein half-life is very short as c-Myc protein turnover via ubiquitination and proteosomal degradation occurs rapidly allowing quiescent cells to maintain low c-Myc protein levels. However, in response to serum

stimulation, quiescent cells enter the cell cycle and through the Ras/Raf/MEK/ERK kinase cascade c-Myc protein can be stabilized by phosphorylation on Serine 62 (S62) (Sears et al. 1999). Concurrently Ras can activate the PI3K/AKT pathway to inhibit GSK3β, which has been shown to phosphorylate c-Myc on Threonine 58 (T58) following S62 phosphorylation (Sears et al. 2000). In late G_1 Ras activity diminishes and the suppression of GSK3β activity is relieved allowing T58 phosphorylation to occur. This promotes the prolylisomerase, Pin1, to convert Proline 63 in c-Myc from a *cis* to *trans* conformation setting c-Myc up for protein phosphatase type 2A, specifically PP2A-B56α, to remove the stabilizing S62 phosphorylation (Yeh et al. 2004; Arnold and Sears 2006). Singly T58 phosphorylated c-Myc has been shown to be recognized by the SCF^{Fbw7} ubiquitin machinery, which multi-ubiquitinates c-Myc marking it for degradation by the 26S proteosome (Welcker et al. 2004; Yada et al. 2004).

Axin1 is a multi-domain scaffold protein that coordinates several different protein complexes that are involved in regulating Wnt, TGFβ, SAPK/JNK, and p53 signaling (Zeng et al. 1997; Hart et al. 1998; Ikeda et al. 1998; Kishida et al. 1998; Kishida et al. 1999; Sakanaka and Williams 1999; Zhang et al. 1999; Ikeda et al. 2000; Neo et al. 2000; Furuhashi et al. 2001; Zhang et al. 2001; Levina et al. 2004; Rui et al. 2004; Liu et al. 2006; Marikawa 2006; Li et al. 2007; Lin and Li 2007). Similar to deletion of *c-myc*, homozygous disruption of full-length *fused/axin1* results in embryonic lethality at day E9.5 due to truncation of the

forebrain, neural tube defects, and axis duplication in mice (Perry et al. 1995; Vasicek et al. 1997). Thus far Axin1 has been characterized to be a tumor suppressor as numerous gene mutations have been identified throughout *axin1* from a number of different cancers. These mutations likely compromise the ability of Axin1 to form complexes with DVL, MEKK, GSK3β, PP2A, APC, β-catenin, and even Axin1 with itself (Satoh et al. 2000; Webster et al. 2000; Dahmen et al. 2001; Moreno-Bueno et al. 2002; Shimizu et al. 2002; Taniguchi et al. 2002; Baeza et al. 2003; Jin et al. 2003; Miao et al. 2003; Daa et al. 2004; Zhou and Gao 2007). Importantly, it has been shown in some cases of hepatocellular cancers (HCCs) harboring mutations in *axin1*, re-introduction of wild-type Axin1 expression resulted in increased apoptosis (Satoh et al. 2000). This strongly suggests that Axin1 maybe an important molecular target in HCC as well as other cancers with compromised Axin1 function. Altogether, these studies highlight the importance of maintaining normal expression of wild-type Axin1.

One the most well characterized functions for Axin1 is in negatively regulating Wnt signaling by forming a degradation complex for β-catenin that contains GSK3β and APC (Hart et al. 1998; Ikeda et al. 1998). It has been shown that Axin1 expression can inhibit β-catenin/TCF/Lef transcription (Sakanaka et al. 1998). Interestingly, it has also been shown that *c-myc* is a target gene of β-catenin/TCF/Lef transcription (He et al. 1998) and in this manner Axin1 can negatively regulate c-Myc expression. Furthermore, analysis of some

human tumors revealed a correlation between increased β-catenin protein levels and increased *c-myc* expression (Shiina et al. 2002; Ozaki et al. 2005). However, studies of other human tumors showed that aberrant β-catenin/TCF/Lef transcription in human tumors does not always result in increased c-Myc protein levels despite an increase in *c-myc* expression (Wang et al. 2002). These findings suggest that the regulation of c-Myc expression likely occurs at multiple levels.

We now report an additional regulatory role for Axin1 to negatively regulate c-Myc protein levels at the posttranslational level. I find that Axin1 associates with GSK3β, PP2A-B56α, Pin1, and c-Myc to form a degradation complex for c-Myc analogous to that formed for β-catenin by Axin1 mediated coordination of GSK3β, APC and β-catenin. Furthermore, I show that GSK3β and PP2A association with Axin1 as well as Pin1 activity are important for the formation of the Axin1 mediated c-Myc degradation complex. Importantly, I show that acute Axin1 expression negatively regulates c-Myc protein levels, but does not affect *c-myc* mRNA levels. In contrast, shRNA-mediated knock-down of Axin1 expression stabilizes c-Myc and increases its accumulation. Lastly, we have found a mutant form of Axin1 from pre-B ALL SupB15 cells with an in-frame deletion within the GSK3β binding domain that disrupts GSK3β association with Axin1. I show that GSK3β association with Axin1 is critical for the ability of Axin1 to suppress c-Myc dependent transcription thereby inhibiting an important function of c-Myc oncogenicity. Altogether, our results add to our understanding

as to how Axin1 acts as a critical tumor suppressor by negatively regulating the potent oncoprotein, c-Myc, at least in part through promoting its turnover.

Results:

Axin1 associates with c-Myc, GSK3β, PP2A-B56α, and Pin1. Due to the high level of regulation and rapid turnover of c-Myc protein I hypothesized that some of the proteins involved in regulating c-Myc protein turnover might be organized into a complex by associating with a scaffolding protein. We along with other groups have previously reported critical roles for GSK3β and PP2A-B56α in the negative regulation of c-Myc protein levels through the regulation of T58 and S62 phosphorylation (Lutterbach and Hann 1994; Pulverer et al. 1994; Sears et al. 2000; Yeh et al. 2004; Arnold and Sears 2006). These key players in c-Myc protein turnover helped to focus our attention to relevant scaffold proteins. Of particular interest was the scaffold protein Axin1, which has been shown to associate with GSK3β and PP2A-B56α (Behrens et al. 1998; Fagotto et al. 1999; Li et al. 2001). To examine whether Axin1 associates with c-Myc I transiently transfected 293 cells with expression vectors for c-Myc and either V5-empty or V5-Axin1. I then immunoprecipitated V5-Axin and found that c-Myc coimmunoprecipitated in the presence, but not absence of V5-Axin1 (Figure 3.1A). Not surprisingly, I found that endogenous GSK3β and PP2A-B56α also coimmunoprecipitated with V5-Axin1 (Figure 3.1A) as both have previously been reported to associate with Axin1. Interestingly, I also found the prolyl-isomerase, Pin1, which we previously reported to have an important role in negatively regulating c-Myc protein levels (Yeh et al. 2004) coimmunoprecipitated with V5-Axin1 (Figure 3.1A). To confirm the association between c-Myc and Axin1, I

performed the reverse immunoprecipitation by co-expressing V5-Axin1 and either HA-c-Myc or empty control in 293 cells. After αHA immunoprecipitation I found that V5-Axin1 coimmunoprecipitated in the presence of HA-c-Myc, but not in the negative control (data not shown). These results show that Axin1 associates with c-Myc, GSK3β, PP2A-B56α and Pin1.

I next assessed whether endogenous c-Myc and Axin1 associated and found that endogenous Axin1 coimmunoprecipitated with endogenous c-Myc, but not with another endogenous transcription factor, Sp1 (Figure 3.1B). I also found that endogenous GSK3β, PP2A-B56α, and Pin1 coimmunoprecipitated with c-Myc, but not with Sp1 (Figure 3.1B). Although Sp1 and c-Myc have been shown to bind to the promoter region of hTERT and cooperate to drive the expression of hTERT (Kyo et al. 2000), it appears that c-Myc and Sp1 protein levels are regulated by different mechanisms and I do not observe their direct interaction in this assay. These findings demonstrate that Axin1 and c-Myc associate at endogenous levels and suggest that Axin1 coordinates the formation of a complex comprised of GSK3β, PP2A-B56α and Pin1 to promote turnover of c-Myc protein.

Recently it has been proposed that other proteins containing the same or similar sequence of amino acids that control c-Myc protein stability and turnover, would be candidates for degradation by similar mechanism as c-Myc (Escamilla-Powers and Sears 2007). One such protein is cyclin E, which has been shown to be subject to ubiquitin mediated turnover (Clurman et al. 1996). Moreover, cyclin

E is regulated in a similar manner as c-Myc by Ras signaling and the E3/F-box protein, Fbw7 (Minella et al. 2005). I therefore tested whether cyclin E associated with Axin1 by cotransfecting 293 cells with CMV-driven cyclin E and either control or V5-Axin1. Cyclin E was coimmunoprecipitated by αV5 in the presence of V5-Axin1, but not in the absence of V5-Axin1 (Figure 3.1C). This finding suggests that other proteins containing a similar degron sequence as c-Myc could be Axin1 target proteins.

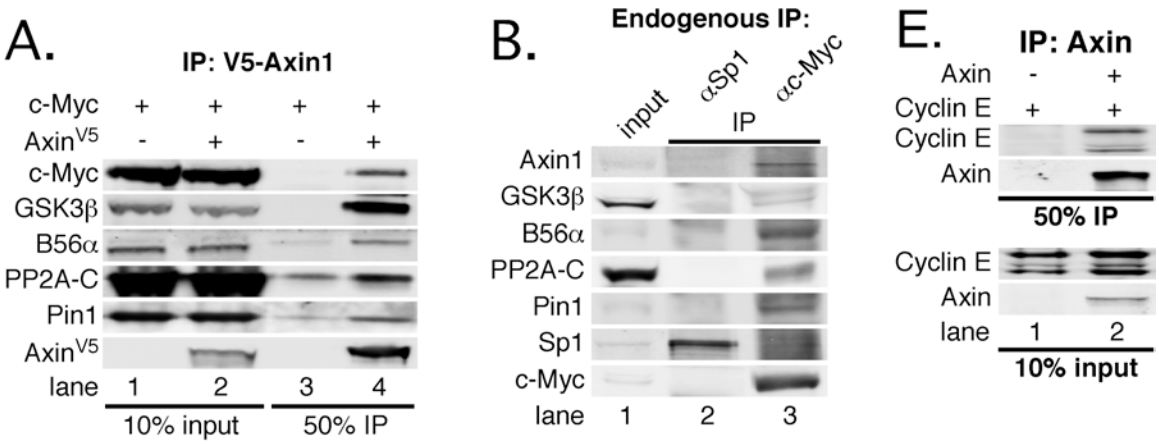


Figure 3.1: Axin1 associates with c-Myc, GSK3β, PP2A-B56α, and Pin1.

(A) Ectopically expressed c-Myc coimmunoprecipitates with Axin1 along with endogenous GSK3β, PP2A-B56α and Pin1. 293 cells were transiently transfected with CMV-driven V5-Axin and c-Myc as indicated as well as CMV-β-gal. Cleared lysates were collected, volumes adjusted for β-gal activity, and immunoprecipitated with αV5. Inputs and immunoprecipitates were analyzed by western blot with the indicated antibodies. (B) Endogenous Axin1 associates with c-Myc, but not with another transcription factor, Sp1. Cleared lysates were immunoprecipitated with αSp1 for endogenous Sp1 or αC33 for endogenous c-Myc. Inputs and immunoprecipitates were analyzed by western blot with indicated antibodies. (C) Axin1 associates with Cyclin E, containing a similar degron sequence as c-Myc. 293 cells were cotransfected with CMV-driven β-gal and cyclin E plus either control or V5-Axin1. Cleared lysates were collected, volumes adjusted for β-gal activity, and subjected to immunoprecipitation with αV5. Input and IP samples were analyzed by western blot analysis with the indicated antibodies.

Knockdown of Axin1 results in increased c-Myc protein levels. To test whether Axin1 negatively regulates c-Myc protein levels I designed an shRNA to Axin1 and confirmed the ability of this shRNA to knockdown Axin1 by co-expressing V5-Axin1 with either pE-H1/TO-scramble or pE-H1/TO-shAxin in 293 cells. As shown in Figure 3.2A (top panel) the shRNA to Axin1 robustly reduces V5-Axin1 protein levels (lane 2) as compared to control (lane 1). I also found that endogenous c-Myc protein levels increase upon knockdown of endogenous Axin by expressing either pE-H1/TO-scramble or pE-H1/TO-shAxin in 293 cells (Figure 3.2A, bottom panel). However, it is possible that knockdown of Axin1 could cause an increase in c-Myc protein levels through an increase in *c-myc* mRNA as it has previously been shown that *c-myc* is a target gene of β-catenin/Tcf/Lef transcription (He, TC 1998) and knockdown of Axin1 would effectively increase β-catenin/Tcf/Lef transcription. I analyzed *c-myc* mRNA levels and found that shRNA mediated knockdown of Axin1 increased *c-myc* mRNA levels as compared to control (data not shown). Therefore, I tested whether ectopic CMV driven HA-c-Myc protein levels were affected by knockdown of Axin1. To date there are no reports that the β-catenin/Tcf/Lef transcription complex affects CMV driven expression. I found that HA-c-Myc protein levels increased significantly upon knockdown of Axin1 (Figure 3.2B, bottom panel). This finding strongly suggests that Axin1 can regulate c-Myc protein levels at the post-translational level.

Axin1 expression promotes c-Myc ubiquitination. My previous experiments suggested that Axin1 regulates c-Myc protein levels through a post-translational mechanism. Therefore, I assessed whether Axin1 regulates c-Myc protein stability by co-expressing CMV driven V5-c-Myc with vector-expressed shRNAs to either scramble (negative control), Axin1, or B56α (positive control) in 293 cells. Again CMV driven V5-c-Myc was used to eliminate the potential affect of β-catenin/Tcf/Lef driven transcription. Transfected cells were maintained for 24 hours in DMEM supplemented with 2% FBS and then treated with 100μg/mL cycloheximide to inhibit protein synthesis. I found that knockdown of Axin1 dramatically increases c-Myc protein stability (>2 hours) as compared to scramble control shRNA (32.2 min.) (Figure 3.2C, compare top V5-c-Myc panel to middle V5-c-Myc panel). As a positive control I also found that c-Myc protein stability increased upon knockdown of B56α (>2 hours) (Figure 3.2C, bottom V5-c-Myc panel), which we previously demonstrated to increase c-Myc protein stability (Arnold and Sears 2006). From this finding we concluded that Axin1 can negatively regulate c-Myc protein levels by negatively regulating c-Myc protein stability.

Axin1 enhances the association of GSK3β and PP2A with c-Myc. Altogether the above findings suggest that Axin1 negatively regulates c-Myc protein levels by promoting the formation of a degradation complex for c-Myc containing GSK3β and PP2A-B56α similar to that coordinated by Axin1 for β-catenin

containing GSK3β and APC (Li et al. 2001; Dajani et al. 2003). To examine whether Axin1 promotes the association of GSK3β and PP2A with c-Myc I coexpressed HA-c-Myc with either pE-H1/TO-scramble or pE-H1/TO-shAxin in 293 cells. Following αHA immunoprecipitation I found that despite a significant increase in HA-c-Myc immunoprecipitated upon knockdown of Axin1 there was less GSK3β and PP2A-C coimmunoprecipitated as compared to control (Figure 3.2B, compare lane 3 to lane 4). This result strongly suggests that Axin1 promotes the association of c-Myc with GSK3β and PP2A.

To confirm my observations of Axin1 enhancing the association between GSK3β and PP2A with c-Myc, I assessed whether overexpression of Axin1 would increase the association of GSK3β, PP2A and additionally Pin1 with c-Myc. I cotransfected 293 cells with CMV-driven HA-Axin1 and V5-c-Myc as indicated in Figure 3.2D and then immunoprecipitated from cleared lysates using αV5. GSK3β, PP2A and Pin1 were coimmunoprecipitated in the presence, but not absence of V5-c-Myc (Figure 3.2D, compare lane 5 to lane 4). Importantly, I found that expression of HA-Axin1 increased the amount of GSK3β, PP2A and Pin1 that coimmunoprecipitated with V5-c-Myc (Figure 3.2D, compare lane 5 and 6). Not surprisingly, I found that HA-Axin1 coimmunoprecipitated with V5-c-Myc, but not in the absence of V5-c-Myc (Figure 3.2D, compare lane 6 to lane 4). These findings demonstrate that Axin1 enhances the association of GSK3β, PP2A and Pin1 with c-Myc thereby coordinating a potential degradation complex for c-Myc.

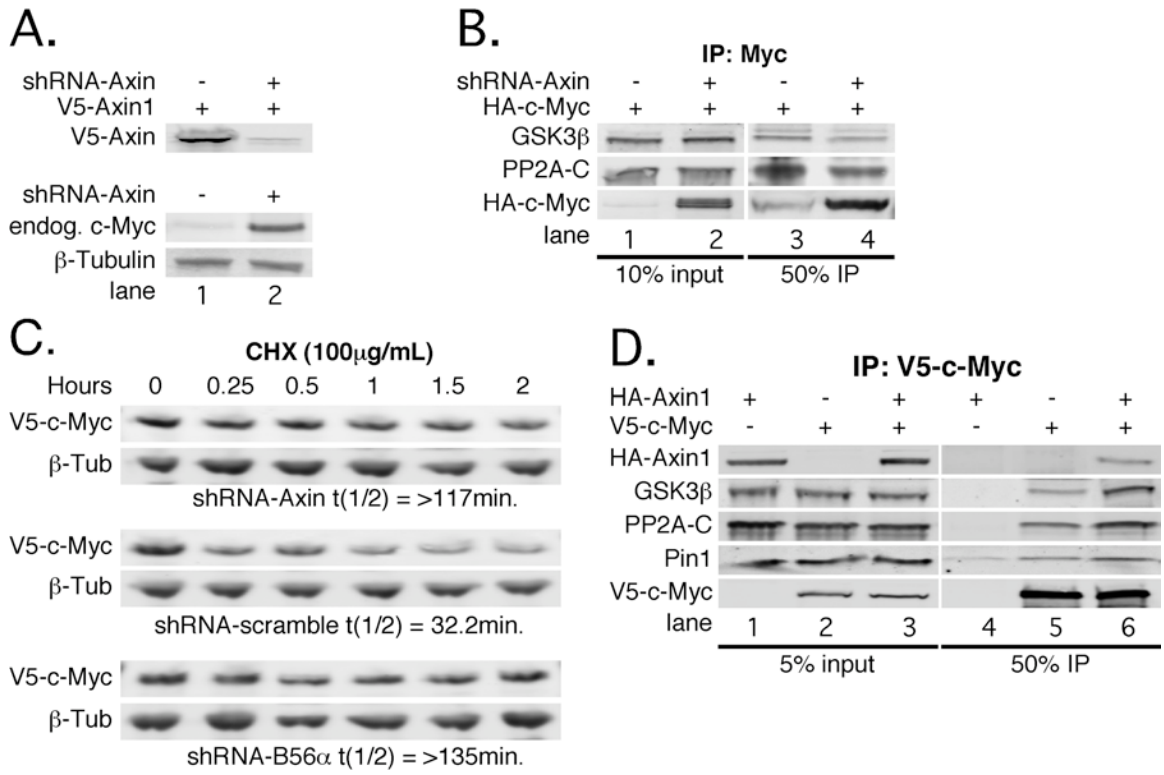


Figure 3.2: Knockdown of Axin1 increases c-Myc protein levels and stability and Axin1 promotes the association of GSK3β and PP2A with c-Myc.

(A) Knockdown of Axin1 increases c-Myc protein levels. 293 cells were transfected with CMV driven β-gal, V5-Axin1 and either vector driven scramble or Axin1 targeted shRNA. Cellular lysates were collected and analyzed by western blot with indicated antibodies 72hrs post-transfection. (B) Knockdown of endogenous Axin1 results in reduced GSK3β and PP2A association with c-Myc. CMV driven β-gal, HA-c-Myc and either vector driven scramble or Axin1 targeted shRNA constructs were transfected into 293 cells and cellular lysates were collected 72hrs post-transfection. Cellular lysates volumes were adjusted based on β-gal activity and analyzed by western blot with indicated antibodies. (C) Knockdown of Axin1 increases c-Myc protein stability. 100mm dishes of 293 cells were cotransfected with CMV-driven β-gal, V5-c-Myc and either vector driven shRNA to scramble, Axin1 or B56α. Each transfection was split into six 60mm dishes 24 hours post-transfection in DMEM supplemented with 10% FBS and then maintained in DMEM supplemented with 2% FBS for 48 hours. Cells were treated with 100μg/mL CHX to inhibit protein synthesis and whole cell lysates were collected at indicated time points. Samples were analyzed by western blot analysis with indicated antibodies. C-Myc protein levels were quantitated relative to β-Tubulin protein levels using LI-COR software and c-Myc protein half-life calculations were done using Excel. (D) Overexpression of Axin1

increases GSK3β, PP2A and Pin1 association with c-Myc. 293 cells were transfected with CMV-driven β-gal, HA-Axin1 and V5-c-Myc as indicated followed by immunoprecipitation with αV5 in cleared lysates, volumes adjusted for β-gal activity. Western blot performed with indicated to antibodies.

Acute expression of V5-Axin1 reduces endogenous c-Myc protein levels.

Our results from the knockdown of Axin1 resulting increased c-Myc protein levels as well as the ability of Axin1 to promote the association of GSK3β, PP2A and Pin1 with c-Myc suggest that Axin1 negatively regulates c-Myc protein levels. We wanted to determine whether Axin1 expression could reduce c-Myc protein levels. To do this I generated 293tr-V5-Axin1 cells, which express a tet-repressor protein and have stably integrated V5-Axin1 expression under the control of a CMV promoter containing tet-repressor binding elements to prevent V5-Axin1 expression in the absence of Doxycyclin (Dox). Since Axin1 regulates β-catenin/Tcf/Lef transcription I determined the effect over time of Dox induced expression of V5-Axin1 on endogenous *c-myc* mRNA levels in the 293tr-V5-Axin cells. I found no effect on *c-myc* mRNA levels out to 12 hours of V5-Axin1 expression (Figure 3.3A, yellow bars). However, I did observe a robust and statistically significant reduction in *c-myc* mRNA levels by 24 hours Dox treatment (Figure 3.3A, yellow bars). I next asked what affect Dox-induced expression of V5-Axin1 had on endogenous c-Myc protein levels over time. Interestingly, I found that very acute expression of V5-Axin1, one hour of Dox treatment, consistently reduced endogenous c-Myc protein levels by approximately 50% (Figure 3.3B, lane 2 compared to lane 1). As a positive

control for Axin1 post-translational function on known target proteins I checked endogenous β-catenin protein levels and found β-catenin protein levels to be consistently reduced by approximately 50% with one hour of Dox treatment (Figure 3.3B, lane 2 compared to lane 1). Intriguingly, Dox-induced expression of V5-Axin1 for five or more hours, consistently resulted in higher endogenous c-Myc protein levels (Figure 3.3B, lanes 4, 6, 8, and 10 compared to lanes 3, 5, 7, and 9, respectively and quantitated in Figure 3.3A, blue bars). I also found similar results for endogenous β-catenin protein levels which either remained unchanged or increased with five or more hours of Dox-induced expression of V5-Axin1 (Figure 3.3B, lanes 4, 6, 8, and 10 compared to lanes 3, 5, 7, and 9, respectively). These results are striking and suggest that overexpression of Axin1 can have a dominant negative effect on the ability of Axin1 to post-translationally regulate target proteins. Furthermore, we would have expected that reduced *c-myc* mRNA levels by inhibition of β-catenin/Tcf/Lef transcription would result in lower c-Myc protein levels by 24 hours post-Dox-induced expression of V5-Axin1. However, we observe the opposite result with increased c-Myc protein levels following 24 hours of Dox-induced V5-Axin1 expression. These results strongly suggest that Axin1 can regulate c-Myc protein levels at both the transcriptional and post-translational level.

