Regulation of c-Myc phosphorylation, protein stability and oncogenic activity in breast cancer

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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
DIX	Dishevelled Interaction Domain
DMEM	Dulbecco's Modified Eagle's Medium
Dox	Doxycycline
DVL	Dishevelled
ERK	Extracellular Receptor Kinase
FBS	Fetal Bovine Serum
GSK3β	Glycogen Synthase Kinase 3β
HAT	Histone acetyltransferase
HCC	Hepatocellular Cancer
HDAC	Histone deactylase
HER2	Human epidermal growth factor receptor -2
HLH	Helix Loop Helix

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
p53	Tumor Protein 53
PI3K	Phosphoinositide-3-Kinase
Pin1	Prolyl Isomerase 1
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
pT58	Phosphorylation at Threonine 58
pS62	Phosphorylation at Serine 62
RTK	Receptor Tyrosine Kinase
S62	Serine 62
Small T	SV40 Small T Antigen
SCC	Squamous Cell Carcinoma

SCF	Skp/Cullin/F-box
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
S-Myc	Suppressor Myc
SV40	Simian Virus 40
T58	Threonine 58
TAD	Transactivation Domain
TCF	T Cell Specific Factor
TGFβ	Transforming Growth Factor β
UTR	Untranslated Region
WNT	Wingless
WT	Wildtype
β-gal	β-Galactosidase
βTRCP	F-box and WD repeat domain containing 11

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Abstract

Overexpression of the c-Myc transcription factor occurs in the majority of human breast cancer. Gene amplification and increased transcription are only found in a subset of those with overexpression of c-Myc protein. How c-Myc overexpression occurs in the rest of human breast cancer is still not clear. This thesis seeks to investigate the possibility and mechanisms of