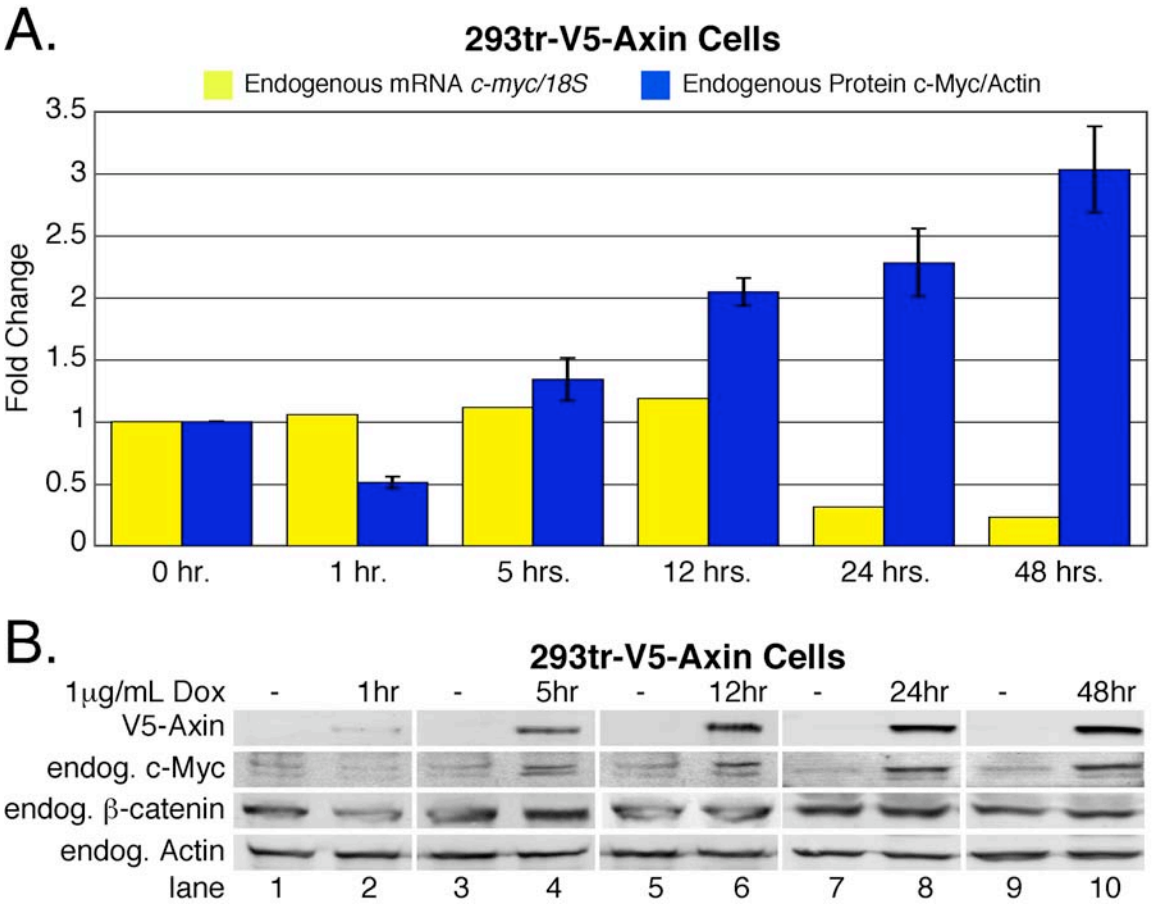


Figure 3.3: Acute expression of Axin1 negatively regulates c-Myc protein levels prior to affects seen on *c-myc* mRNA levels.

(A) Quantitation of changes in *c-myc* mRNA and c-Myc protein levels upon expression of Axin1. 293tr-V5-Axin1 cells were stimulated for indicated times with 1μg/mL Dox and qRT-PCR analysis was used to measure endogenous *c-myc* mRNA levels relative to *18S* RNA levels and western blot with indicated antibodies was done to measure endogenous c-Myc protein levels relative to Actin. Average of two experiments shown in yellow bars for *c-myc/18S* RNA levels and blue bars represent three experiments examining c-Myc protein levels relative to Actin. (B) Acute expression of V5-Axin1 reduces c-Myc protein levels. 293tr-V5-Axin1 cells were stimulated with 1μg/mL Dox to express V5-Axin1 for the indicated time periods. Total cell lysates were collected and analyzed by western with indicated antibodies.

Axin1 expression increases ubiquitination of c-Myc and proteosome inhibition increases c-Myc association with Axin1. In Figure 3.3 I found that Axin1 negatively regulates c-Myc protein levels and I also found that knockdown of Axin1 increased c-Myc protein stability (Figure 3.2C). These findings demonstrate a role for Axin1 to negatively regulate c-Myc protein expression at the post-translational level. An important step in the turnover of c-Myc protein is the multi-ubiquitination of c-Myc by the SCF^{Fbw7} ubiquitin machinery and subsequent degradation by the 26S proteosome (Welcker et al. 2004; Yada et al. 2004). I therefore asked whether Axin1 expression affects c-Myc ubiquitination. 293 cells were cotransfected with HA-ubiquitin, c-Myc, and either V5-empty or V5-Axin1 followed by αHA immunoprecipitation under denaturing conditions to pull out ubiquitinated proteins and c-Myc western blot to detect ubiquitinated c-Myc. As shown in Figure 3.4A, there is a greater than eight fold increase in upper molecular weight forms of c-Myc with expression of Axin1, compare lane 4 to lane 3. I also tested the effects of proteosome inhibition on the association of c-Myc with Axin1. To do this 293 cells were cotransfected with CMV-drive β-gal, c-Myc, and either V5-empty or V5-Axin1 as indicated in Figure 3.4B and indicated samples were treated with a combination of 10μM each MG115 and MG132 for 4 hours prior to collection. As expected based on Axin1's role in promoting c-Myc ubiquitin mediated degradation, c-Myc association with Axin1 was increased with proteosome inhibition (figure 3.4B compare lane 4 to 3). Furthermore I found the presence of an upper molecular weight c-Myc band

consistent with ubiquitinated c-Myc that is associated with V5-Axin1 upon proteosome inhibition (Figure 3.4 lane 4, upper arrow). It is difficult for us to speculate as to the relative increases in the total amount of c-Myc protein upon proteosome inhibition since my input samples appear overloaded based on the abnormal banding patterns observed (Figure 3.4B, bottom panels, lanes 1 and 4), which is due to increased protein amounts seen with proteosome inhibition. Altogether, my findings suggest that c-Myc is degraded from a complex with Axin1 and proteosome inhibition blocks this so that c-Myc remains bound to Axin1. Altogether, these findings strongly suggest that Axin1 promotes c-Myc ubiquitination and consequently negatively regulates c-Myc protein stability.

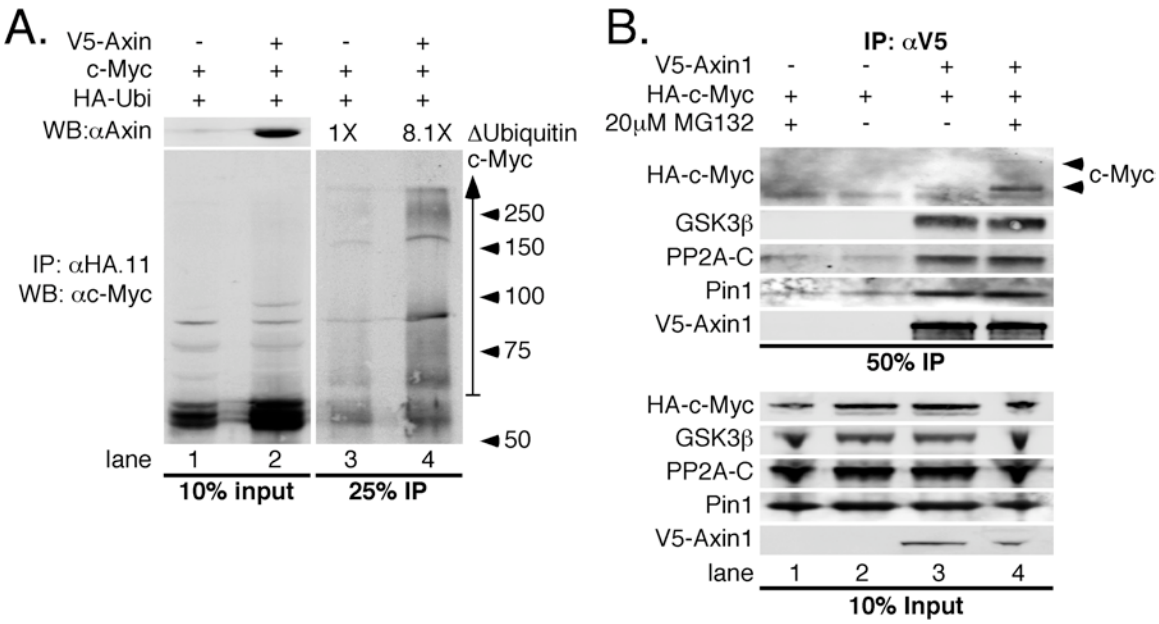


Figure 3.4: Axin1 promotes c-Myc ubiquitination and proteosome inhibition increases c-Myc association with Axin1.

(A) Axin1 increases c-Myc ubiquitination. 293 cells were cotransfected with CMV-driven β-gal, V5-Axin1, c-Myc and HA-ubiquitin as indicated. Cells were treated with combination 10μM each MG115 and MG132 for 4 hours prior to collection. Cleared cellular lysates were collected under denaturing conditions, volumes adjusted for β-gal activity, and immunoprecipitated with αHA.11. Western blot analysis was done with indicated antibodies and quantitation of upper molecular weight c-Myc species was done with LI-COR software. (B) Proteosome inhibition increases c-Myc association with Axin1. 293 cells were cotransfected with CMV-driven β-gal, V5-Axin1, and HA-c-Myc as indicated. Cells were treated with combination 10μM each MG115 and MG132 for 4 hours prior to collection. Cleared cellular lysates were collected under non-denaturing conditions and subjected to immunoprecipitation with αV5 to pull out ubiquitinated proteins. Input and IP samples were analyzed by western blot with indicated antibodies.

Axin1 has increased association with GSK3β and PP2A under low serum conditions. It has previously been shown that c-Myc protein is unstable in quiescent cells in the absence of serum or mitogen stimulation. In contrast, serum stimulation transiently stabilizes c-Myc allowing c-Myc protein to

accumulate (Sears et al. 1999; Sears et al. 2000; Sears 2004). Since serum levels have such a robust effect on c-Myc protein levels we asked whether different serum levels would affect the ability of Axin1 to promote the turnover of c-Myc protein. Specifically, I examined endogenous c-Myc protein levels in 293tr-V5-Axin1 cells under 10%, 2%, and 0.2% FBS conditions either untreated or treated with Dox to induce V5-Axin1 expression. These serum levels were chosen because each shows a different cellular phenotype. In 10% FBS, 293tr-V5-Axin1 cells rapidly proliferate and grow until density arrested. Whereas, in 2% FBS these cells proliferate slowly and in 0.2% FBS serum they cease to proliferate. I found that expression of V5-Axin1 in the 293tr-V5-Axin1 cells efficiently reduced endogenous c-Myc protein levels under 2% and 0.2% FBS serum conditions, but not under 10% FBS serum conditions (Figure 3.5, input panel). Furthermore, I immunoprecipitated V5-Axin1 under 10%, 2%, and 0.2% FBS serum conditions and found that both GSK3β and PP2A readily coimmunoprecipitated with V5-Axin1 under 2% and 0.2% FBS serum conditions, but not under 10% serum conditions (Figure 3.5, upper panels). These findings demonstrate that the Axin1 mediated c-Myc degradation complex forms under low serum conditions when c-Myc protein levels are more rapidly turned over. Moreover, these findings demonstrate that mitogenic signaling affects Axin1 function by affecting the ability of GSK3β and PP2A to associate with Axin1.

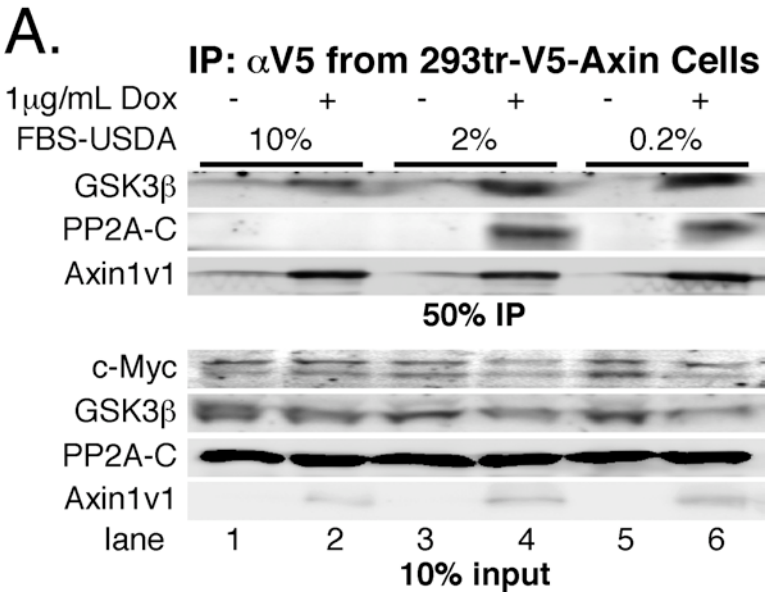


Figure 3.5: Mitogenic signaling affects the association of GSK3β and PP2A with Axin1.

293tr-V5-Axin1 cells were maintained in DMEM supplemented with 10%, 2%, or 0.2% FBS with or without 1mg/mL Dox as indicated. Cleared lysates were collected and subjected to immunoprecipitation with aV5. Input and IP samples were subjected to western blot analysis with the indicated antibodies.

Knockdown of Axin1 decreases T58 phosphorylation and increases S62 phosphorylation of c-Myc. We and others have previously shown that GSK3β can phosphorylate T58 of c-Myc (Lutterbach and Hann 1994; Pulverer et al. 1994) and inhibition of GSK3β results in decreased T58 phosphorylation of c-Myc (Sears et al. 2000). We have also shown that PP2A-B56α dephosphorylates S62 and inhibition of PP2A-B56α results in increased S62 phosphorylation of c-Myc (Yeh et al. 2004; Arnold and Sears 2006). Since Axin1 enhances the association of GSK3β and PP2A-B56α with c-Myc (Figure 3.2B and D) we were interested in determining whether knockdown of Axin1 would affect the phosphorylation status of c-Myc. To examine possible changes in c-Myc

phosphorylation upon knockdown of Axin1, 293 cells were cotransfected with CMV-driven V5-c-Myc and vector driven shRNA to either scramble, Axin1 or B56α. I found that knockdown of Axin1 substantially decreased T58 phosphorylation and moderately increased S62 phosphorylation relative to total c-Myc (Figure 3.6A). As a positive control and consistent with my previous results I found that knockdown of B56α resulted in increased T58 and S62 phosphorylation of c-Myc relative to total c-Myc (Figure 3.6A and 2.7). This finding suggests that Axin1 coordinates GSK3β and PP2A-B56α with c-Myc to promote a switch in c-Myc phosphorylation from a S62 phosphorylated to T58 phosphorylated form. By promoting the switch in c-Myc phosphorylation status towards a T58 phosphorylated form, Axin1 helps to set c-Myc up for recognition and multi-ubiquitination by the SCF^{Fbw7} ubiquitin ligase machinery. Consistent with this hypothesis I demonstrated in Figure 3.4A that Axin1 expression increased the multi-ubiquitination of c-Myc. Altogether, my results indicate a critical role for Axin1 to negatively regulate c-Myc protein levels by regulating association with the kinase and phosphatase that control its phosphorylation status at T58 and S62 and thus promoting its ubiquitin mediated turnover.

$c\text{-}Myc^{WT}$, but not $c\text{-}Myc^{T58A}$ or $c\text{-}Myc^{S62A}$ protein levels are negatively regulated by Axin1. T58 phosphorylation of c-Myc has been shown to be required for c-Myc recognition and protein turnover by the SCF^{Fbw7} ubiquitin ligase machinery (Welcker et al. 2004; Yada et al. 2004) and $c\text{-}Myc^{WT}$ shows

increased T58 phosphorylation compared to S62 phosphorylation under quiescent cell conditions (Sears et al. 2000). We have previously characterized two point mutants of c-Myc, c-Myc^{T58A} and c-Myc^{S62A}, which affect both c-Myc phosphorylation and protein stability (Sears et al. 2000). In contrast to c-Myc^{WT}, c-Myc^{T58A} protein shows increased constitutive S62 phosphorylation and increased protein stability, and it is resistant to turnover by SCF^{Fbw7}, while c-Myc^{S62A} lacks phosphorylation at both T58 and S62 and is also resistant to turnover by SCF^{Fbw7} (Welcker et al. 2004). Interestingly, we have found that c-Myc^{S62A} is relatively unstable, but can be slightly more stable than c-Myc^{WT} (Sears et al. 2000). This would suggest that c-Myc^{S62A} protein turnover is regulated by another mechanism. Since T58 and S62 phosphorylation of c-Myc are critical for the regulation of c-Myc protein stability we wanted to assess the ability of Axin1 to regulate the turnover of the T58 and S62 phosphorylation mutants of c-Myc. To do this I transiently transfected 293tr-V5-Axin1 cells with CMV driven constructs for HA-c-Myc^{WT}, HA-c-Myc^{T58A}, or HA-c-Myc^{S62A}. Dox was added as indicated for 3 hours to allow for acute expression of V5-Axin1 as indicated (Figure 3.6B). I found that acute expression of V5-Axin1 decreased HA-c-Myc^{WT} protein levels consistently by 60% as compared to control (Figure 3.6B, compare lane 1 and 2), but no affect was observed by V5-Axin1 expression on HA-c-Myc^{T58A} or HA-c-Myc^{S62A} protein levels (Figure 3.6B, compare lanes 3 to 4 and 5 to 6). Since both phosphorylation mutant lack T58 phosphorylation, these results suggest that T58 phosphorylation is important for Axin-mediated c-Myc turnover

and, combined with my previous results showing that Axin1 expression increases c-Myc ubiquitination, strongly suggest that Axin1 promotes the modification of c-Myc to a single T58 phosphorylated form, so that the SCF^{Fbw7} ubiquitin ligase machinery can multi-ubiquitinate c-Myc thereby promoting its turnover by the 26S proteosome.

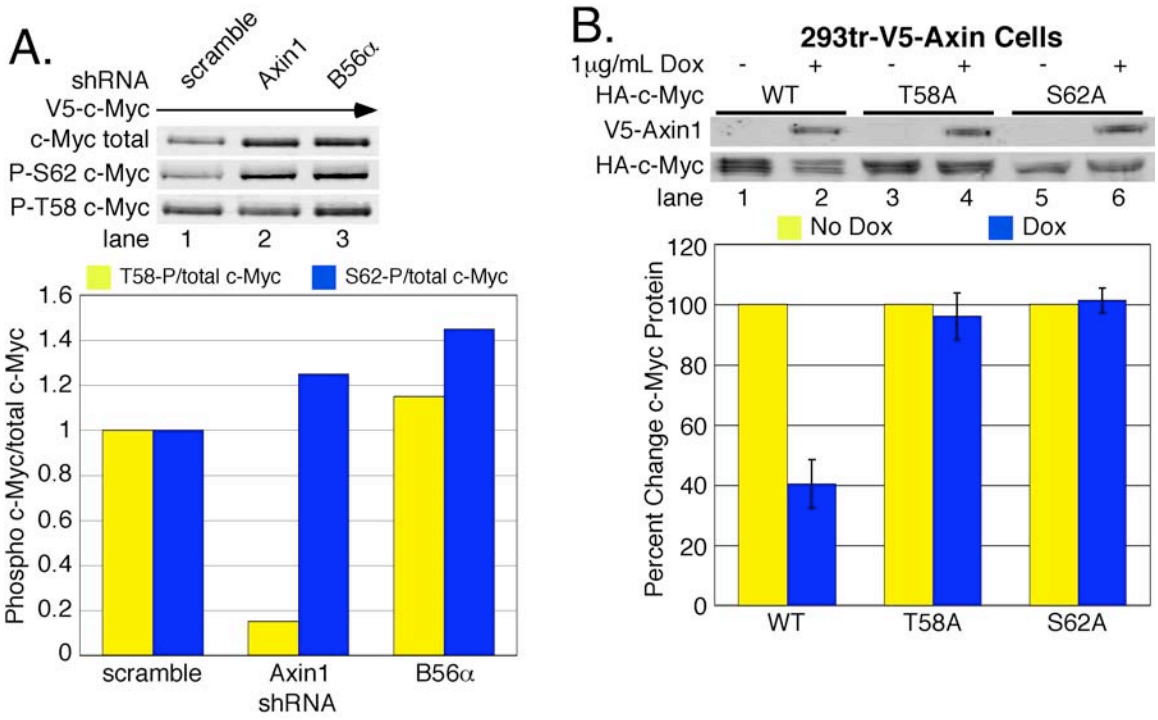


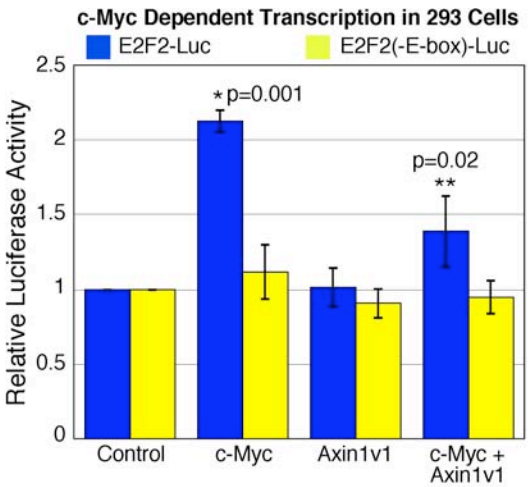
Figure 3.6: Knockdown of Axin1 decreases T58 phosphorylation and increases S62 phosphorylation of c-Myc and Axin1 expression reduces c-Myc^{WT}, not c-Myc^{T58A} or c-Myc^{S62A} protein levels.

(A) Knockdown of Axin1 results in increased S62, but decreased T58 phosphorylation of c-Myc. 293 cells were cotransfected with CMV-driven β-gal and V5-c-Myc plus either vector driven shRNA to scramble, Axin1, or B56α as indicated. Whole cell lysates were collected, volumes adjusted to βgal activity and analyzed by western blot analysis with αV5 for total c-Myc, αS62 or αT58 antibodies. Western results were quantitated using LI-COR software and c-Myc T58-P or S62-P levels were graphed relative to total c-Myc levels using Excel. (B) Axin1 expression reduces c-Myc^{WT}, but not c-Myc^{T58A} or c-Myc^{S62A} protein levels. 293tr-V5-Axin1 cells were transfected with CMV-driven β-gal and either HA-c-Myc WT, T58A or S62A as indicated. Expression of V5-Axin1 was induced

with 1μg/mL of Dox as indicated and total cell lysates were subjected to western blot analysis with indicated antibodies. Western blot shown is representative of three independent experiments, which were quantitated using LI-COR software and averages with standard deviations were graphed using Excel.

Axin1 reduces c-Myc dependent transcription. Since c-Myc is a transcription factor that drives the expression of numerous genes involved in cellular proliferation, growth, differentiation, and apoptosis (Dang et al. 1999) we asked whether Axin1 expression would regulate c-Myc dependent transcription. To assess the affect of Axin1 expression on c-Myc dependent transcription I used two luciferase reporter plasmids, which have the E2F2 promoter containing consensus c-Myc binding E-box elements (E2F2-Luc) or an E2F2 promoter with point mutations in the E-box elements [E2F2(-E-box)-Luc] to prevent c-Myc binding (Sears et al. 1997). In order to eliminate the effect by Axin1 regulation on β-catenin/TCF/Lef transcription and consequent effects on c-Myc protein levels and transcriptional activity I transfected 293 or U2OS cells with CMV driven c-Myc and Axin1 along with either the E2F2-Luc or E2F2(-E-box)-Luc reporter plasmids as indicated in Figure 3.7. As previously observed, ectopically expressed c-Myc alone increased E2F2-driven luciferase activity by 2.1 fold in 293 cells (Figure 3.7, left graph, second set of bars). This is consistent with numerous other reports on the weak transcription activating potential of c-Myc and it was dependent upon intact c-Myc E-box binding sites as the E2F2(-E-box) mutant promoter was not activated. Ectopic Axin1 expression alone in 293 cells had no affect on transcription driven by the E2F2 promoter, but I did observe a consistent and statistically significant reduction of ectopically driven c-Myc

dependent E2F2 promoter activity by Axin1 expression in 293 cells (Figure 3.7, left graph, third and fourth set of bars). I observed similar results in U2OS cells. However, c-Myc expression alone causes a significantly more robust increase of c-Myc dependent transcriptional activity in U2OS cells as compared to that observed in 293 cells (Figure 3.7, right graph, column two as compared to left graph, column two). The significant increase in c-Myc dependent transcription in the U2OS cells is most likely due to the deletion of ARF in these cells, as ARF has been shown to inhibit c-Myc transcriptional activity (Qi et al. 2004; Amente et al. 2006). Interestingly, I see a consistent reduction of luciferase activity in U2OS cells with Axin1 expression alone as compared to control (Figure 3.7, right graph, third column), possibly due to the more robust activity of endogenous c-Myc on the E2F2-luc construct. More importantly I observe complete ablation of ectopically driven c-Myc dependent transcription from the E2F2 promoter by Axin1 expression (Figure 3.7, right graph, fourth column). Altogether, these results demonstrate a potent ability for Axin1 to inhibit c-Myc dependent transcription.



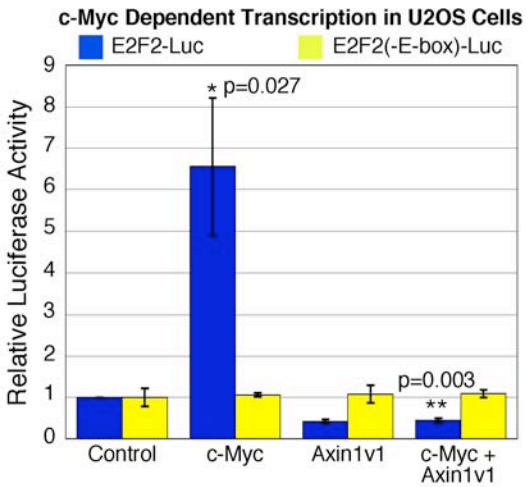


Figure 3.7: Axin1 negatively regulates c-Myc dependent transcription.
293 or U2OS cells were cotransfected with CMV-driven β-gal and either E2F2-Luc or control E2F2(-E-box)-Luc together with either CMV-driven empty, c-Myc, and/or Axin1v1 expression vectors as indicated and maintained in DMEM supplemented with 0.2% FBS for 48 hours. Cleared cellular lysates were collected and analyzed for β-gal and luciferase activity. Luciferase activity was corrected for transfection efficiency by β-gal activity and three independent experiments were graphed with averages and standard deviations using Excel.

C-Myc requires multiple domains in Axin for association that are distinct from domains required for β-catenin association. To determine what region of Axin1 that c-Myc associates with I initially used V5-tagged N-terminal (Exons 1-5) and C-terminal (Exons 5-10) expression constructs for Axin1 diagramed in Figure 3.8A to grossly narrow my search to the N- or C-terminus of Axin1. 293 cells were cotransfected with CMV driven c-Myc and either empty control, V5-Axin1, V5-Axin$^{(Ex1-5)}$ or V5-Axin$^{(Ex5-10)}$. Following αV5 immunoprecipitation I found that c-Myc coimmunoprecipitated with full-length V5-Axin, but not with either V5-Axin$^{(Ex1-5)}$ or V5-Axin$^{(Ex5-10)}$ (Figure 3.8B, left panel). This suggests that domains in both the N-terminal and C-terminal regions of Axin1 are required for c-Myc association with Axin1. One possibility is that the association of both GSK3β and PP2A are important for c-Myc association with Axin1. Consistent with this hypothesis, I found that GSK3β coimmunoprecipitated with V5-Axin$^{(Ex1-5)}$ and full-length V5-Axin, but not with V5-Axin$^{(Ex5-10)}$. Whereas, PP2A-C coimmunoprecipitated with V5-Axin$^{(Ex5-10)}$ and full-length V5-Axin, but not with V5-Axin$^{(Ex1-5)}$. Both of these findings are consistent with previous mapping studies showing that GSK3β associates within exons three and four of Aixn1 and PP2A

associates within exons seven and eight (Fagotto et al. 1999). I also generated a deletion mutant of Axin1 missing exons three to eight to completely remove both GSK3β and PP2A binding domains within Axin1. Following αV5 immunoprecipitation from 293 cells that were cotransfected with c-Myc and either empty control, V5-Axin1 or V5-Axin$^{(\Delta Ex3-8)}$, I found that c-Myc, GSK3β and PP2A-C only coimmunoprecipitated in the presence of full-length V5-Axin1 (Figure 3.8B, right panel). Therefore, I focused my attention to the middle region of Axin1 to determine more precisely the regions required for c-Myc association with Axin1.

To examine the middle region of Axin1 for c-Myc association more extensively I generated three more V5-Axin1 fragments, V5-Axin1^{Ex1-6}, V5-Axin1^{Ex1-7}, and V5-Axin1^{Ex1-8} to sequentially extend the N-terminal V5-Axin1^{Ex1-5} fragment that did not associate with c-Myc in the above experiments (Figure 3.8B, left panel). Each of these V5-Axin1 fragments, V5-Axin1^{Ex1-5}, full-length V5-Axin1, or empty control was cotransfected with CMV-c-Myc into 293 cells. I found that just adding exon 6 to the V5-Axin^{Ex1-5} fragment resulted in the association of c-Myc with V5-Axin^{Ex1-6} (Figure 3.8C). In contrast, endogenous β-catenin bound all N-terminal fragments of V5-Axin1 examined (Figure 3.8C), which is consistent with previous reports mapping the β-catenin binding domain to exon 5 in Axin1 (Ikeda et al. 1998; Xing et al. 2003). I also found that GSK3β bound to all N-terminal fragments of V5-Axin1 (Figure 3.8C) consistent with the finding that GSK3β associates with exons three and four in Axin1. Altogether these findings demonstrate that c-Myc requires central N- and C-terminal

domains as well as exon 6 for association with Axin1 and β-catenin associates with different domains of Axin1.

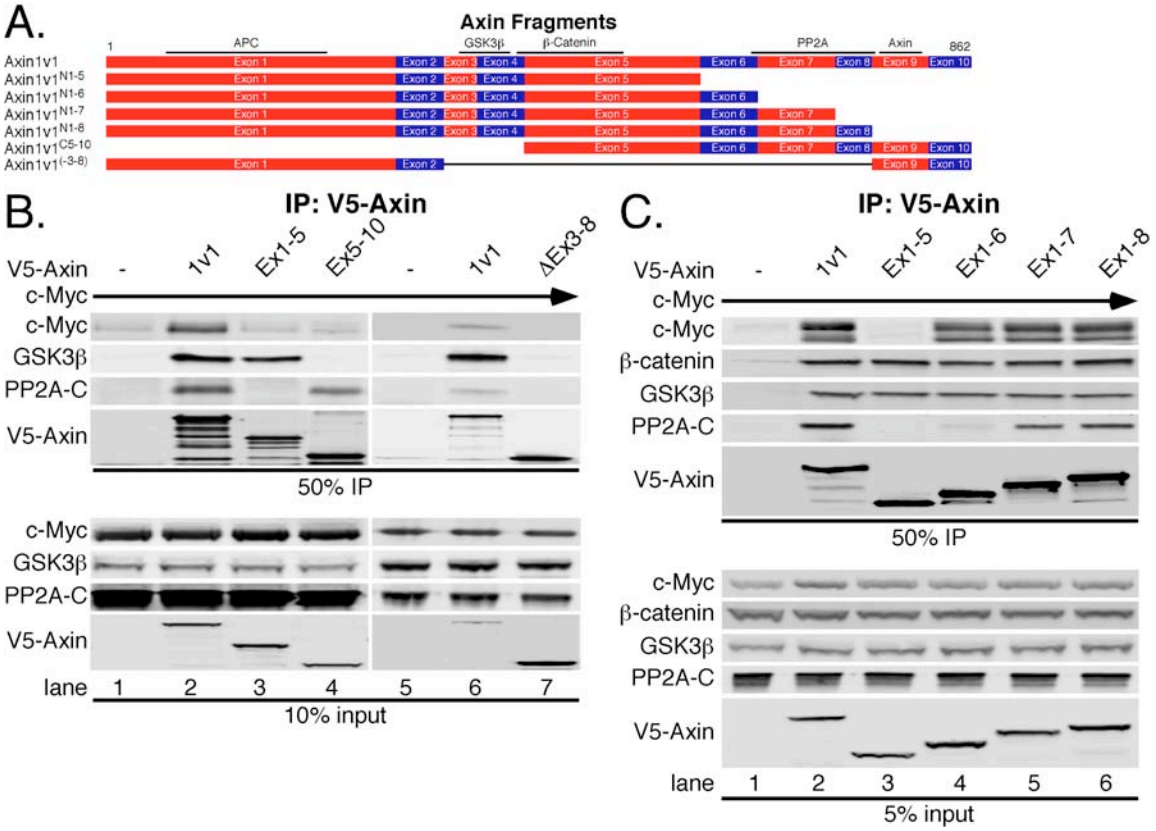


Figure 3.8: c-Myc and β-catenin associate with different domains of Axin1.

(A) Diagram of full-length Axin1 and Axin1 fragments. Exons along with reported binding domains are indicated. (B) c-Myc requires Exons 3 to 8 for association with Axin. 293 cells were cotransfected with CMV-driven β-gal, c-Myc and either empty, V5-Axin1v1, V5-Axin1^{Ex1-5}, V5-Axin1^{Ex5-10}, or V5-Axin$^{\Delta Ex3-8}$ as indicated. Cleared lysates were collected, volumes adjusted for β-gal activity, and subjected to immunoprecipitation with αV5. Input and IP samples were subjected to western blot analysis with indicated antibodies. (C) c-Myc requires exon 6 and associates with different domains of Axin1 compared to β-catenin. 293 cells were cotransfected with CMV-driven β-gal, c-Myc and either empty, V5-Axin1v1, V5-Axin1^{Ex1-5}, V5-Axin1^{Ex1-6}, V5-Axin1^{Ex1-7} or V5-Axin1^{Ex1-8} as indicated. Cleared lysates were immunoprecipitated with αV5 in volumes adjusted for β-gal activity. Western blot analysis was done on input and IP samples with indicated antibodies.

PP2A association with Axin1 enhances c-Myc association with Axin1. The association of c-Myc with V5-Axin^{Ex1-6} was approximately half as robust as that observed with full-length V5-Axin1 (Figure 3.8C, compare lane 2 to 4), suggesting that a domain(s) in the C-terminus of Axin1 are important for full association of c-Myc with Axin1. In line with this idea, the sequential addition of exons seven and eight resulted in a further increase of c-Myc associated with the V5-Axin^{Ex1-7} and V5-Axin^{Ex1-8} fragments (Figure 3.8C, compare lane 4, 5 and 6). PP2A has been shown to associate with Axin1 within exons seven and eight (Hsu et al. 1999) and consistent with these reports I find that PP2A-C associates with V5-Axin^{Ex1-7} and V5-Axin^{Ex1-8} (Figure 3.8C, lane 5 and 6). In contrast, I do not observe any association of PP2A-C with V5-Axin^{Ex1-5} and very little association of PP2A-C with V5-Axin^{Ex1-6} (Figure 3.8C, lanes 3 and 4). These findings suggest that PP2A association with Axin1 enhances the association of c-Myc with Axin1.

To further examine the importance of the PP2A association with respect to enhancing the association of c-Myc with Axin1 we cloned the naturally occurring variant two of Axin1 in which exon eight is spliced out and I generated a deletion mutant of Axin1 missing both exons seven and eight, removing most of the reported PP2A binding domain. I cotransfected 293 cells with CMV-driven HA-c-Myc with either control, V5-Axin1v1, V5-Axin1v2 or V5-Axin$^{\Delta Ex7-8}$. Following αV5 immunoprecipitation I found that both HA-c-Myc and PP2A-C association with V5-Axin significantly decreased as exons seven and eight were sequentially

removed (Figure 3.9A). Altogether these findings demonstrate that the association of PP2A significantly enhances the association of c-Myc with Axin1.

GSK3β association with Axin1 is critical for the association of c-Myc with Axin1. It is extremely important to note that the C-terminal fragment V5-Axin^{Ex5-10} containing the putative exon 6 binding site for c-Myc as well as the PP2A binding domain did not associate with c-Myc. This demonstrates that the N-terminal region of Axin1 is also required for c-Myc association with Axin1. Due to the important function of GSK3β in regulating c-Myc protein turnover we hypothesized that the association of GSK3β with Axin1 is critical for the association of c-Myc with Axin1. Therefore, I examined the affect of removing the GSK3β binding domain from Axin1 with respect to the association between c-Myc and Axin1 by generating a deletion mutant of Axin1 missing exons three and four, previously shown to encompass the GSK3β binding domain. I cotransfected 293 cells with CMV-driven c-Myc and either control, V5-Axin1 or V5-Axin$^{\Delta Ex3-4}$ followed by αV5 immunoprecipitation. GSK3β did not associate with V5-Axin$^{\Delta Ex3-4}$, but readily associated with full-length V5-Axin1 as compared to control (Figure 3.9B). Importantly, I found that c-Myc association with Axin1 was drastically reduced with V5-Axin$^{\Delta Ex3-4}$ close to the levels seen in the negative control lane (Figure 3.9B, compare lane 3 to lane 1). It is possible that a minor amount of c-Myc appears to associate with V5-Axin$^{\Delta Ex3-4}$ as a result of this mutant's ability to dimerize with endogenous full-length Axin1. In contrast, c-Myc

robustly associated with full-length Axin1 (Figure 3.9B, lane 2). These findings support our hypothesis that GSK3β association with Axin1 is critical for the association of c-Myc with Axin1.

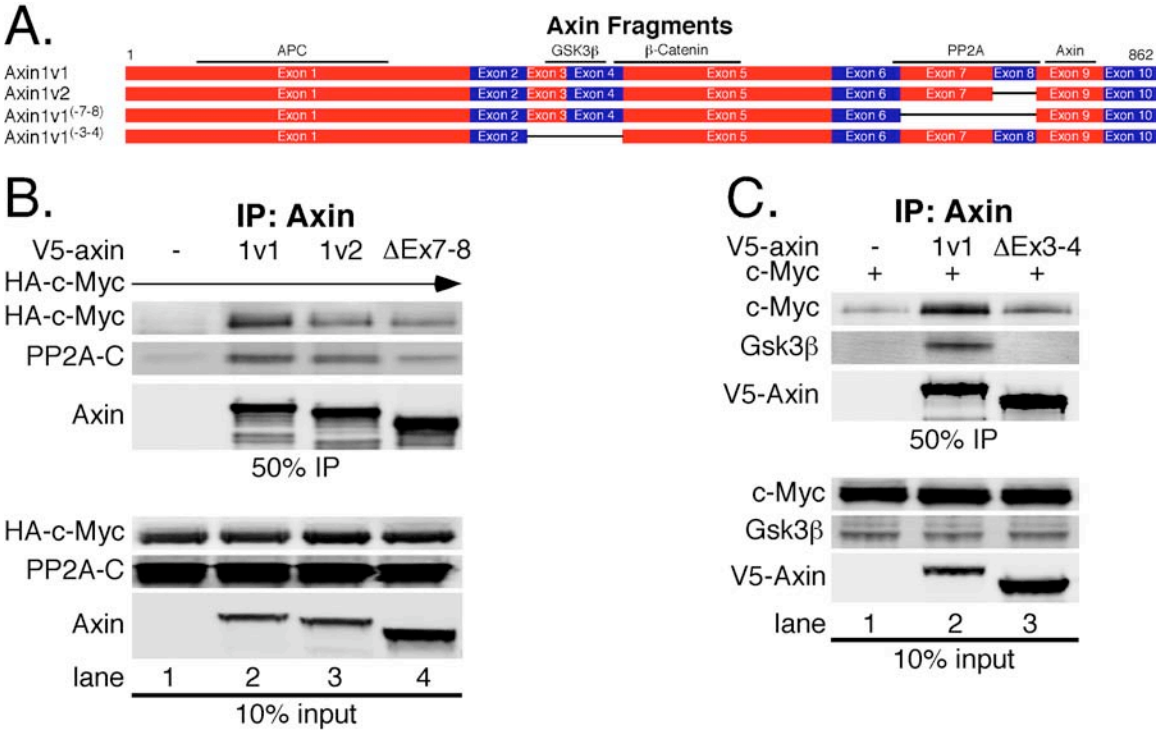


Figure 3.9: GSK3β and PP2A binding domains within Axin1 enhance the association of c-Myc with Axin1.

(A) Diagram of full-length Axin1 and Axin1 fragments. Exons along with reported binding domains are indicated. (B) PP2A association with Axin1 affects c-Myc association with Axin1. 293 cells were cotransfected with CMV-driven β-gal, HA-c-Myc and either empty, V5-Axin1v1, V5-Axin1v2 or V5-Axin1$^{\Delta Ex7-8}$ as indicated. Cleared lysates were immunoprecipitated with αV5 in volumes adjusted for β-gal activity and then input and IP samples were subjected to western blot analysis with indicated antibodies. (C) GSK3β association with Axin1 affects c-Myc association with Axin1. 293 cells were cotransfected with CMV-driven β-gal, HA-c-Myc and either empty, V5-Axin1v1 or V5-Axin1$^{\Delta Ex73-4}$ as indicated. Cleared lysates were immunoprecipitated with αV5 in volumes adjusted for β-gal activity and then input and IP samples were subjected to western blot analysis with indicated antibodies.

Axin1 localization is affected by the domains present within Axin1. In the previous sections I used several fragments of Axin1 to characterize the ability of Axin1 to form the c-Myc degradation complex depending upon the domains present in Axin1. We decided to examine the localization of these different forms of Axin1 to see whether the different domains of Axin1 affected its localization, which may affect the ability of Axin1 to form different complexes. 293 cells were transfected with CMV-driven V5-Axin1v1, V5-Axin1v2, V5-Axin1^{N1-5}, V5-Axin1^{N1-6}, V5-Axin1^{N1-7}, V5-Axin1^{N1-8}, V5-Axin1$^{\Delta Ex7-8}$, V5-Axin1^{C5-10}, V5-Axin1$^{\Delta Ex3-4}$ or V5-Axin1^{SupB15}, a deletion mutant identified in the leukemia cell line SupB15, discussed in more detail below. Localization of the different forms of Axin1 was visualized by immunoflouresence. Consistent with previous reports, the localization of the naturally occurring forms of Axin1v1 and Axin1v2 are predominantly cytoplasmic with very faint nuclear staining (Figure 3.10, panels 1 and 6) (Anderson et al. 2002). In contrast, the N-terminal fragments of Axin1 showed increased nuclear staining, in particular the N1-5 and N1-6 fragments appeared to have consistently higher nuclear staining than the N1-7 and N1-8, N-terminal fragments of Axin1 (Figure 3.10, compare panels 2 and 3 to 4 and 5). It is important to note that both V5-Axin1^{N1-5} and V5-Axin1^{N1-6} have the same localization pattern (Figure 3.10, panels 2 and 3), yet only V5-Axin1^{N1-6} associates with c-Myc (Figure 3.8C). This strongly suggests that the difference in the association of c-Myc with these two different fragments of Axin1 is not due to differences in localization, but rather to direct binding ability.

Although the association of c-Myc with Axin1 may not be significantly affected by localization, it is clear from my data that domains in both the N-terminal and C-terminal regions of Axin1 can affect Axin1 localization. This is demonstrated in Figure 3.10, panels 2 and 8, which show more diffuse localization of V5-Axin1 fragments, N1-5 and C5-10. As shown above in Figure 3.9C, the GSK3β binding domain within the N-terminal region of Axin1 is important for the association of c-Myc with Axin1. It also appears that the GSK3β binding domain is important for the cytoplasmic localization of Axin1 since removal of exons three and four containing the GSK3β binding domain results in diffuse Axin1 localization (Figure 3.10, panel 9). Consistently, a mutant form of Axin1 derived from a leukemia pre-B ALL cell line, SupB15, containing an in-frame deletion that results in the removal of the GSK3β binding domain also shows diffuse localization (Figure 3.10, panel 10). On the other end, removal of part or the majority of the PP2A binding domain by expressing V5-Axin1v2 or V5-Axin1$^{\Delta Ex7-8}$ does not seem to measurably affect Axin1 localization (Figure 3.10, panels 6 and 7). However, the C-terminal region of Axin1 contains two nuclear export signals (NES) and three nuclear localization signals (NLS) (Figure 1.6). In particular, exons five and six each contain a NLS, which may explain the stronger nuclear localization of the two Axin1 fragments, V5-Axin1^{N1-5} and V5-Axin1^{N1-6} (Figure 3.10, panels 2 and 3). On the other hand, exon seven contains a NES, which likely explains the more cytoplasmic localization of both V5-Axin1^{Ex1-7} and V5-Axin1^{Ex1-8} as compared to V5-Axin1^{N1-5} and V5-Axin1^{N1-6} (Figure 3.10, panels 4

and 5). Full-length V5-Axin1 contains one more NES and NLS each that reside in exon 9 and this results in the predominant cytoplasmic localization of Axin1. Altogether, these results indicate that the binding of GSK3β combined with the nuclear export and localization signals normally localize Axin1 predominantly to the cytoplasm.

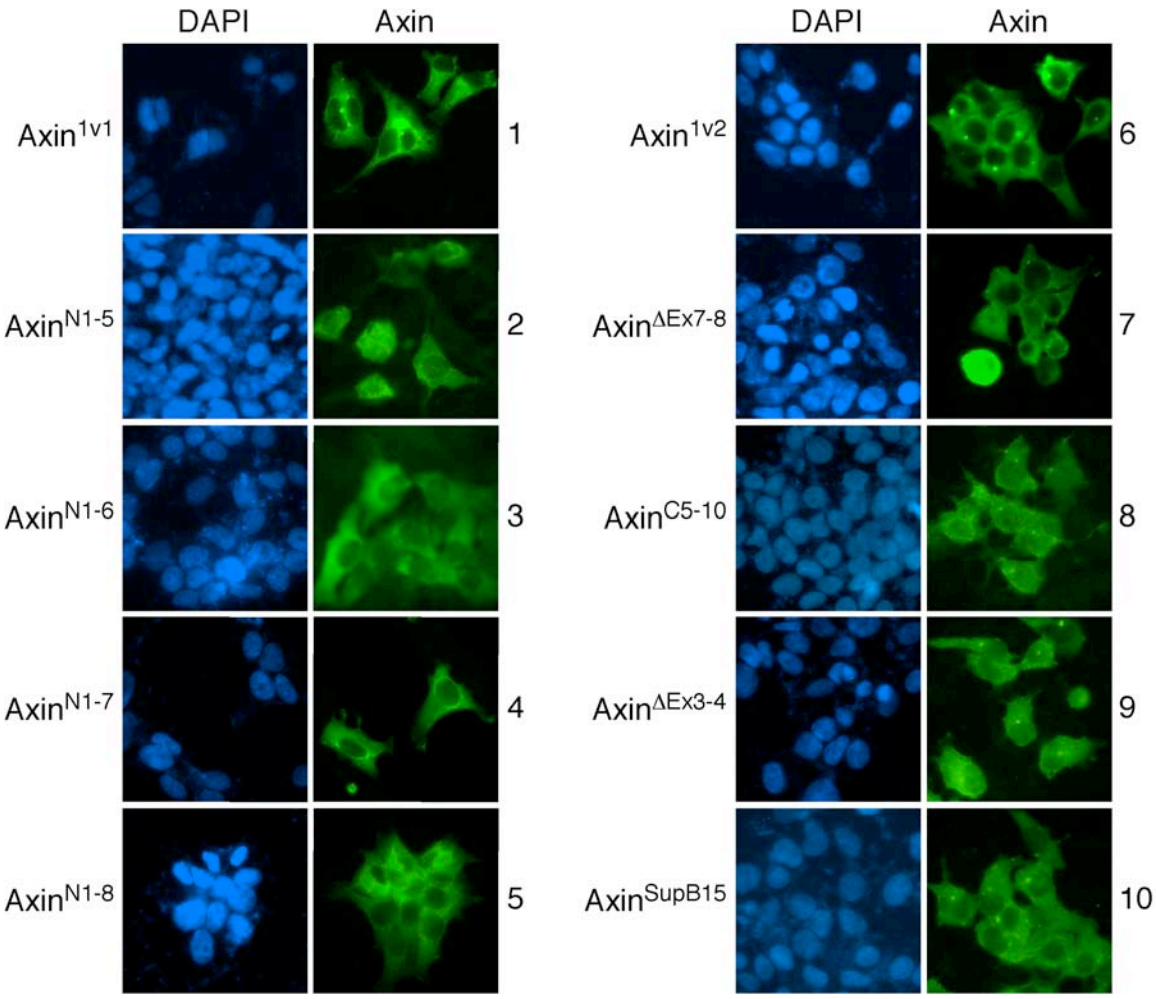


Figure 3.10: Axin1 localization is affected by the domains present within Axin1.

293 cells were transfected with CMV-driven V5-Axin1v1, V5-Axin1v2, V5-Axin1^{N1-5}, V5-Axin1^{N1-6}, V5-Axin1^{N1-7}, V5-Axin1^{N1-8}, V5-Axin1$^{\Delta Ex7-8}$, V5-Axin1^{C5-10}, V5-Axin1$^{\Delta Ex3-4}$ or V5-Axin1^{SupB15} as indicated. Cells were fixed, permiabalized, and

probed as indicated with DAPI for nuclei and αV5 and FITC secondary for Axin and then visualized by de-convolution microscopy.

Pin1 activity enhances the association of c-Myc, GSK3β and PP2A with Axin1. We demonstrated in Figure 3.1A that Pin1 associated with V5-Axin1 and our previous results have identified a role for Pin1 in promoting c-Myc protein turnover (Yeh et al. 2004). Interestingly, my attempts to identify a binding domain for Pin1 in Axin1 using all of my deletion mutants of Axin1 did not show any clear binding region for Pin1 in Axin1. In fact Pin1 appeared to associate throughout Axin1 raising the possibility that Pin1 binds indirectly and may have a role in regulating Axin1 function in addition to its role in promoting c-Myc protein turnover. To explore this idea I examined the effect of Pin1 activity on the association of c-Myc and GSK3β with Axin1 *in vitro* using a purified rabbit retic-lysate system (Promega). I initially coexpressed T7 driven V5-Axin1 and HA-c-Myc *in vitro* followed by immunoprecipitation with αV5 and found that Axin1 and c-Myc associate (Figure 3.11A, lane 2 compared to negative control lane 1). Notably, this finding suggests that Axin1 and c-Myc can directly interact independent of GSK3β and PP2A since GSK3β and key components of the PP2A holoenzyme such as the structural A and B56α subunits are not expressed in the rabbit retic-lysate system. Although the catalytic C subunit of PP2A is expressed in the retic-lysate, it does not associate with Axin1 as determined by coimmunoprecipitation (data not shown). I next expressed GSK3β and Pin1 in addition to V5-Axin1 and HA-c-Myc *in vitro* as indicated followed by αV5

immunoprecipitation. I found that GSK3β and c-Myc associated with Axin1 in the absence of Pin1 (Figure 3.11A, lane 3). However, the association of c-Myc and especially GSK3β with Axin1 increased with Pin1 expression (Figure 3.11A, lane 4). Interestingly, Pin1 did not associate with Axin1 *in vitro* (Figure 3.11A, lane 4), whereas Pin1 does associate with Axin1 *in vivo* (Figure 3.1A, 3.11B and data not shown). Also, I did not evaluate PP2A association with Axin1 in these *in vitro* assays as it has previously been shown that functional PP2A-holoenzymes are not efficiently formed in rabbit retic-lysates due to the requirement of several PP2A modifying enzymes in this system. These findings indicated that Pin1 activity plays an important role in the formation of the Axin1-mediated c-Myc degradation complex by increasing the association of GSK3β and c-Myc with Axin1.

To further evaluate the role of Pin1 in the formation of the Axin1 mediated c-Myc degradation complex I designed an shRNA to knockdown Pin1. 293 cells were cotransfected with CMV-driven HA-c-Myc and V5-Axin1 along with either scramble shRNA or Pin1 shRNA as indicated in Figure 3.11B. Pin1 was knocked down to approximately 45% as compared to control (Figure 3.11B, lower panel, input, compare lane 3 to either lane 1 or 2). Following immunoprecipitation with αV5 I found that GSK3β, PP2A-C, Pin1 and HA-c-Myc all associated with V5-Axin1 (Figure 3.11B, lane 2) similar to that seen in Figure 3.1A. Importantly, knockdown of Pin1 decreased the amount of GSK3β, PP2A-C and HA-c-Myc associated with V5-Axin1 (Figure 3.11B, compare lane 2 to lane 3). Not

surprisingly, Pin1 association with V5-Axin1 was also diminished (Figure 3.11B, lane 3). Altogether, my *in vitro* and *in vivo* cell culture findings strongly indicate that Pin1 plays a critical role in facilitating the formation of the Axin1-mediated c-Myc degradation complex.

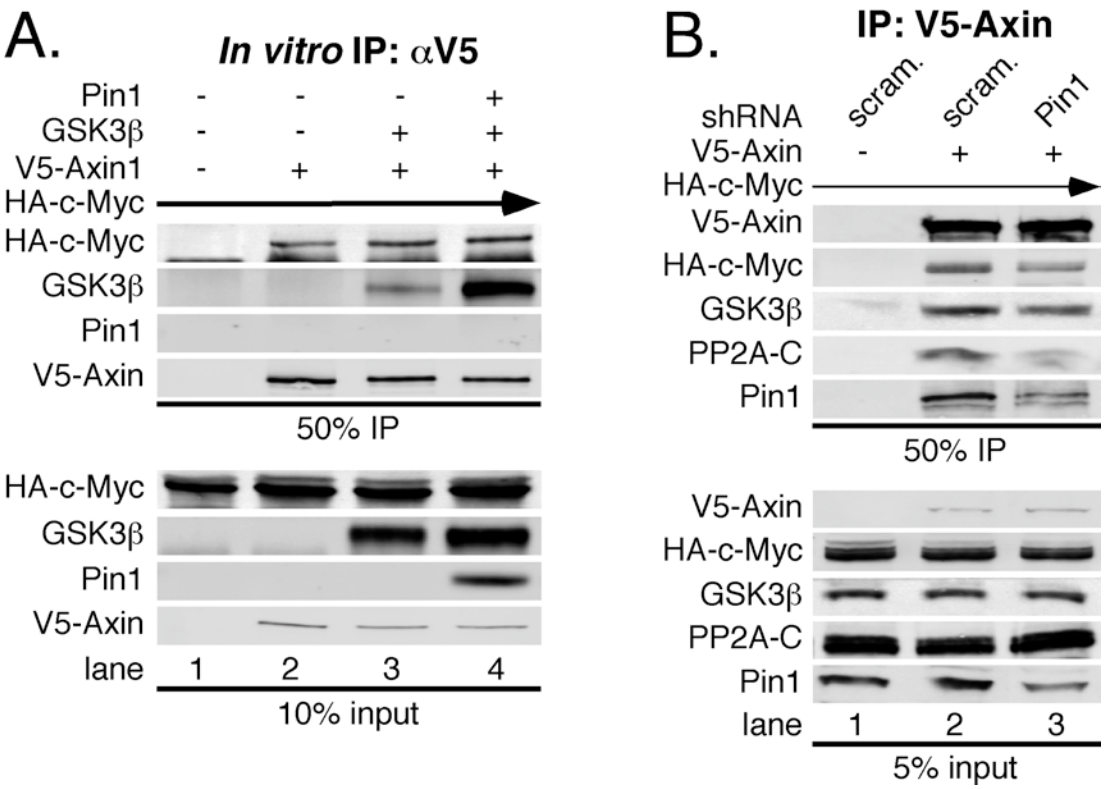


Figure 3.11: Pin1 expression increases the association of GSK3β, PP2A and c-Myc with Axin1.

(A) Pin1 activity *in vitro* increases the association of GSK3β and c-Myc with Axin1. T7-driven HA-c-Myc, V5-Axin1, GSK3β and/or Pin1 were expressed using the Quick Coupled Transcription/Translation System (Promega) as indicated. *In vitro* reactions were subsequently immunoprecipitated with αV5 and input and IP samples were analyzed by western blot analysis with indicated antibodies. (B) Knockdown of Pin1 decreases the association of c-Myc, GSK3β and PP2A with Axin1. 293 cells were cotransfected with CMV-driven β-gal, HA-c-Myc and/or V5-Axin1 and expression constructs for either scrambled control of shRNA targeting Pin1 as indicated. Cells were maintained for five days in DMEM supplemented with 2% FBS. Cleared lysates were collected, volumes adjusted by β-gal activity and subjected to immunoprecipitation with αV5. Input and IP samples were analyzed by western blot with indicated antibodies.

Expression of c-Myc promotes formation of the Axin1 mediated c-Myc degradation complex. Having found that c-Myc and Axin1 association is affected by the ability of GSK3β and PP2A to associate with Axin1 as well as

Pin1 expression, we asked whether c-Myc expression levels can also affect the formation of the Axin1 mediated degradation complex for c-Myc. 293 cells were transfected with CMV-driven control or HA-c-Myc and either control or V5-Axin1 as indicated (Figure 3.12). Following immunoprecipitation with αV5 I found that in the absence of ectopic HA-c-Myc there was significantly less GSK3β and no detectable PP2A-C associated with V5-Axin1 as compared to that seen in the presence of transfected and immunoprecipitated HA-c-Myc (Figure 3.12, compare lane 4 to lane 8). Of note, I observed that the GSK3β coimmunoprecipitating with V5-Axin1 (Figure 3.12, lane 8) lines up with the phosphorylated form of GSK3β in the input samples (Figure 3.12, lane 6). We do not know what site in GSK3β is phosphorylated and therefore, we do not know the functional consequences of this finding. These findings suggest that c-Myc association with Axin1 enhances the association of GSK3β and PP2A with Axin1. It is also possible that c-Myc may drive the expression of some key components found within the degradation complex. In support of this theory we have found that c-Myc increases the expression of the PP2A regulatory B subunit, B56α (Deanne Tibbits, data not shown). Altogether, c-Myc likely enhances the formation of the degradation complex by both stabilizing the association of GSK3β and PP2A with Axin1 as well as increasing the expression of some key components found within the degradation complex.

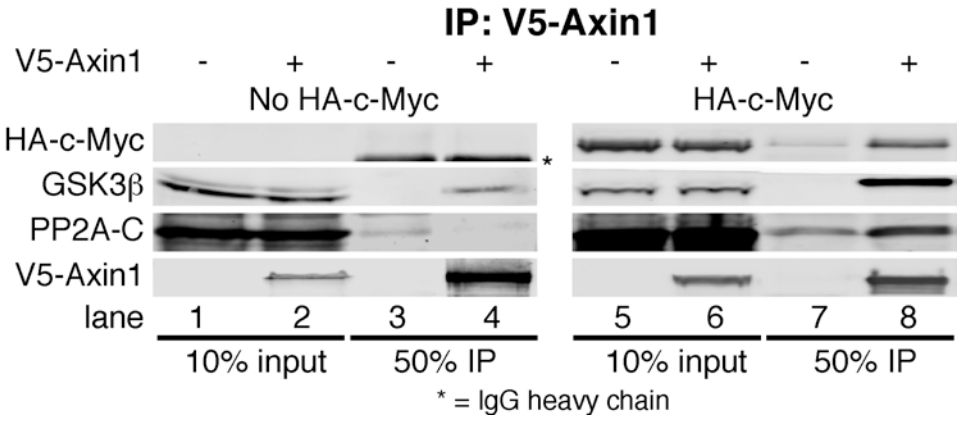


Figure 3.12: Expression of c-Myc increases the association of GSK3β and PP2A with Axin1.

CMV-driven β-gal plus empty, HA-c-Myc and/or V5-Axin1 were cotransfected into 293 cells. Cleared lysates were collected, volumes adjusted by β-gal and immunoprecipitated with αV5. Western blot analysis was performed on input and IP samples with indicated antibodies.

Putative CK1α phosphorylation sites affect c-Myc association with Axin1.

Previous work by a collaborator of ours, Dr. Virshup from the Huntsman Cancer Institute demonstrated that CK1α-mediated phosphorylation of Axin1 increased the association of GSK3β with Axin1. They generated two phosphorylation mutant constructs of Axin1, which have point mutations in putative CK1α phosphorylation sites. Since c-Myc association with Axin1 is affected by the association of GSK3β with Axin1, I tested whether these putative CK1α phosphorylation sites affected c-Myc association with Axin1. I cotransfected 293 cells with CMV-driven c-Myc and either control, HA-tagged wild-type Axin1, HA-tagged phosphorylation four site mutant (4M), or HA-tagged two site mutant (2M) constructs (Figure 3.13A, diagram). Following αHA immunoprecipitation I found that c-Myc, GSK3β, and PP2A readily associated with wild-type HA-Axin1, but c-

Myc association with both phosphorylation point mutant constructs of HA-Axin1 was significantly reduced, as was the association of GSK3β and PP2A (Figure 3.13B, compare lanes three and four to lane two). This finding suggests that CK1α-mediated phosphorylation of Axin1 is important for the formation of the Axin1-mediated c-Myc degradation complex.

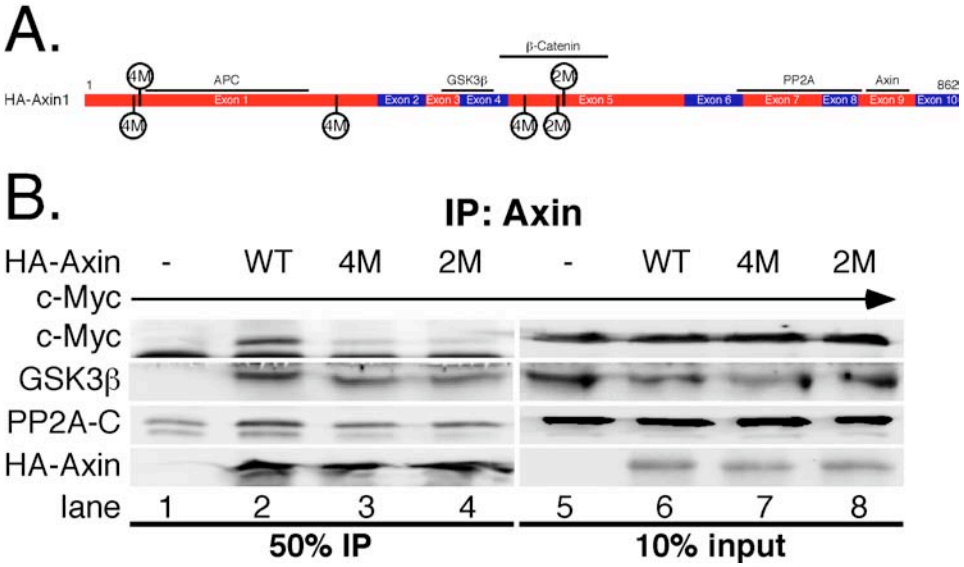


Figure 3.13: Intact CK1α phosphorylation sites are important for c-Myc, GSK3β, and PP2A association with Axin1.

293 cells were cotransfected with β-gal, c-Myc and either empty, HA-Axin1 WT, 4M, or 2M as indicated. Cleared lysates were collected, volumes adjusted based on β-gal activity and immunoprecipitated with αHA.11. Input and IP samples were subjected to western blot with indicated antibodies.

Axin1 associates with transactivation domain of c-Myc and Axin1 association with c-Myc significantly increases with S62 phosphorylation. Since GSK3β, Pin1 and PP2A-B56α modulate the phosphorylation of T58 and S62, which reside in the transactivation domain (TAD) of c-Myc (Figure 3.13A, diagram) I examined whether Axin1 associates with the TAD of c-Myc. 293 cells

were cotransfected with Axin1 and either empty control, V5-c-Myc^{WT} or V5-c-Myc$^{\Delta TAD}$ followed by immunoprecipitation with αV5. I found that Axin1 only coimmunoprecipitated in the presence of V5-c-Myc^{WT}, but not V5-c-Myc$^{\Delta TAD}$ (Figure 3.14B, compare lane 2 to 3) showing that Axin1 associates with the TAD of c-Myc. This finding is consistent with our previous result showing that PP2A-B56α associates with the TAD of c-Myc (Arnold and Sears 2006).

Since the transactivation domain of c-Myc contains the two highly conserved phosphorylation sites, T58 and S62 (Figure 3.14A, diagram), that we and others have previously characterized to regulate c-Myc protein stability, we wanted to assess whether phosphorylation of these sites affects Axin1 association with c-Myc. To do this I coexpressed CMV-driven Axin1 with either empty control, V5-c-Myc^{WT}, V5-c-Myc^{T58A} or V5-c-Myc^{S62D} in 293 cells. αV5 immunoprecipitates were washed stringently to challenge the interaction of Axin1 with the different forms of c-Myc. I found that Axin1 strongly associated with c-Myc^{T58A} as compared to c-Myc^{WT} (Figure 3.14C, compare lane 3 to lane 2). Previous studies of c-Myc^{T58A} show it has significantly higher S62 phosphorylation levels as compared to c-Myc^{WT} due to the inability of PP2A to dephosphorylate this mutant. Therefore, this finding suggests that constitutive S62 phosphorylation increases the association of Axin1 with c-Myc. In support of this hypothesis, Axin1 associated with the S62 phosphorylation mimic, c-Myc^{S62D} to a greater degree as compared to c-Myc^{WT} (Figure 3.13C, lane 4 compared to lanes 2 and 3). To determine whether lack of S62 phosphorylation diminishes the

association of Axin1 with c-Myc I coexpressed CMV-driven V5-Axin1 with control, HA-c-Myc^{WT}, or HA-c-Myc^{S62A}. Following stringent washing of αHA immunoprecipitates I found that Axin1 again associated with c-Myc^{WT} (Figure 3.14D, lane 2). However, Axin1 did not associate with c-Myc^{S62A} any more than that observed in the negative control (Figure 3.14D, compare lane 3 to lane 1). These findings suggest that c-Myc S62 phosphorylation may be important for recruitment of Axin1 to c-Myc.

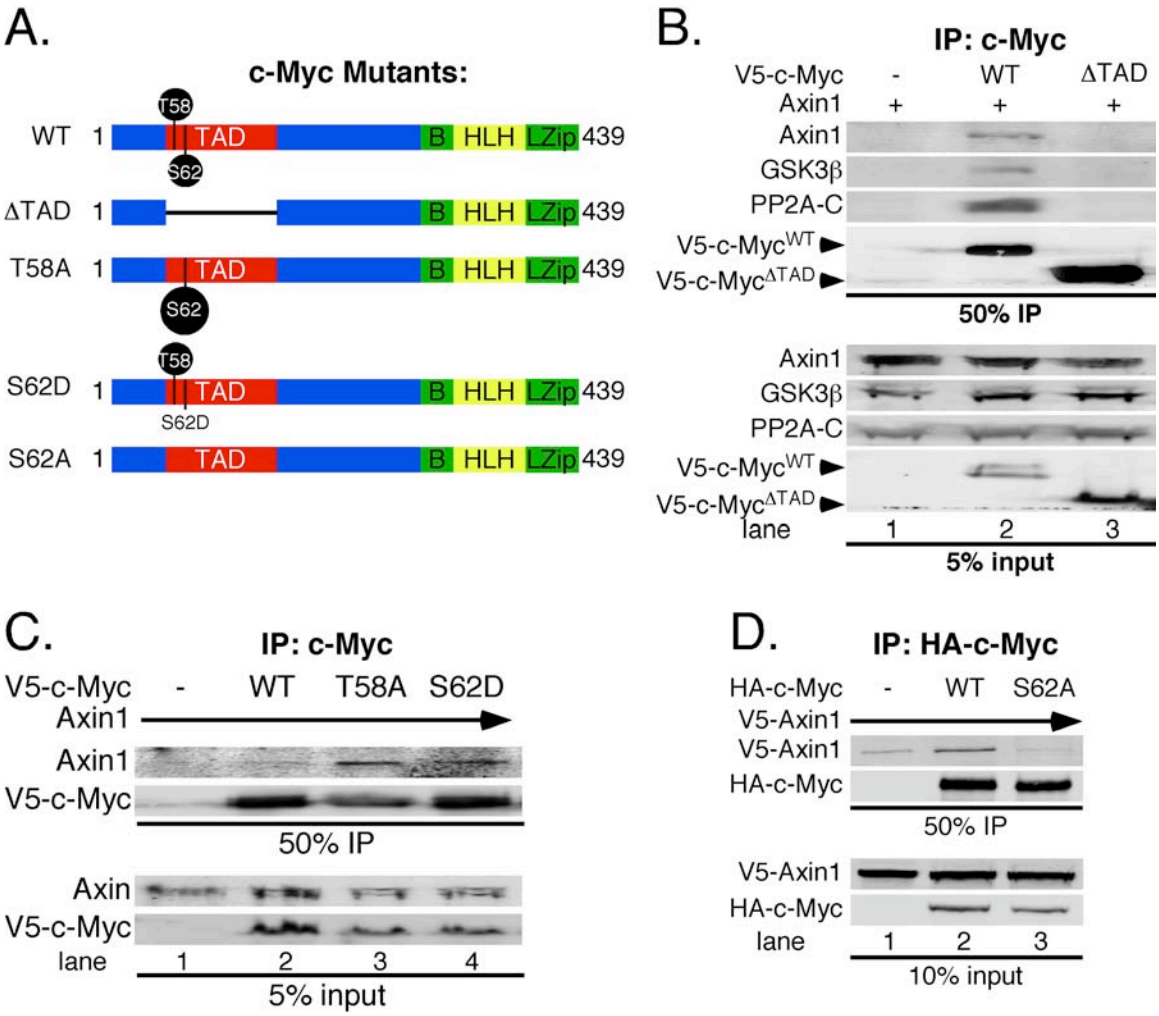


Figure 3.14: Axin1 association with c-Myc requires Serine 62 within the transactivation domain of c-Myc.

(A) Diagram of c-Myc WT, ΔTAD, T58A, S62A, S62D and S62A showing the transactivation domain (TAD), relative phosphorylation at T58 and S62, and basic (B) helix-loop-helix (HLH) leucine zipper (Lzip) domain. (B) Axin1, GSK3β and PP2A associate with the transactivation domain of c-Myc. CMV-driven β-gal, Axin1, and either empty, V5-c-Myc, or V5-c-Myc$^{\Delta TAD}$ were cotransfected into 293 cells as indicated. Cleared lysates were collected, volumes adjusted based on β-gal activity, and immunoprecipitated with αV5. Input and IP samples were analyzed by western blot with indicated antibodies. (C) Constitutive serine 62 phosphorylation on c-Myc increases the association of Axin1 with c-Myc. 293 cells were cotransfected with β-gal, Axin1, and either empty, V5-c-Myc WT, T58A or S62D as indicated. Cellular lysates were cleared, volumes adjusted based on β-gal, and immunoprecipitated with αV5. Input and IP samples were analyzed by western blot with indicated antibodies. (D) Serine 62 phosphorylation on c-Myc is required for Axin1 to associate with c-Myc. CMV-driven β-gal, V5-Axin1, and either empty, HA-c-Myc WT or S62A as indicated. Cleared cellular lysates were collected, volumes adjusted by β-gal activity and immunoprecipitated with αHA.11. Western blot analysis was performed on input and IP samples with indicated antibodies.

Axin1 expression suppresses peak c-Myc protein accumulation in cells entering G_1 from G_0. We and others have previously shown that cells exiting a quiescent, G_0 state and entering the cell cycle display a peak accumulation of c-Myc protein levels at 3-6 hours in early G_1, followed by a reduction in c-Myc protein levels to approximately 30% of peak levels by the end of G_1. The accumulation of c-Myc protein in early G_1 is due to both transcriptional up-regulation of *c-myc* mRNA levels as well as increased c-Myc protein stability (Sears 2004). To examine whether this accumulation of c-Myc protein levels in early G_1 could be suppressed by Axin1 expression I infected IMR90 human primary fibroblast cells with Lenti-virus to express GFP or V5-Axin1 and Adenovirus to express CMV driven HA-c-Myc as indicated in Figure 3.15. Consistent with our previous findings c-Myc protein levels followed a “bell-

shaped" curve with peak c-Myc protein levels between 3-6 hours after stimulation in early G_1 and reduced c-Myc protein levels by 12 hours in late G_1 (Figure 3.15, left middle panel). In contrast, the "bell-shaped" peak accumulation of c-Myc protein normally seen in early G_1 was suppressed by expression of V5-Axin1 (Figure 3.15 right middle panel). This finding demonstrates that ectopic Axin1 expression suppresses the normal accumulation of c-Myc protein in early G_1, which could possibly result in delayed entry into S phase.

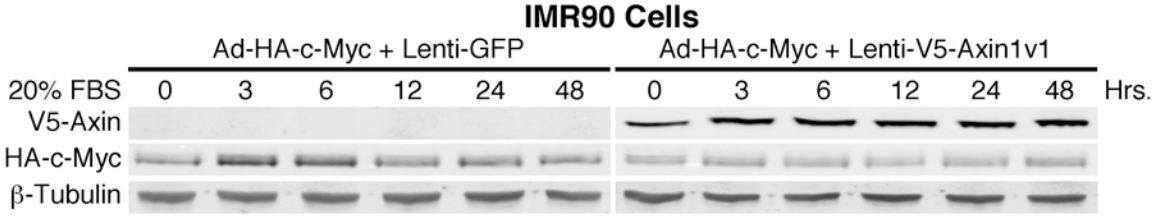


Figure 3.15: Axin1 can suppress c-Myc peak protein levels as cells enter G_1.

IMR90 cells were infected with Lenti-virus containing CMV-driven GFP or V5-Axin1, starved 48 hour in DMEM supplemented with 0.2% FBS, and then infected with Adenovirus containing CMV-HA-c-Myc for 16 hours. Cells were then stimulated with 20% FBS and whole cell lysates were collected at indicated time-points. Samples were analyzed by western blot with indicated antibodies.

Identification of an Axin1 mutation in the Pre-B ALL SupB15 cell-line that disrupts the association between GSK3β and Axin1, reduces c-Myc/Axin1 binding and fails to suppress c-Myc dependent transcription. We have previously characterized several leukemia cell-lines and found that c-Myc protein stability was significantly increased in the majority of these cell-lines. In particular c-Myc protein stability was increased nearly 3.5 fold in the pre-B ALL cell-line, SupB15, as compared to another leukemia cell-line, HL60, which

showed a normal turnover rate for c-Myc protein (Malempati et al. 2006). Moreover, it was shown that c-Myc association with GSK3β was disrupted in the SupB15 cell-line as compared to the HL60 cell-line. Interestingly, sequencing of *c-myc* mRNA from the SupB15 cell-line revealed no mutations within the region of c-Myc in which GSK3β interacts with and phosphorylates c-Myc. *Gsk3β* mRNA was not analyzed for mutations in the SupB15 cell-line since there are no reported naturally occurring mutations in *gsk3β*. Although, we clearly observed a disruption in the association of c-Myc with GSK3β the mechanism for the disruption remained unknown. However, following our identification of Axin1 as a scaffold protein that associates with GSK3β and c-Myc, combined with the numerous reports of mutations in *axin1* in solid tumors, we decided to sequence *axin1* mRNA from our previously characterized leukemia cell-lines. We found an in-frame deletion within the GSK3β binding domain of Axin1 in the SupB15 cell-line (Figure 3.16A). Sequencing of the entire mutant *axin1* transcript also revealed that the transcript we cloned was the naturally occurring splice variant two of *axin1*, which has exon eight removed. I showed in Figure 3.9A that variant two of Axin1 shows reduced association with PP2A and c-Myc. Therefore, I assessed the ability of this mutant form of Axin1 variant two from the SupB15 cells to associate with GSK3β, PP2A, and c-Myc. 293 cells were cotransfected with CMV-driven c-Myc and either control, V5-Axin1 variant one (1v1), V5-Axin1 variant two (1v2), V5-Axin1v1$^{\Delta Ex3\text{-}4}$, and V5-Axin1v2^{SupB15}. Following immunoprecipitation with αV5 I found that c-Myc did not associate with V5-

Axin1v2, V5-Axin1v1$^{\Delta Ex3-4}$, or V5-Axin1v2^{SupB15} as robustly compared to wild-type V5-Axin1v1 Figure 3.16B, top panel). I also found that GSK3β did not associate with V5-Axin1v1$^{\Delta Ex3-4}$ or V5-Axin1v2^{SupB15} at all, but did associate with V5-Axin1v1 and V5-Axin1v2 (Figure 3.15B, middle panel). These findings demonstrate that the mutant form of Axin identified from the SupB15 cells lacks the ability to associate with GSK3β and has reduced association with c-Myc, which could explain why the association between c-Myc and GSK3β is disrupted in SupB15 cells. Moreover, we have previously shown that inhibiting GSK3β function by expressing a dominant negative GSK3β results in the accumulation of c-Myc protein and decreased T58 phosphorylation (Sears et al. 2000). In an analogous manner it is likely that disruption of the association between Axin and GSK3β could also result in increased c-Myc protein levels due to the inability of GSK3β to efficiently associate with c-Myc and facilitate its turnover.

Given the important role GSK3β plays in negatively regulating c-Myc protein levels I asked whether the mutant form Axin from SupB15 cells would have an affect on c-Myc dependent transcription. I therefore transfected 293 cells as previously described with either E2F2-Luc or E2F2(-Ebox)-Luc and either CMV-driven control or c-Myc as well as V5-Axin1v1, V5-Axin1v2, V5-Axin$^{\Delta Ex3-4}$ or V5-Axin^{SupB15} as indicated in Figure 3.16C. Consistent with my previous results in Figure 3.7, right panel, c-Myc expression alone induced a greater than six fold increase in luciferase activity from the E2F2-Luc construct as compared to control. Also consistent with my previous results, V5-Axin1v1 expression

robustly suppressed the c-Myc dependent increase in luciferase activity from the E2F2-Luc construct. Interestingly, expression of V5-Axin1v2 also significantly reduced c-Myc dependent luciferase activity (Figure 3.16C). However, neither V5-Axin$^{\Delta Ex3\text{-}4}$ nor V5-Axin^{SupB15} suppressed c-Myc dependent luciferase activity significantly (Figure 3.16C). This result shows that independent of whether variant one or two (presence or absence of exon eight) of Axin1 is expressed, it is the association of GSK3β with Axin that is critical for Axin1's ability to suppress c-Myc dependent transcription. Furthermore, our previous report showing increased c-Myc protein stability in the SupB15 cell-line, suggests that the association of GSK3β with Axin is also critical for Axin1's ability to negatively regulate c-Myc protein levels.

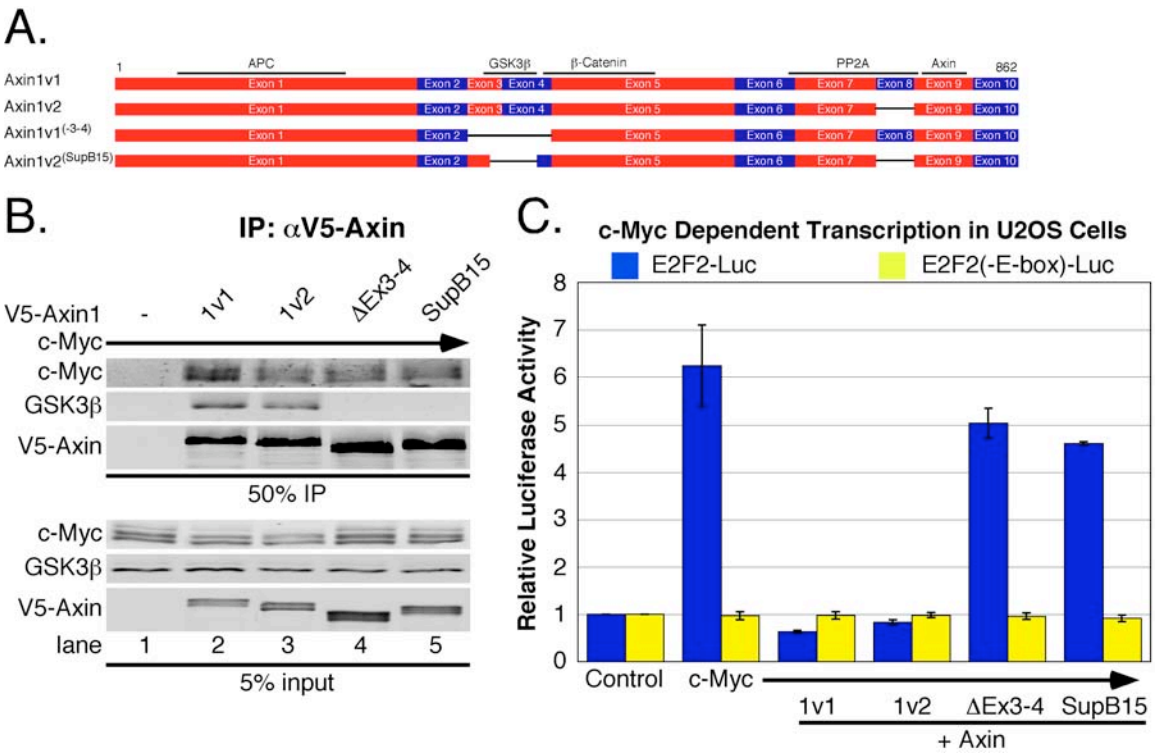


Figure 3.16: Identification of mutant Axin1 from PreB-ALL SupB15 cell-line that no longer negatively regulates c-Myc dependent transcription.

(A) Diagram of Axin1 constructs used. Exons and deleted regions of Axin1 are shown along with reported binding domains of known Axin1 interacting proteins. (B) Mutant form of Axin1 identified from SupB15 cells shows reduced association with c-Myc. 293 cells were transfected with CMV-driven β-gal, c-Myc and either empty, V5-Axin1v1, 1v2, ΔEx3-4, and SupB15 mutant as indicated. Cleared lysates were collected, volumes adjusted by β-gal, and immunoprecipitated with αV5. Western blot analysis was done on input and IP samples with indicated antibodies. (C) Axin1 mutants unable to associate with GSK3β do not suppress c-Myc dependent transcription. 293 cells were cotransfected with CMV-driven β-gal and either E2F2-Luc or E2F2(-E-box)-Luc and either empty or c-Myc plus either V5-Axin1v1, 1v2, ΔEx3-4, or SupB15 mutant as indicated. Cells were maintained in DMEM supplemented with 2% FBS for 48 hours prior to collection. Cellular lysates were analyzed for β-gal and luciferase activity. Luciferase activity was adjusted to β-gal activity from three separate experiments and average adjusted luciferase activates with standard deviations were graphed using Excel.

Switch in Axin1 splice variant expression in breast cancer cell-lines. This section will be brief, as we do not fully understand the importance of our findings

in breast cancer cell-lines. Having seen a number of reported mutations in Axin1 from breast cancers we sequenced *axin1* from several breast cancer cell-lines and found no mutations. However, we did find a statistically significant switch in the splice variants of Axin1 being expressed in a some breast cancer cell-lines that have stable c-Myc protein. In particular, we found that MCF7 and SKBR3 cells have significantly higher expression of *axin1v2* as compared to *axin1v1* (Figure 3.17B). This finding was confirmed by qRT-PCR (Xiaoli, data not shown) and interestingly, these two cell-lines also have stabilized c-Myc protein (Xiaoli, data not shown). I have consistently found that both c-Myc and PP2A associate with Axin1v2 approximately half as well as compared to Axin1v1 (Figure 3.17C). On the other hand, GSK3β association with Axin1v2 only showed a slight reduction in association with Axin1v2 as compared to Axin1v1 (Figure 3.17C). It is possible that Axin1v2 is less efficient at degrading c-Myc protein due to decreased association with c-Myc and PP2A, which could help explain the increased stability of c-Myc in these breast cancer cell-lines. However, I also observe that Axin1v2 can robustly suppress c-Myc dependent transcription from a reporter construct (Figure 3.16C). Therefore, we are not clear about the significance of the switch in the expression levels between *axin1v2* and *axin1v1* in breast cancer cell-lines.

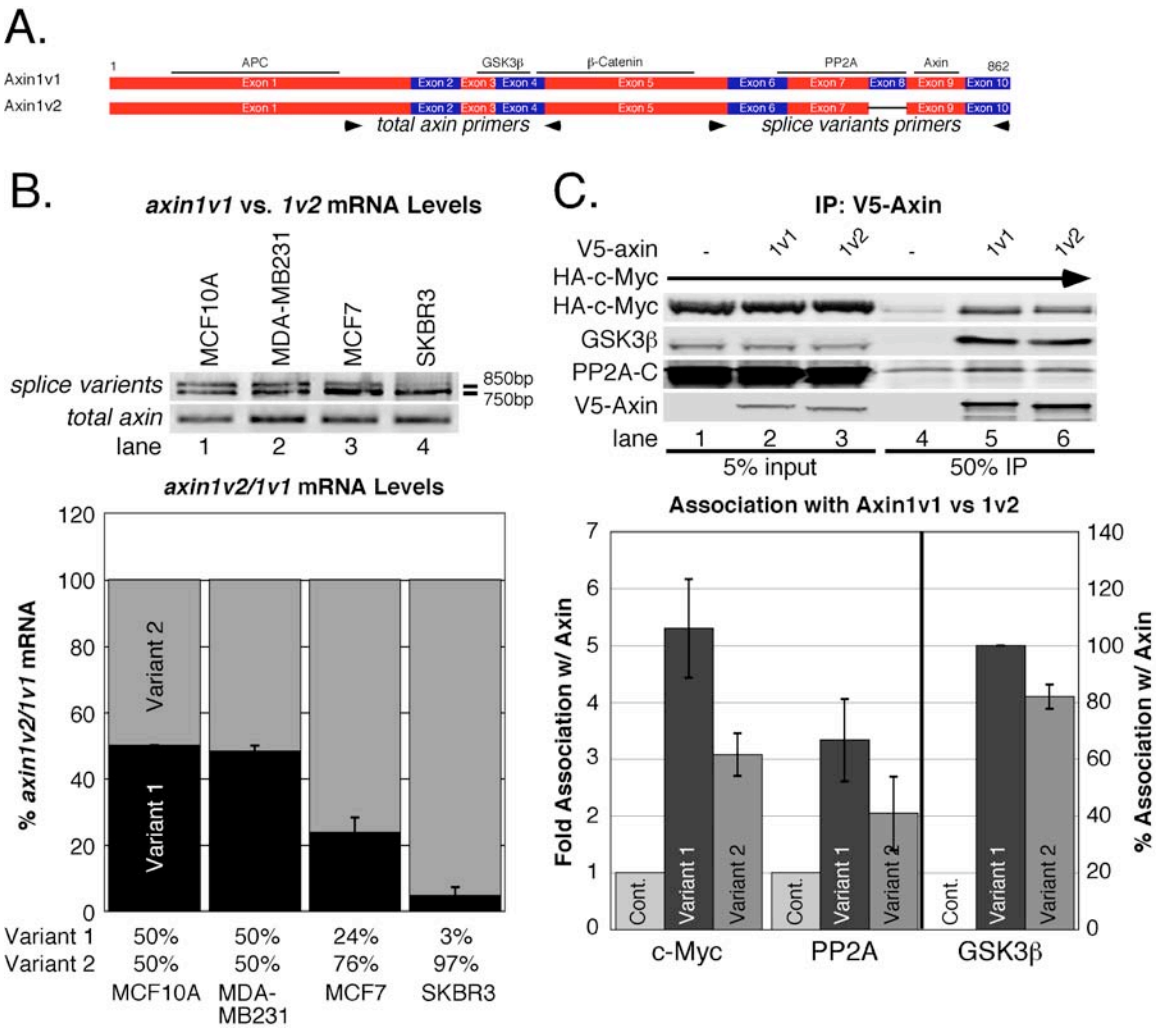


Figure 3.17: Switch in expression of *axin1* splice variants.

(A) Diagram of Axin1v1 and Axin1v2. Exons and binding domains of known Axin1 interacting proteins are shown along with primer binding sites for detecting total and splice variants of Axin1. (B) Switch towards *axin1v2* compared to *axin1v1* in breast cancer cell-lines with stabilized c-Myc protein. RNA was isolated from MCF10A, MDA-MB231, MCF7 and SKBR3 cells and cDNA was generated for semi-quantitative PCR analysis using total and variant primers. Average ratio of *axin1v1* versus *axin1v2* was quantitated and graphed with standard deviations from three separate experiments. (C) Axin1v1 associates with GSK3β, PP2A and c-Myc more robustly than Axin1v2. 293 cells were cotransfected with CMV-driven β-gal, HA-c-Myc and either empty, V5-Axin1v1 or Axin1v2 as indicated. Cleared lysates were collected, volumes adjusted based on β-gal activity, and immunoprecipitated with αV5. Input and IP samples analyzed by western blot with indicated antibodies. Western blot data from three separate experiments was quantitated using LI-COR software and averages with

standard deviations were graphed using Excel for the association of c-Myc, GSK3β, and PP2A with Axin1.

Discussion:

We previously characterized critical roles for GSK3β, PP2A-B56α, and Pin1 in promoting c-Myc protein turnover (Sears et al. 2000; Yeh et al. 2004; Arnold and Sears 2006). We report here that the scaffold protein, Axin1, coordinates GSK3β, PP2A-B56α, and Pin1 into a degradation complex for c-Myc. Moreover, each of these components facilitates formation of the complex. I demonstrate that Axin1 negatively regulates c-Myc protein levels by negatively regulating c-Myc protein stability. Importantly, the naturally occurring forms of Axin1 potently suppress c-Myc dependent transcription thereby suppressing a critical function of c-Myc oncogenicity. Altogether our findings greatly enhance our understanding of both an important tumor suppressor, Axin1 and a potent oncoprotein, c-Myc.

GSK3β association with Axin1 is critical for Axin1-mediated regulation of c-Myc. Given the important role Axin1 plays in other signaling pathways such as Wnt, TGFβ, SAPK/JNK and p53 signaling, the addition of c-Myc as an Axin1 target protein further highlights the importance of Axin1 function. To date Axin1 has been shown to function as a tumor suppressor in all of these pathways. Additionally, numerous mutations have been identified in Axin1 that likely affect both naturally occurring transcriptional variants of Axin1 in terms of their ability to coordinate protein complexes involved in regulating the above signaling pathways. We demonstrate here that deletion of the GSK3β binding domain

from Axin1 abolished the association of GSK3β with Axin1 and significantly decreased the association of c-Myc with Axin1 as well as significantly impairing the ability of Axin1 to suppress c-Myc dependent transcription. As previously highlighted in the introduction, there are a number of mutations that have been identified from a variety of solid human tumors that fall within the GSK3β binding domain of Axin1 (Salahshor and Woodgett 2005). Some of these mutations in *axin1* identified from breast cancers have been shown to specifically disrupt the association of GSK3β with Axin1 (Webster et al. 2000). Moreover, zebrafish harboring a single point mutation, called the masterblind mutation, that falls within the GSK3β binding domain of Axin1 have a striking phenotype in which the eyes and telencephalon are reduced or absent (Heisenberg et al. 2001). This mutation has been shown to disrupt the association between GSK3β and Axin1 and can be rescued by expression of wild-type Axin1 or overexpression of GSK3β. Altogether, my results combined with previous reports clearly show the importance of GSK3β association with Axin1 for its function, including its negative regulation of c-Myc.

Mitogen signaling affects the formation of the Axin1 mediated degradation complex formation. We have previously shown that mitogenic signaling is very important for regulating c-Myc protein stability. More specifically, c-Myc protein stability is increased by mitogenic activation of the Ras/Raf/MEK/ERK kinase cascade, which phosphorylates c-Myc on S62. Concurrently, Ras can also

activate PI3/Akt signaling which can inhibit GSK3β from phosphorylating c-Myc on T58, thereby holding c-Myc in a signally S62 phosphorylated state where c-Myc protein is found to be stable. However, once Ras activity diminishes GSK3β can phosphorylate c-Myc at T58, followed by dephosphorylation of S62 through the combined activity of Pin1 and PP2A-B56α. Singly T58 phosphorylated c-Myc is unstable as it is readily recognized by the SCF^{Fbw7} ubiquitin machinery, which multi-ubiquitinates c-Myc marking it for degradation by the 26S proteosome. I demonstrate here that mitogenic signaling also affects the ability of Axin1 to form the c-Myc degradation complex containing GSK3β, PP2A-B56α and Pin1. It has previously been shown that the mitogenic stimulated kinase MEKK1 and GSK3β competitively associate with Axin1 resulting in a "switch" of Axin1 mediated complexes (Zhang et al. 2001). It is therefore plausible that mitogen stimulation (10% FBS) activates MEKK1 and promotes the association of MEKK1 with Axin1 thereby preventing GSK3β association with Axin1. We have already shown that preventing the association of GSK3β with Axin1 has a profound affect on the ability of Axin1 to suppress c-Myc dependent transcription. In contrast, it is likely that under low mitogen stimulation conditions (2% and 0.2% FBS) MEKK1 is not activated and does not interfere with Axin1/GSK3β association allowing for the c-Myc degradation complex to form. I also demonstrated that PP2A association with Axin1 is significantly inhibited upon mitogenic stimulation indicating that PP2A association with Axin1 may also be affected by some mitogenic regulatory mechanism. Altogether, these findings demonstrate that c-Myc protein turnover

mediated by the formation an Axin1/GSK3β/PP2A complex is negatively regulated by mitogenic signaling.

T58 and S62 phosphorylation affect the ability of Axin1 and c-Myc to associate as well as the ability of Axin1 to turnover c-Myc protein levels. Axin1 interacts with the transactivation domain of c-Myc containing the highly conserved T58 and S62 residues that under go hierarchal phosphorylation and thereby regulate c-Myc protein stability (Sears et al. 2000; Yeh et al. 2004; Escamilla-Powers and Sears 2007). We have previously demonstrated that point mutants of c-Myc, which prevent phosphorylation of these sites, affect c-Myc protein stability. We found that a point mutant of c-Myc that prevents T58 phosphorylation, c-Myc^{T58A} is significantly more stable and oncogenic than c-Myc^{WT} (Sears et al. 2000; Yeh et al. 2004). Conversely, a point mutant of c-Myc that prevents S62 phosphorylation, c-Myc^{S62A} is either as stable or slightly more stable, but less oncogenic than c-Myc^{WT}. Interestingly, I find the association of Axin1 with c-Myc^{T58A} is significantly more robust than that observed with c-Myc^{WT} (Figure 3.13C). However, c-Myc^{T58A} protein levels are not reduced by Axin1 expression (Figure 3.6). This suggests that c-Myc^{T58A} acts as a "substrate trap" for Axin1.

We have previously suspected that c-Myc^{T58A} acts as a "substrate trap" when I identified PP2A-B56α as the PP2A holoenzyme that promotes dephosphorylation of S62 on c-Myc and demonstrated increased association

between B56α and c-Myc^{T58A} (Arnold and Sears 2006). It is possible that by sequestering Axin1 along with PP2A-B56α, c-Myc^{T58A} may inhibit Axin1 and PP2A-B56α function in other pathways. Of particular interest is the role that Axin1 and PP2A-B56α play in the induction of apoptosis where Axin1 regulates SAPK/JNK and p53 induced apoptosis and PP2A-B56α has been shown to prevent Bcl2 activation thereby preventing survival signaling. By this mechanism, c-Myc^{T58A} may inhibit the induction apoptosis, which is consistent with studies showing that under conditions where c-Myc^{WT} induces apoptosis, c-Myc^{T58A} does not (Chang et al. 2000; Hemann et al. 2005).

In contrast to c-Myc^{T58A}, c-Myc^{S62A} does not associate with Axin1 (Figure 3.13D) and expression of Axin1 does not reduce c-Myc^{S62A} protein levels (Figure 3.6). We and others have previously demonstrated that c-Myc^{S62A} is not phosphorylated on T58 since T58 phosphorylation by GSK3β requires prior phosphorylation at S62. Moreover, c-Myc^{S62A} is not recognized by the SCF^{Fbw7} (Welcker et al. 2004) and therefore, c-Myc^{S62A} is likely degraded by another mechanism. Altogether we speculate that S62 phosphorylation of c-Myc is required for the association of Axin1 with c-Myc and T58 phosphorylation is required for Axin1 to turnover and release c-Myc protein.

***axin1v1* verses *axin1v2* splice variants show altered expression correlating with c-Myc stability in breast cancer cell-lines.** There are two naturally occurring splice variants of Axin1 where variant 2 lacks part of the PP2A binding

domain. While little is known about the regulation of these splice variants, there appears to be a correlation between the relative increase in the expression of *axin1v2* compared to *axin1v1* in breast cancer cell-lines as c-Myc protein stability increases. For example, SKBR3 cells have the lowest expression of *axin1v1* at 3% of total *axin1* expressed and have the longest c-Myc protein half-life at ~80min. Whereas, c-Myc protein half-life in MCF7 is ~47min and the expression of *axin1v1* is 24% of the total *axin1* expressed. Lastly, the more "normal" breast cancer cell-line, MCF10A exhibits 50% expression of *axin1v1* compared to total *axin1* and a half-life for c-Myc of ~21min (analysis of c-Myc half-life in breast cancer cell-lines examined by Xiaoli, data not shown). Thus far the best explanation we have is that Axin1v1 associates with GSK3β and PP2A more robustly than Axin1v2 and thus is likely more potent at facilitating c-Myc degradation (Figure 3.17C). However, Axin1v2 can still robustly suppress c-Myc dependent transcription as measured by a reporter plasmid driven by the E2F2 promoter nearly to the same degree as Axin1v1 (Figure 3.16C). Thus, Axin1v2 may also function to suppress c-Myc oncogenicity, although it may not be as active for promoting c-Myc turnover. This observation clearly needs to be addressed further and is currently being explored by Xiaoli Zhang.

Chapter Four :

Summary and Discussion

PP2A-B56α regulation of c-Myc

Identifying PP2A-B56α is a PP2A holoenzyme with tumor suppressor function. The identification of a particular PP2A holoenzyme, PP2A-B56α that negatively regulates c-Myc protein levels and transcriptional activity is described in chapter two. This is an important finding since it is now becoming clear that PP2A has both oncogenic and tumor suppressor potential and it is the PP2A holoenzyme, in particular the incorporation of a particular regulatory B subunit that determines whether a PP2A holoenzyme will have oncogenic or tumor suppressor capacity. Accordingly, identification and characterization of PP2A-B56α negative regulation of c-Myc protein levels and transcriptional activity demonstrate a tumor suppressor function for this PP2A holoenzyme. Moreover, a tumor suppressor function for PP2A-B56α is also strongly supported by its negative regulation of β-catenin protein levels and of the anti-apoptotic function of Bcl2 (Seeling et al. 1999; Ruvolo et al. 2002). Furthermore, mutations in the PP2A-A subunit have been identified in human breast tumors that specifically disrupt the ability of the B56α regulatory B subunit to associate with the A subunit and form PP2A-B56α holoenzymes (Ruediger et al. 2001b). Altogether, these findings clearly support a tumor suppressor role for PP2A-B56α.

In support of the conclusion that PP2A-B56α has important tumor suppressor function, RNAi knockdown of B56α aids in the transformation of human primary cells (Sarah Byers, data not shown). It is important to note that knockdown of B56α alone does not transform cells as these cells also stably

express SV40 Large T antigen (inhibits p53 and RB), H-Ras, and hTERT. However, these cells have previously been used to demonstrate that global inhibition of PP2A by expression of SV40 small t antigen is required, in addition to the inhibition of p53 and RB as well as activation of Ras and hTERT, to transform these cells. Furthermore, this system was used to identify the regulatory B subunit, B56γ as a regulatory B subunit with tumor suppressor function (Chen et al. 2004). Altogether, the *in culture* studies of B56α strongly suggest that PP2A-B56α is a PP2A holoenzyme with tumor suppressor function.

Several studies have now begun to examine the role of PP2A-B56α *in vivo* and from these studies it is becoming clear that B56α has important roles in development. It has been shown that B56α has an important role in axis formation in *xenopus* through its role in Wnt signaling and the regulation of β-catenin (Seeling et al. 1999; Li et al. 2001). Unfortunately, there is not a B56α knockout mouse yet to evaluate the requirement for B56α in the development of mammals, which is why we are currently in the process of generating this knockout mouse. We will also be able to evaluate the tumor suppressor function of B56α in these mice. Clearly, the next step in understanding the importance of PP2A-B56α in tumorigenesis will center on *in vivo* experiments.

PP2A holoenzyme formation is critical for PP2A activity. In the course of identifying and characterizing PP2A-B56α regulation of c-Myc protein levels and transcriptional activity we also gained insight into the importance of holoenzyme

formation for PP2A activity. Our previous findings examined the regulation of PP2A activity on c-Myc protein levels utilizing global inhibitors of PP2A. In chapter two I followed up on these findings by examining the affects of increasing PP2A activity on c-Myc protein levels. I found that globally increasing PP2A activity by overexpression of a HA-tagged C subunit, decreased c-Myc protein levels maximally to 60% as compared to control (Figure 2.1A and B). In this experiment we saw indications that PP2A activity was limited and most likely the limiting factor was the presence of the regulatory B subunit, which directs PP2A activity towards c-Myc. In support of this, I showed that disrupting the ability of regulatory B subunits to incorporate into a PP2A holoenzyme by expression of SV40 small t, could not be overcome by co-overexpression of the HA-tagged C subunit that I previously showed to reduce c-Myc protein levels (Figure 2.1). I also showed that the combined expression of the HA-tagged C and B56α potently reduced elevated c-Myc protein levels to approximately 20% of control levels, whereas the individual expression either HA-tagged C or B56α did not have a great effect on elevated c-Myc protein levels (Figure 2.6). More recently it has been shown that PP2A holoenzyme formation is critical to PP2A activity as C subunit isolated by immunoprecipitation of the A or B subunits had considerably higher catalytic activity as compared to C subunit isolated directly (Hombauer et al. 2007). Altogether, these findings demonstrate the importance of PP2A holoenzyme formation, especially the formation of PP2A-B56α holoenzymes in cancers with elevated c-Myc protein expression.

PP2A-B56α holoenzyme function can be disrupted by several mechanisms. Interestingly, we have been informed by a collaborator of ours that B56α is suppressed at the protein level in human melanoma tumors that exhibit elevated expression of c-Myc protein as compared to normal melanocytes (Dr. Misha Nikiforov, data not shown). Furthermore, we have been informed by another group using conditional expressing *c-myc* mice that in all c-Myc driven lymphomas from these mice, the promoter region of *B56α* was hypermethylated, presumably silencing the expression of B56α. Surprisingly, our initial analysis of human leukemia cell-lines that have stabilized c-Myc showed an increase in B56α expression at the protein level using β-tubulin as a reference for relative expression as compared to control cell-lines with unstable c-Myc (Deanne Tibbitts, data not shown). However, further evaluation of B56α expression relative to the expression of the A and C subunits of PP2A revealed that the leukemia cell-lines with stabilized c-Myc had lower relative expression as compared to control cell-lines with unstable c-Myc. This finding suggested that the stochiometeric expression of B56α relative to the A and C subunit expression levels was in fact lower in leukemia cell-lines with stabilized c-Myc as compared to cell-lines with unstable c-Myc protein. We also found that the incorporation of B56α into a PP2A holoenzyme form appears to be compromised in the leukemia cell-lines with stabilized c-Myc as compared to control cells with unstable c-Myc (Deanne Tibbitts, data not shown). It is possible that this apparent disruption of PP2A-B56α holoenzyme formation maybe due to the stochiometeric shift in

expression of B56α as compared to expression of the A and C subunits. Therefore, the exact mechanism(s) for the overall decrease in PP2A-B56α holoenzymes will need to be further explored. One possible mechanism that should be explored first is sequencing of the A subunit as it has been demonstrated that disruption of PP2A holoenzymes specifically containing the B56α regulatory subunit due to mutations in the A subunit are observed in human tumors (Ruediger et al. 2001b). These findings demonstrate that PP2A-B56α holoenzyme formation can be disrupted by several mechanisms, which may have important consequences in human cancer.

An alternative mechanism that could lead to disruption of PP2A-B56α holoenzyme activity is a "substrate trap" mechanism by which B56α and/or PP2A-B56α holoenzymes can be abnormally sequestered. In chapter two I found that B56α had increased affinity for c-Myc^{T58A} (Figure 2.5B) and it was previously shown that c-Myc^{T58A} is resistant to dephosphorylation by PP2A (Yeh et al. 2004). It is possible that c-Myc^{T58A} acts as a "substrate trap" for B56α. The idea of sequestering B56α by a "substrate trap" mechanism was also proposed when it was shown that the oncogenic form of p63, ΔNp63 interacted with B56α resulting in increased β-catenin protein levels (Patturajan et al. 2002). Interestingly, we and others have previously shown that c-Myc^{T58A} is qualitatively different from c-Myc^{WT}, as c-Myc^{T58A} has reduced apoptotic activity in cell culture and in vivo and is more oncogenic in vivo (Chang et al. 2000; Yeh et al. 2004; Hemann et al. 2005). While examination of differences in the transcriptome of

cells expressing c-Myc^{T58A} verses c-Myc^{WT} by microarray analysis do reveal differences (data not shown), it is not clear whether these differences are a direct result of altered c-Myc^{T58A} transcriptional activity. Therefore, it is possible that c-Myc^{T58A} exerts its increased oncogenicity and differences in the transcriptome in part through its ability to sequester PP2A-B56α away from targets such as Bcl2 and β-catenin. The ability to prevent PP2A-B56α from inactivating Bcl2 could increase survival as Bcl2 activity has been shown to promote survival. Moreover, by preventing PP2A-B56α from negatively regulating β-catenin, c-Myc^{T58A}, could cause changes in the transcriptome through increased β-catenin/Tcf/Lef transcriptional activity. Also, β-catenin is a critical factor for stem cell maintenance and therefore disrupting the normal regulation of β-catenin could increase cancer stem cell populations. Preliminary findings from conditional expressing c-Myc^{T58A} mice do show an increased upper molecular weight Bcl2 band consistent with phosphorylated and active Bcl2 as compared to conditionally expressing c-Myc^{WT} mice (Xiaoyan Wang, data not shown). The effect of c-Myc^{T58A} expression on β-catenin protein levels and transcriptional activity is currently being examined. Altogether, our identification of PP2A-B56α as a negative regulator of c-Myc protein levels and transcriptional activity as well as our growing understanding of the mechanisms by which disruption of PP2A-B56α holoenzyme occurs in cells with stable c-Myc supports a role for PP2A-B56α as a PP2A holoenzyme with tumor suppressor function.

PP2A-B56α regulates multiple oncogenes and may therefore be a keystone tumor suppressor. As mentioned above, PP2A-B56α has been reported to negatively regulate β-catenin protein levels and suppress Bcl2 anti-apoptotic function, and we now report a role for PP2A-B56α in negatively regulating c-Myc protein levels and transcriptional activity, summarized below in figure 4.1. This places PP2A-B56α in a critical role regulating several potent oncoproteins, highlighting the importance of maintaining normal PP2A-B56α function. As discussed above we and others have found in human tumors and cancer cell-lines that in some cases B56α expression is impaired, PP2A-B56α holoenzyme formation is disrupted, or the PP2A-B56α holoenzyme can be sequestered by a "substrate trap" mechanism. While B56α plays a critical role in regulating several proto-oncoproteins, it has been reported that the expression of *B56α* is quite low (Martens et al. 2004). Moreover, I also found that endogenous B56α expression is limited towards c-Myc (Figure 2.1 A and B). Therefore, disruption of PP2A-B56α activity by any of the above mechanisms allows for the possible co-deregulation of other oncoproteins, appearing as multiple "hits" in the progression of tumorogenisis. Given the multiple tumor suppressor roles for PP2A-B56α, it appears that PP2A-B56α may provide critical tumor suppressor activity.

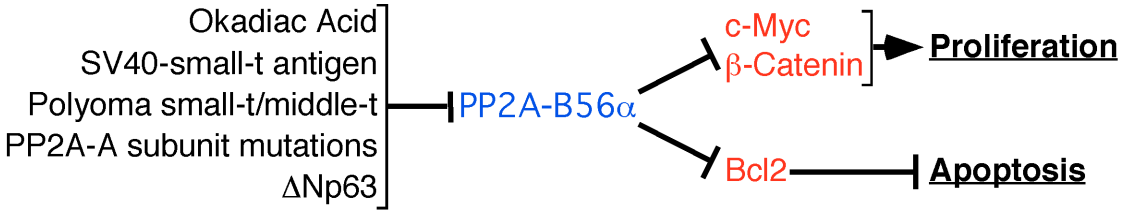


Figure 4.1: PP2A-B56α regulates multiple potent oncoproteins.

Inhibition of PP2A-B56α could cause the co-deregulation of multiple proto-oncoproteins that include β-catenin and Bcl2. The biological outcomes of inhibiting PP2A-B56α function would be increased proliferation and decreased apoptosis.

Axin1 coordinates a degradation complex for c-Myc

Axin1 coordinates a degradation complex for c-Myc that includes GSK3β, Pin1, and PP2A-B56α. The regulation of c-Myc expression at the post-translational level involves a number of proteins, which sequentially modify the phosphorylation status of c-Myc on two highly conserved residues, T58 and S62. To date we have focused of the signaling pathways that activate or repress the activity of the proteins involved in regulating c-Myc phosphorylation at T58 and S62, which control c-Myc expression. It is now becoming clear that signaling pathways and transcription factors are regulated by the coordination of involved proteins into complexes. These protein complexes are physically coordinated by scaffold proteins, which are multi-domain, structural proteins. Therefore, an important aspect regarding the regulation of c-Myc by multiple proteins centered on determining whether these proteins were coordinated into a complex by a scaffold protein. Based on published data, the scaffold protein Axin1 was shown to associate with GSK3β and PP2A-B56α (Behrens et al. 1998; Fagotto et al. 1999; Li et al. 2001). Both GSK3β and PP2A-B56α play critical roles in regulating the phosphorylation status of c-Myc at T58 and S62, which regulate c-Myc protein stability and protein levels. Consequently, I tested whether Axin1 and c-Myc associated and found that Axin1 and c-Myc interact both *in vitro* and in cell culture. I also found that in addition to interacting with GSK3β and PP2A-B56α, the prolyl-isomerase, Pin1 interacted with Axin1 in cell culture experiments (Figure 4.2). Interestingly, I found that expression of Pin1, GSK3β, PP2A-B56α

and c-Myc could all facilitate the formation of the complex mediated by Axin1 (Figure 4.2). I showed that Axin1 knockdown increased c-Myc protein stability and protein levels while ectopic Axin1 expression increased ubiquitination of c-Myc. Importantly, I also found that expression of both naturally occurring splice variants of Axin1 could suppress c-Myc transcriptional activity. Moreover, we identified a mutant form of Axin1 in a leukemia pre-B ALL cell-line, SupB15, that has stabilized c-Myc protein. Our previous work examining possible causes for the increase in c-Myc protein stability in SupB15 cells revealed that the association between c-Myc and GSK3β was impaired in this cell line (Malempati et al. 2006). Interestingly, the mutation we identified in Axin1 in SupB15 cells contains an in-frame deletion in the GSK3β binding domain. Although we have not been able to reintroduce wild-type Axin1 back into these cells, we have found that this mutant form of Axin1 from SupB15 cells does not associate with GSK3β, and c-Myc shows reduced association with this mutant form of Axin1 as compared to wild-type Axin1. Importantly, the SupB15 version of Axin1 was no longer able to suppress c-Myc dependent transcription as measured by an E2F2-reporter construct. In total, our identification and characterization of Axin1 as a scaffold protein coordinating a degradation complex for c-Myc greatly adds to our understanding regarding the highly complex regulation of c-Myc expression and also identifies a new role for the tumor suppressor function of Axin1.

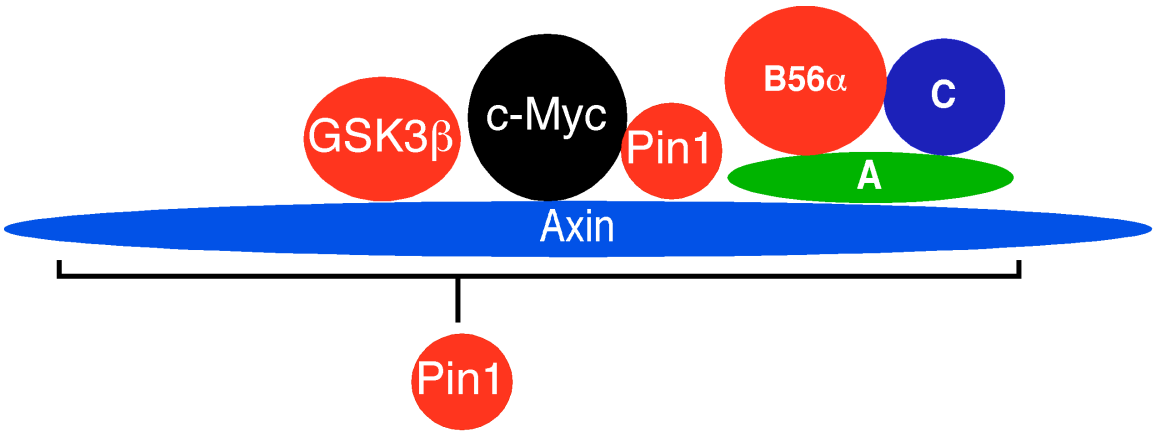


Figure 4.2: Axin1 coordinates c-Myc degradation complex.
Axin1 is a multidomain scaffold protein that associates with GSK3β, PP2A-B56α and Pin1 to form a degradation complex for c-Myc.

GSK3β assoaiation with Axin1 is critical for Axin1 regulation of c-Myc. Our results indicate that the interaction between GSK3β and Axin1 can be critical to the ability of Axin1 to suppress c-Myc dependent transcription (Figure 3.7). Moreover, we identified a mutant form of Axin1 from a leukemia pre-B ALL cell-line that does not bind GSK3β and does not suppress c-Myc dependent transcription efficiently (Figure 3.15). The interaction between GSK3β and Axin1 has also been shown to be important for the ability of Axin1 to promote the turnover of β-catenin. Furthermore, a number of mutations have been identified from a wide variety of human cancers that likely impair Axin1 interaction with GSK3β. Recently, another group examining the role of Axin1 in regulating c-Myc has informed us that disrupting GSK3β function inhibits the ability of Axin1 to promote T58 phosphorylation of c-Myc (Dr. Amati, personal communication). Phosphorylation of T58 in c-Myc is a critical step in promoting the degradation of

c-Myc since the F-box, Fbw7 recognizes T58 phosphorylated c-Myc and multi-ubiquitinates c-Myc marking it for degradation by the 26S proteosome. One caveat to the association of GSK3β with Axin1 is that the binding domain for GSK3β in Axin1 likely has inhibitory affects on GSK3β function (Chou et al. 2006), yet GSK3β associated with Axin1 is clearly active since it efficiently phosphorylates β-catenin. How the activation of GSK3β associated with Axin1 occurs is currently being examined. Never the less, GSK3β association and activity are critical to both the turnover of c-Myc protein and suppression of c-Myc dependent transcription.

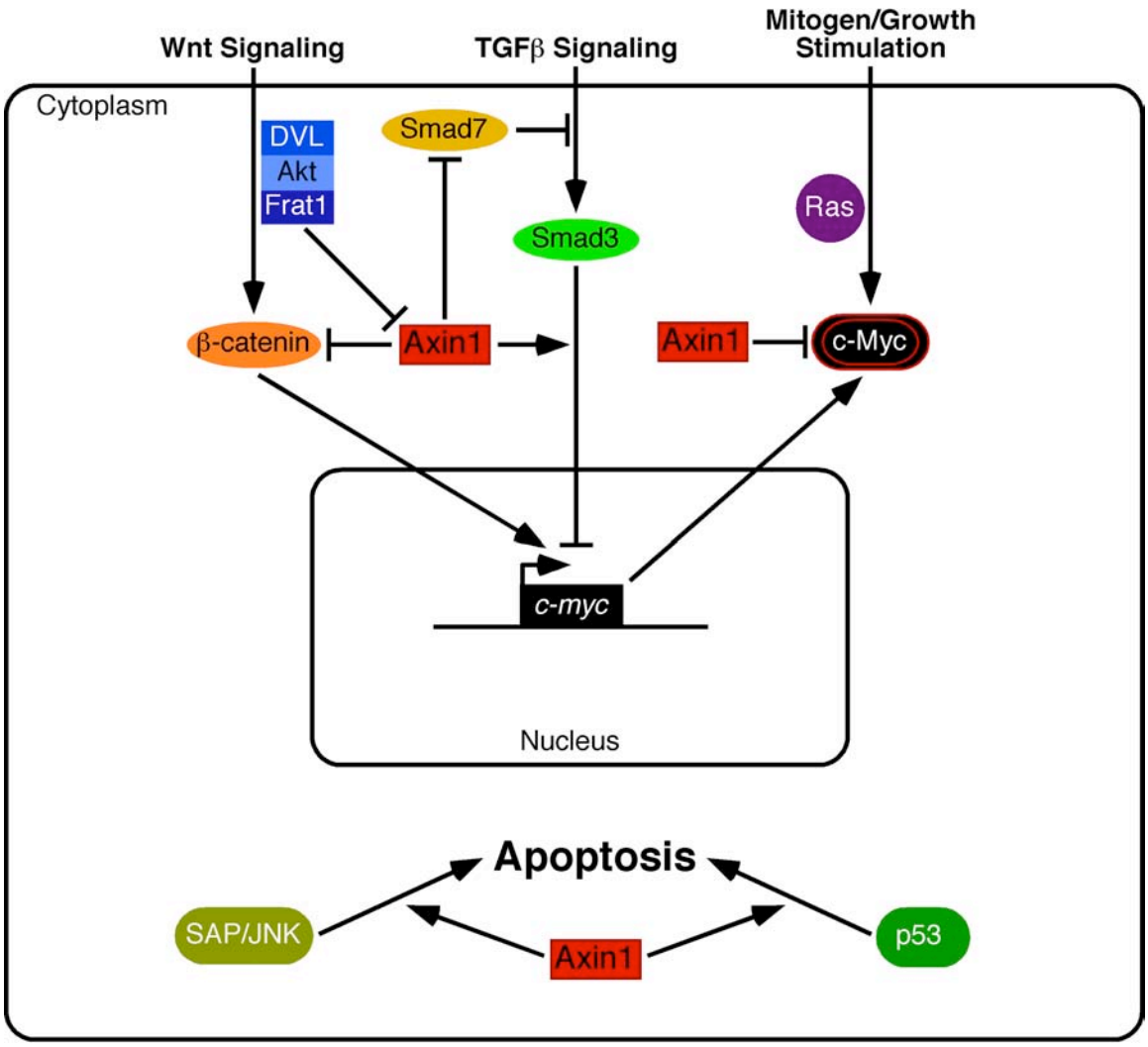


Figure 4.3: Axin1 regulates multiple signaling pathways.

Axin1 regulates several signaling pathways and transcription factors such as Wnt/β-catenin, TGFβ, SAPK/JNK, p53 and c-Myc. Axin1 has been shown to negatively regulate expression of *c-myc* mRNA by regulating Wnt and TGFβ signaling. DVL, Akt, and Frat1 antagonize Axin1 regulation of Wnt signaling, whereas Smad7 antagonizes TGFβ signaling. We now report a role for Axin1 to regulate c-Myc protein expression at the post-translational level.

Axin1 is a key regulator of multiple signaling pathways, which could allow for crosstalk between these pathways and feedback regulation. In addition to regulating c-Myc protein turnover, Axin1 has been shown to regulate several

other signaling pathways including Wnt, TGFβ, SAPK/JNK, and p53 signaling, summarized above in figure 4.3. The idea that Axin1 simply regulates these pathways independent of one another is unlikely since these pathways are intricately intertwined in order to coordinately regulate proliferation, growth, differentiation and apoptosis. More recently, there are reports of crosstalk and feedback loops between different Axin1 regulated pathways, which depend on the ability of Axin1 to function in multiple pathways, summarized below in Figure 4.4. For example, Axin1 turnover of elevated β-catenin protein levels was shown to depend in large part upon the ability Axin1 to interact with p53 and form an alternative β-catenin degradation complex that includes Siah/SIP/Skp1/Ebi instead of the Wnt based APC, GSK3β, and PP2A-B56α degradation complex (Liu et al. 2001; Matsuzawa and Reed 2001). It has also been shown that the ability of Axin1 to turnover β-catenin protein is important for Axin1 activation of SAPK/JNK signaling (Neo et al. 2000). Furthermore, it has been demonstrated that ectopic Axin1 expression in colorectal cancer cell-lines can reduce total Ras protein levels when β-catenin is wild-type, but not when β-catenin is mutant and resistant to Axin1 mediated turnover (Jeon et al. 2007), discussed in more detail below. The ability of Axin1 to co-regulate multiple signaling pathways in a codependent manner allows for exquisite stochiometric regulation of these different pathways. This exquisitely sensitive role in coordinating complex biological processes could play a critical role in the activity of Axin1 during development as it has been shown that homozygous disruption of full-length

axin1 in mice is embryonic lethal, see Chapter 1. Moreover, the expression of *axin1* is very low (Salahshor and Woodgett 2005) and therefore loss or sequestering of Axin1 function, similar to the loss of PP2A-B56α, could result in the deregulation of multiple oncoproteins resulting in the appearance of multiple hits in tumorigenesis.

In addition to the simultaneous regulation of several pathways by Axin1, there are also likely feedback loops in play that involve Axin2, a homologue of Axin1. The main feedback loop summarized below in Figure 4.4, involves the induction of *axin2* mRNA expression by elevated expression of β-catenin in human tumor cells as compared to surrounding normal tissue or by ectopic expression of β-catenin *in culture cells* (Jho et al. 2002; Leung et al. 2002; Lustig et al. 2002). Axin2 has been shown to associate with APC and GSK3β to reduce β-catenin protein levels by forming an analogous degradation complex for β-catenin as that coordinated by Axin1 (Behrens et al. 1998). A recent report has also indicated that elevated expression of c-Myc in mice with inducible *c-myc* transgenes increases the expression of *axin2* mRNA (Sansom et al. 2007). Therefore, one question is whether Axin2 will also negatively regulate c-Myc expression at the post-translational level by forming a degradation complex analogous to that formed by Axin1 for c-Myc. This question is currently being investigated by Charlie Morgan in the Sears lab and initial observations indicate that c-Myc expression in 293 cells does not induce Axin2 expression whereas β-catenin expression does.

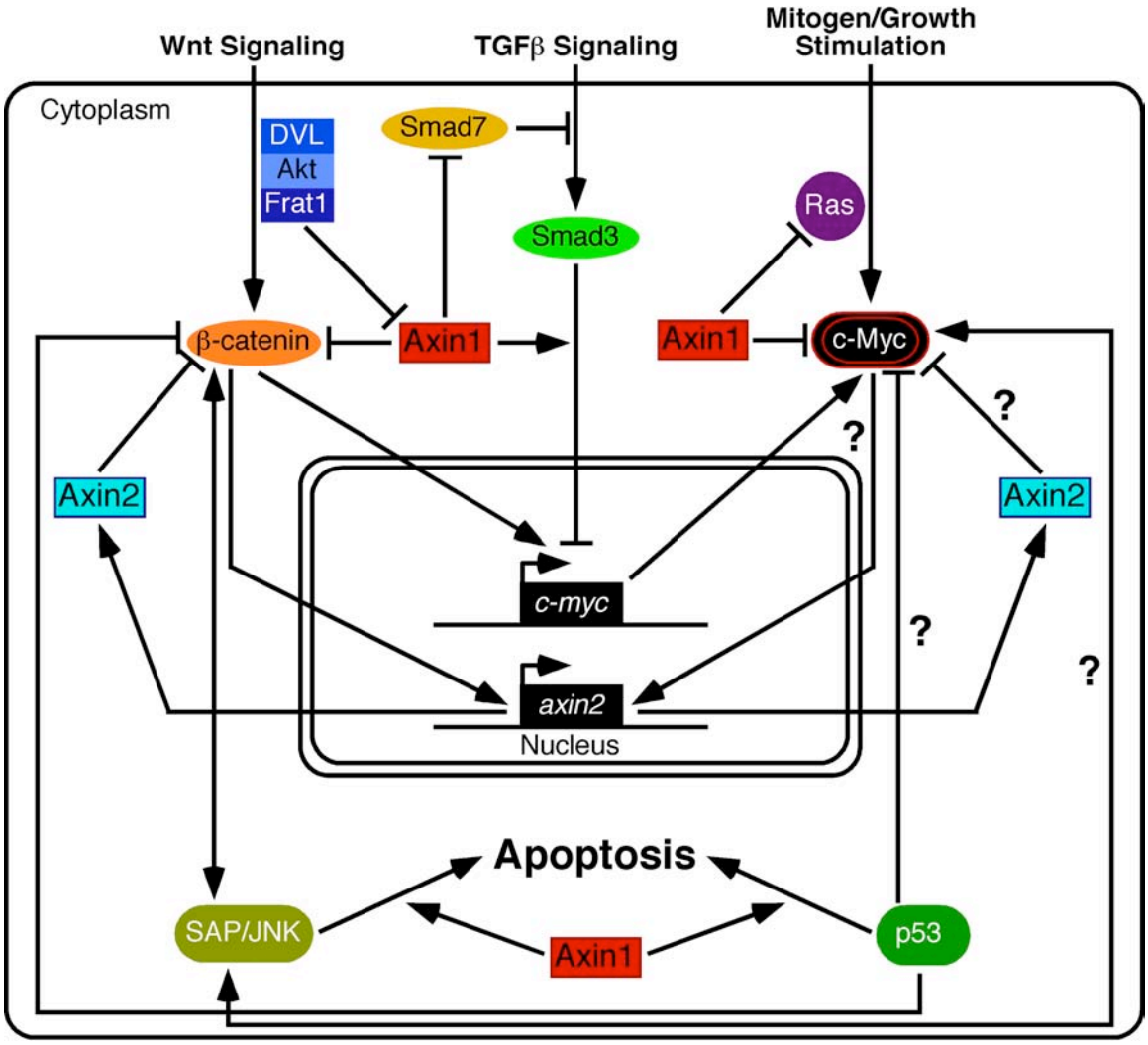


Figure 4.4: Possible crosstalk and feedback signaling through Axin1.

Expanding on Figure 4.3, β-catenin is reported to increase the expression of *axin2*, which can feedback to reduce β-catenin protein levels. The ability of SAPK/JNK signaling to induce apoptosis can depend upon the ability of Axin1 to regulate β-catenin. It has been demonstrated that p53 can coordinate a novel degradation complex for β-catenin. Furthermore, Axin1 can enhance the activation of p53. Ectopic expression of Axin1 has been shown to reduce total Ras protein levels dependent upon the ability of Axin1 to turnover β-catenin. New questions now arise with respect to Axin1 regulation of c-Myc expression and whether this also involves induction and regulation by Axin2 and whether it is coordinated with Axin1 regulation of other signaling pathways.

Ras and c-Myc protein expression maybe coordinated through Axin1. As discussed above, Axin1 likely regulates multiple targets simultaneously in order to tightly regulate biological processes such proliferation, growth, apoptosis, and differentiation. A recent report demonstrated that ectopic Axin1 expression reduced total Ras protein levels in colorectal cancer cell-lines with wild-type β-catenin. However, in colorectal cancer cell-lines with mutant β-catenin that cannot be turned over by ectopic expression of Axin1, total Ras protein levels remained unchanged (Jeon et al. 2007). I now find a similar result that Axin1 turnover of c-Myc is coupled to reducing total Ras protein levels, specifically, total Ras protein levels are reduced under conditions where Axin1 can promote the turnover of c-Myc^{WT} and (Figure 4.5, compare lanes 1 and 2). However, the inability of Axin1 to reduce c-Myc^{T58A} protein levels results in the inability of Axin1 to negatively regulate total Ras protein levels (Figure 4.5, compare lanes 3 and 4). Axin1 also cannot promote c-Myc^{S62A} degradation, but c-Myc^{S62A} does not interact with Axin1, and thus c-Myc^{S62A} expression does not appear to affect the reduction of Ras levels by Axin1 (Figure 4.5, lanes 5 and 6). It is not clear how Axin1 promotes the turnover of Ras protein or how this is dependent upon the ability of Axin1 to negatively regulate oncoproteins such as β-catenin and c-Myc. However, it is possible that Axin1 co-regulation of β-catenin or c-Myc protein turnover and reduction in total Ras protein levels involves a competitive mechanism where elevated expression of one substrate sequesters Axin1 from another. This could act as a potential protective mechanism where elevated

oncoprotein levels that cannot be reduced result in sustained elevated Ras levels, which can induce senescence. This possibility clearly needs to be explored further and could shed some light on how mechanistically, oncoproteins activate Ras to induce senescence.

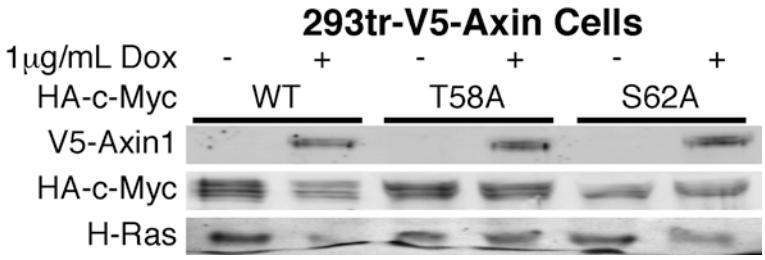


Figure 4.5: Axin1-mediated reduction of Ras protein levels is dependent upon the ability of Axin1 to bind and turnover elevated c-Myc.
60mm dishes of 293tr-V5-Axin1 cells were transfected with CMV driven β-gal and either c-Myc^{WT}, c-Myc^{T58A} or c-Myc^{S62A} overnight. Plates were split the following day into two 60mm plates and 24 hours post-split plates were treated with 1μg/mL Dox as indicated for four hours.

C-Myc^{T58A} can act as a "substrate trap" for Axin1, similar to that observed for PP2A-B56α. As discussed above c-Myc^{T58A} acts as a substrate trap for PP2A-B56α and the same appears to be the case for Axin1. I found that Axin1 has a significantly higher affinity for c-Myc^{T58A} (Figure 3.13) and Axin1 expression was not able to decrease c-Myc^{T58A} protein levels, whereas c-Myc^{WT} protein levels were reduced by an average of ~40% (Figure 3.6). The ability of c-Myc^{T58A} to sequester Axin1 through a "substrate trap" mechanism could have a dramatic impact on Axin1's ability to regulate other target pathways and proteins, summarized below in Figure 4.3. Interestingly, the expression of Axin1 appears to be quite low in cells as it is a limiting factor in the turnover of β-catenin protein (Hart et al. 1998). Therefore, elevated expression of c-Myc^{T58A} could quite easily

shift the pool of Axin1 away from its other targets. As previously mentioned, c-Myc^{T58A} has significantly increased oncogenic potential due to its ability to suppress the induction of apoptosis. By sequestering Axin1 away from its roles in positively regulating the induction of apoptosis through SAPK/JNK and p53 signaling, c-Myc^{T58A} could promote the survival of cells. Moreover, c-Myc^{T58A} could prevent the ability of Axin1 to negatively regulate β-catenin protein levels and transcriptional activity thereby increasing proliferation. Lastly, the ability of Axin1 expression to down-regulate Ras protein levels appears to be compromised by c-Myc^{T58A} expression (Figure 4.5) in a similar manner to that seen with stable point mutants of β-catenin (Jeon et al. 2007). Elevated Ras protein levels often leads to senescence in normal cells, but Ras point mutations that inhibit this function of Ras are very common in human cancers, which maybe why c-Myc^{T58A} and mutant forms of Ras such as H-Ras cooperate in the transformation of primary human cells (Yeh et al. 2004). The potential effects of c-Myc^{T58A} expression on Axin1 function in regulating other known targets such as β-catenin, SAPK/JNK, p53 and Ras are currently being explored in the lab. In addition, the possibility that stabilized c-Myc^{WT} could also potentially sequester Axin1 is intriguing since we have found c-Myc^{WT} to be aberrantly stabilized in some human leukemia and breast cancer cell lines and primary patient tumor samples. Therefore, understanding how different oncoproteins can inhibit normal Axin1 function will give us critical insight into the mechanisms by which

tumorigenesis can begin to arise from the deregulation of a few potent oncoproteins.

Chapter Five :

Materials and Methods

Plasmids and RNAi

Construction of expression plasmids CMV-empty, CMV-βgal, CMV-Myc, pCEP-small-T-antigen, pD40-His/V5-c-Myc, pD40-His/V5-c-Myc^{T58A}, and pD40-His/V5-c-Myc^{S62A}, as well as reporter constructs, E2F2-Luc, and E2F2(-E-box)-Luc have previously been described (Sears et al. 1997; Yeh et al. 2004). pD40-His/V5-c-Myc^{T58E} and pD40-His/V5-c-Myc^{S62D} were generated using mutation primers (primer table) and TOPO cloning (Invitrogen) into pDEST40 mammalian expression vector (Sears et al. 1997; Yeh et al. 2004). pD40-His/V5-c-Myc$^{\Delta TAD}$ was created by digesting CMV-Myc with PstI to remove amino acids 40-179, containing the transactivation domain, followed by LR cloning into pDEST40. CMV-HA-Myc was made by PCR amplifying the C-terminus of c-Myc to include an in-frame HA-tag (primer table). This PCR product was used to replace the existing C-terminus of c-Myc in CMV-Myc by restriction cloning using SacII and XbaI sites. pD30-PP2A-FLAG-A, pD30-PP2A-HA-C, pD40-His/V5-B55α and pD40-His/V5-B56α were created by PCR amplification of gene coding sequences from a liver cDNA library followed by LR cloning into either pDEST30 mammalian expression vector (PP2A-FLAG-A and PP2A-HA-C) or pDEST40 mammalian expression vector (B55α and B56α). Expression vectors for pCEP4HA-B56α, β, γ1, γ3, δ1and ε as well as HA-Axin1 WT, 4M, and 2M were a generous gift from Dr. David Virshup (Huntsman Cancer Institute, University of Utah). The

pCAN-E4orf4 expression vector was kindly provided to us by Dr. Clodagh O'Shea (University of California San Francisco, CA).

Full-length, human Axin1 was PCR amplified with Pfu Ultra (Stratagene) from a human liver cDNA library using primers outlined in Axin1 primer table. Axin1 fragments were PCR amplified using primers outlined in Axin1 primer sequence table, from cloned full-length, human Axin1 with Pfu Ultra. N- and C-terminal fragments of Axin were directly cloned into pENTR-D-TOPO vector (Invitrogen) according to manufacturer's protocol. Internal deletion mutants of Axin1 were generated using primers containing either BamHI or BglII restriction sites (Axin1 primer sequence table), digested with BamHI or BglII, and purified via gel extraction kit, QIAEX II (Qiagen). Purified, digested PCR products were ligated using T4 DNA ligase (Roche) and TOPO cloned into pENTR-D-TOPO vector (Invitrogen). All pENTR-D-Axin vectors were LR cloned into pcDNA-Dest40 (Invitrogen) for tagging with V5/6Xhis and expression in both *in vitro* and mammalian experiments. Alternatively, full-length Axin1 with stop codon was cloned into pT-REX-Dest30 (Invitrogen) via LR clonase (Invitrogen) to be expressed in mammalian cells without a tag.

SiRNA SMARTpools for scramble control, PP2A-Cα (PPP2CA) and B55α (PPP2R2B) were purchased from Dharmacon (Lafayette, CO). ShRNA expression vectors were generated using OligoEngine (Seattle, WA) software to identify target sequences to each Axin1, Pin1, PP2A-A, B56α,-β,-γ,-δ and -ε (target sequences in primer table). Oligos encoding the sense and antisense

shRNA sequences were cloned into the pSUPER (OligoEngine) or pENTR-H1/TO (Invitrogen) shRNA expression vector by manufacturer's protocol.

Table 5.1: Primer and shRNA target sequences.

Myc Primers	**Sequence**
pD40-His-c-Myc^{T58E}	5'-GGAAGAAATTCGAGCTGCTTCCGGAGCCGCCCCTGTCCCCGAGCC-3' (mutational primer)
pD40-His-c-Myc^{S62D}	5'-GCTTCCCACCCCGCCCCTGGATCCGAGCCGCCGCTCCGG-3' (mutational primer)
Forward HA-tag-c-Myc	5'-GCCCCGCGGGCACAGCGTCTGCTCC-3' (underlined)
Reverse HA-tag-c-Myc	5'-GCTCTAGATTAAGCGTAATCTGGAACATCGTATGGTACCCTGCACCAGAGTTTCGAAGCTGTTC-3' (underlined)
PP2A Primers	**Sequence**
PP2A-FLAG-A Forward	5'-CACCATGGATTATAAGGACGATGACGATAAGAAGGCGGCGGCCG-3' (TOPO sequence)
PP2A-FLAG-A Reverse	5'-TCAGGCGAGAGACAGAACAGT-3'
PP2A-HA-C Forward	5'-CACCATGTACCCATACGATGTTCCAGACTACGCCATAGACGAGAAGGTGTTCACCAAG-3' (TOPO sequence)
PP2A-HA-C Reverse	5'-TTACAGGAAGTAGTCTGGGGTACG-3'
V5-B55α Forward	5'-CACCTTGGCAGGAGCTGGAGGAG-3' (TOPO sequence)
V5-B55α Reverse	5'-CCCATTCACTTTGTCTTG-3'
V5-B56α Forward	5'-CACCATGTCGTCGTCGTCGC-3' (TOPO sequence)
V5-B56α Reverse	5'-TCCTTCGGCACTTGTATTGCTGAGAATAC-3'

Axin Primers	**Sequence:**
Axin1 Forward TOPO	5'- CACCATGGATATCCAAGAGCAGGGTTTCC-3' (TOPO sequence + Kozak change)
Axin1 Reverse	5'-TCAGTCTACCTTCTCCACTTTGCCG-3'
Axin1 Reverse minus stop	5'-GTCTACCTTCTCCACTTTGCCGATG-3'
Axin1 Exon 2 Forward	5'-CACCATGTATGGATCCTGGCGGGAG-3' (TOPO sequence)
Axin1 Exon 3 Forward	5'-CACCATGAGCGTGGATGGGATCCC-3'
Axin1 Exon 4 Forward	5'-CACCATGTACCGGGTGCCGAAGG-3' (TOPO sequence)
Axin1 Exon 5 Forward	5'-CACCGGATCCATGGAGGAGGAAGGTGA GGAC-3' (TOPO + BamHI sequence)
Axin1 Exon 8 Forward	5'-GGAGGATCCGAGACAAGATCGCAGAGGAAGGTG-3' (BamHI)
Axin1 Exon 2 Reverse	5'-GGCAGATCTCCATCCACGCTGCTGTCC-3' (BglII)
Axin1 Exon 5 Reverse	5'-GGCAGATCTACTGTGGGCGAGGCCATC-3' (BglII)
Axin1 Exon 6 Reverse	5'-GGCAGATCTCCCGTGGCCGGTCCT-3' (BglII)
Axin1 Exon 7 Reverse	5'-CCTCTGCTTGGAGGGTGCTC-3'
Axin1 Exon 8 Reverse	5'-CTCTGTCTCGGAGAGCTCCATG-3'
Axin1 Exon 9 Reverse	5'-TCTGTAGCTGCCCTTTTTGGTCAG-3'
	Target Sequence:
PP2A-A	CCACCAAGCACATGCTACC
B56α	GAACACTGGAATCCGACCA

B56β	TCAGAGCAACCAGCAAGAG
B56γ	TACTGGCCAAAGACTCACA
B56δ	GTGTGTCTCTAGCCCCCAT
B56ε	TTCCAACCCAGCATTGCCA
Axin shRNA	**Target Sequence:**
Axin1	GAGGAAGAAAAGAGAGCCA
Pin1 shRNA	**Target Sequence:**
Pin1	GCCGAGTGTACTACTTCAA

Cell-lines and Transfection

HEK-293 cells were maintained in DMEM supplemented with 10% characterized fetal bovine serum (FBS), 2mM L-glutamine, and 1X penicillin/streptomycin at 37°C and 5% CO_2. Cells were plated to achieve 60-80% confluency 24 hrs post-split for transfection. SMARTpool siRNA transfections were carried out as previously described (Yeh et al. 2004). All other transfections were performed using Metafectene (Biontex, Germany) or HEK-Fectin (BioRAD) according to manufacturer's specifications at a 3:1 ratio of transfection reagent:DNA (75-90% efficiency). Total transfected DNA was held constant by the addition of empty control plasmid. All transfections included 50ng of CMV-βgal to assess transfection efficiencies between experimental conditions. For PP2A-B56α

experiments, transfected cells were maintained in DMEM supplemented with 10% FBS and L-glutamine except shRNA experiments, which were maintained in 2% or 0.2% FBS and L-glutamine for indicated time periods. For Axin experiments cells were transfected in DMEM supplemented with 10% FBS and L-glutamine for 8-12 hours and then the media was changed to DMEM supplemented with 0.2-2% FBS and L-glutamine.

IMR90 cells were maintained in DMEM supplemented with 15% Defined FBS, 2mM L-glutamine, 1mM non-essential amino acids, 1mM sodium pyruvate, and 1X penicillin/streptomycin. Lenti-virus infection of IMR90 cells was done in half-volume of the same media minus penicillin/streptomycin, but with the addition of 6μg/mL polybrene (Specialty Media) and ~10MOI of indicated Lenti-virus for 12 hours. Starve media, DMEM supplemented with 0.2% defined FBS and 1mM L-glutamine, was then added for 48 hours prior to Adenovirus infection. Adenoviral infection of IMR90 cells was done in 10% normal volume of DMEM supplemented with 25mM HEPES pH 7.4 with 10MOI of indicated adenovirus for 90 minutes, rocking plates every 10minutes. Original starve media was replaced for 16 hours and then cells were stimulated by the addition of defined FBS to a final concentration of 20% for cell cycle analysis of c-Myc protein levels.

Breast cancer and leukemia cell-lines were maintained as follows: MCF10A cells were grown in 45% DMEM, 45% F-12 hams, 5% horse serum, 2.5mM L-glutamine, 20ng/mL EGF, 10μg/mL insulin, 500ng/mL hydrocortisone, 100ng/mL cholera toxin, and 1X penicillin/streptomycin. MDA-MB231 cells were

grown in 95% Iscove's DMEM, 5% FBS, 5.9μg/mL insulin, and 1X penicillin/streptomycin. MCF7 cells were grown in 45%DMEM, 45% F-12 hams, 1mM non-essential amino acids, 1mM L-glutamine, and 1X penicillin/streptomycin. SKBR3 cells were grown in DMEM with 10% FBS and 1X penicillin/streptomycin. SupB15 cells were grown in RPMI with 10% defined FBS and 1X penicillin/streptomycin.

Stable 293tr-V5-Axin1 cells were generated by infecting 100mm dish of HEK-293 cells (ATCC) with ~10 MOI Lentivirus encoding the tet-repressor, pLenti6/TR (Invitrogen), in 5mL DMEM supplemented with 10% FBS, 1mM L-glutamine and 6μg/mL polybrene (Specialty Media) for 12 hours. Media was changed to DMEM supplemented with 10% FBS, 1mM L-glutamine and 1X penicillin/streptomycin and grown 36 hours. Media was changed and maintained in DMEM supplemented with 10% FBS, 1mM L-glutamine, 1X penicillin/streptomycin and 5μg/mL Blasticidin (Invitrogen) for 10 days until distinct colonies formed. Six colonies were picked, expanded and screened for their ability to suppress the expression of CMV-driven HA-tagged c-Myc expressed from pT-REX-Dest30-HA-c-Myc by transient transfection as described above. The best suppressing colony was then infected with ~10 MOI Lentivirus expressing V5-Axin1, pLenti4/TO/V5-Dest-Axin1, as described for the tet-repressor infection, described above. Cells were selected in DMEM supplemented with 10% FBS, 1mM L-glutamine, 1X penicillin/streptomycin, 5μg/mL Blasticidin (Invitrogen) and 100μg/mL Zeocin (Invitrogen) for 10 days

until distinct colonies formed. Six colonies were picked, expanded and screened for the ability to only express V5-Axin1 when treated with 1μg/mL Dox. The best clone was then used for further experiments and continually maintained in DMEM supplemented with 10% defined FBS, 2mM L-glutamine, 5μg/mL Blasticidin and 100μg/mL Zeocin.

Antibodies

The c-Myc antibodies, N262, C-33 and agarose-conjugated C-33 as well as B56α and Sp1 (PEP-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA.11 and PP2A-A 6F9 antibodies are from Covance (Berkeley, CA). The rabbit polyclonal to HA tag antibody (rabHA) was obtained from Abcam (Cambridge, MA). The PP2A-Cα antibody was purchased from BD Biosciences (San Jose, CA). The mouse V5 antibody was purchased from Invitrogen (Carlsbad, CA) and the rabbit V5, M2-FLAG, β-catenin, and β-Tubulin antibodies are from Sigma (St. Louis, MO). The c-Myc Serine 62 phospho-specific antibody (αS62phospho) was generated as previously described (Sears et al. 2000). The Threonine T58 phospho-specific (αT58phospho) and GSK3β antibodies were purchased from Cell Signaling Technology (Beverly, MA) and specificity is achieved by blocking with milk (see western blotting). The PP2A-B'α antibody was obtained from Upstate (Lake Placid, NY). Pin1 antibody was purchased from GenWay Biotech (San Diego, CA).

Western Blotting

Total cell lysates were collected in 10X volumes 1.5X Luciferase Buffer from Promega (Madison, WI) with protease and phosphatase inhibitors (10mM sodium fluoride, 100mM sodium vanadate, 10mM β-glycerolphosphate disodium pentahydrate, 1ug/ml aprotinin, 1ug/ml pepstatin, 0.5ug/ml leupeptin, 0.2mg/ml AEBSF, and 1.6mg/ml iodoacetamide). Lysates were freeze-thawed three times, incubated on ice for 20min, and subjected to βgal activity analysis as described in (Sears et al. 1997). 5X SDS Sample Buffer was added to a final concentration of 1.5X. Other samples such input and IP samples were prepared as described in the coimmunoprecipitation section. Total cell lysate sample load volumes were adjusted by βgal activity to account for transfection efficiency coimmunoprecipitation samples were adjusted prior to coimmunoprecipitation. Samples were separated by SDS-PAGE, and transferred to Immobilon-FL (Millipore, Billerica, MA). Membranes were blocked in Odyssey Block Buffer (LI-COR Biosciences, Lincoln, Nebraska), except when probed with anti-Threonine 58 phospo-specific antibody, which used 5% non-fat milk in PBS for blocking. Primary antibodies were diluted in 1:1 Odyssey Block Buffer:PBS, 0.05% Tween or 2.5% milk PBS, 0.05% Tween (P-T58 antibody) at indicated dilutions. Primary antibodies were detected by secondary anti-mouse and anti-rabbit near-infrared fluorescent dyes Alexa Fluor 680 (Molecular Probes, Eugene, OR) and IRDye800 (Rockland, Philadelphia, PA) used at 1:10K dilution in 1:1 Odyssey Block Buffer:1X PBS, 0.05% Tween or 2.5% non-fat milk in PBS, 0.05% Tween

(P-T58 antibody). Immunoblots were scanned using a LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) to visualize proteins, which also allows for simultaneous anti-rabbit and anti-mouse dual wavelength detection. Antibody signals were quantified using LI-COR Odyssey Infrared Imager software version 1.2, which allows for linear signal quantitation over four orders of magnitude. Quantitated protein levels were normalized to control protein levels, which were set to one fold or 100% as indicated. Average protein levels and error bars representing two standard deviations were calculated from three separate experiments using Excel (Microsoft, Redmond, WA). Significant differences were also calculated from three separate experiments by T-test analysis (two-tailed distribution and two-sample unequal variance) using Excel.

RT-PCR analysis

Transfected HEK-293 cells were collected in 1XPBS with 1mM EDTA, 5% of the cells were reserved for βgal assay and western analysis. RNA was isolated from cells exhibiting transfection efficiencies within 5% of each other using TRIzol reagent from Invitrogen (Carlsbad, CA). cDNA was made using the M-MLV Reverse Transcriptase according to manufacturer's protocol (Invitrogen). 2X Immunomix Red from BIOLINE (Randolph, MA) was used for PCR analysis of cDNA (see supplemental information for primer sequence and thermocycler setup).

qRT-PCR analysis

RNA was isolated from 293tr-V5-Axin1 cells collected in 1mL TRIzol reagent (Invitrogen) according to manufacturer's protocol. Isolated RNA was DNase treated in 100mM $MgCl_2$, 10mM DTT, RNasin (Promega), RNase free DNase (Roche) for 15 minutes at 37°C and purified using RNeasy (Qiagen). cDNA was made from DNase treated RNA using M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's protocol with oligo dT primers. qRT-PCR analysis was done using primers for *c-myc* and *18S* as designed by Applied Biosystems on a 7300 qRT-PCR machine (Applied Biosystems) according to manufacturer's preset qRT-PCR cycle conditions.

Cyclohexamide half-life

100mM dishes of HEK-293 cells were cotransfected with 50ng CMV-βgal, 0.5ug pD40-His/V5-c-Myc and 4ug pSUPER-empty or B56α for c-Myc/PP2A-B56α experiments or pENTR-H1/TO-scramble, Axin1, or B56α for c-Myc/Axin1 experiments under 10% FBS serum conditions for 24hrs. Each transfection was split into six 60mM dishes and maintained for 24hrs in DMEM supplemented with 10% FBS and L-glutamine and then starved in DMEM supplemented with 0.2% FBS and L-glutamine for 48hrs. Cells were treated with 100ug/mL cyclohexamide for 5-15 minutes and then the indicated time points were collected.

Coimmunoprecipitation

Cells were resuspended in 10X cell pellet volumes of PP2A-CoIP 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. Cellular lysates were incubated on ice for 20min, sonicated 10 pulses (output = 1 and 10% duty), and cleared by centrifugation at 20K rcf for 10min at 4°C. Cleared lysates volumes were adjusted for transfection efficiency by βgal assay and incubated with either 1:150 dilution of agarose conjugated αC-33, 1:150 conjugated αSp1, 1:500 dilution αHA.11 or 1:750 dilution of αV5 antibodies. Immunoprecipitates were washed 3X with 10X volumes PP2A-CoIP Buffer with 1 minute incubation in buffer during each wash. Where specified, immunoprecipitates were more stringently washed 3X with 10X volumes PP2A-CoIP Buffer with 300mM NaCl.

His-Myc-Ni-NTA Columns

Cells transfected with 6ug pD40-His/V5-conrol, -Myc or -Myc$^{\Delta TAD}$ were resuspended in 10X cell pellet volumes Ni-NTA Lysis Buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole and 0.5% NP-40, pH 8.0 plus protease and phosphatase inhibitors). Cell lysates were then sonicated using a Branson Sonifier 450 sonicator (10X 1sec pulses at 20% duty and output = 2), incubated on ice for 20min, and cleared by centrifugation at 14K rpm for 10min at 4°C. Cleared lysate volumes were adjusted for transfection efficiency according to βgal activity. Qiagen Ni-NTA Beads (Valencia, CA) were added to cleared

lysates and rotated four hours at 4°C. Ni-NTA columns were washed three times with 10X volumes Ni-NTA CoIP Buffer (50mM NaH_2PO_4, 10% glycerol, 150mM NaCl, 20mM Imidazole, 0.2% NP-40, pH 8.0 plus protease and phosphatase inhibitors). Cells transfected with 6ug pD30-PP2A-HA-C were resuspended in 10X cell pellet volumes Ni-NTA CoIP Buffer, incubated on ice for 20 min, and cleared by centrifugation. Ni-NTA columns were incubated with PP2A-HA-C cleared lysate for four hours at 4°C and then washed three times with 10X volumes Ni-NTA Wash Buffer (50mM NaH_2PO_4, 10% glycerol, 150mM NaCl, 30mM Imidazole, 0.2% NP-40, pH 8.0 plus protease and phosphatase inhibitors). Column bound proteins were released by three 0.5X volume elutions with Ni-NTA Elution Buffer (50mM NaH_2PO_4, 10% glycerol, 300mM NaCl, 250mM Imidazole, 0.5% NP-40, pH 6.0 plus protease and phosphatase inhibitors).

Ubiquitin Assay

293 cells were transfected as indicated and 12 hours following transfection cell media was changed to DMEM supplemented with 2% FBS, L-glutamine and 1X penicillin/streptomycin. 36 hours post re-feeding, cells were collected in RIPA lysis buffer (Ingredients), sonicated (20 pulses, output = 2.5, 30% duty), and cleared by centrifugation (20K rcf for 10min at 4 °C). Lysate volumes adjusted based on β-gal activity, input samples collected, and remaining lysate volume immunoprecipitated with conjugated αHA.11 at 1:100 dilution. Immunoprecipitates were washed 4X with RIPA buffer and then subjected to western blot analysis.

Luciferase Assay

Cell pellets were resuspended in 10X volumes 1.5X Luciferase Buffer with protease and phosphatase inhibitors, freeze-thawed three times, incubated on ice for 20min, and cleared by centrifugation at 14k for 10min at 4°C. Samples were subjected to βgal activity analysis. Luciferase activity was detected using a Promega Luciferase Assay Kit and Berthold luminometer (Bundoora, Australia). Luciferase activity was adjusted for βgal activity. Three separate experiments were performed for each luciferase-based assay and adjusted luciferase activities with standard deviations were graphed using Excel.

In vitro Assay

T7-driven HA-c-Myc, V5-Axin1, GSK3β and/or Pin1 were expressed using the T7 Quick Coupled Transcription/Translation kit (Promega) according to manufacture's protocol. *In vitro* reactions were brought up to 300μL total volume with PP2A-CoIP Buffer with protease and phosphatase inhibitors (see coimmunoprecipitation methods). 10% input sample was taken and remaining sample was subjected to immunoprecipitation with αV5 (see coimmunoprecipitation methods). Input and IP samples were analyzed by western blot.

Immunoflouresence

Cells were fixed in 4% paraformadehyde/HEPEs buffer for 10 minutes on ice and then permeabilized with .5% triton in PBS for 10 minutes. Cells were blocked

with 2% BSA in PBS (.05% Tween) for 2 hours on ice. Anti-HA (Cell Signaling) antibody was added at 1:50 for 4 hours on ice followed by 4 washes in block media. Secondary, FITC conjugated antibody (Molecular Probes) was added at 1:500 for 1 hour at room temperature. Immunoflouresence was visualized on a Leica DMR microscope.

Appendix

Contribution to Figures:

Figure 2.1: CMV-E4orf4 was a generous gift from Dr. Clodagh O'Shea at the University of California San Francisco (San Francisco, California).

Figure 2.2 and 2.5: The CMV-4HA-B56 constructs were a generous gift from Dr. David Virshup at the Huntsman Cancer Institute (Salt Lake City, Utah).

Figure 3.9: Deanne Tibbitts cloned CMV-V5-Axin1v2.

Figure 3.10: Sara Tokarz, PhD. performed the staining and deconvolution immunoflourescence for the localization of different fragments of Axin1.

Figure 3.11: Julie Escamilla-Powers cloned the shRNA to Pin1.

Figure 3.13: The CMV-HA-Axin1 wildtype, 2M, and 4M were a generous gift from Dr. David Virshup at the Huntsman Cancer Institute (Salt Lake City, Utah).

Figure 3.16: Deanne Tibbitts cloned CMV-V5-Axin1^{SupB15}.

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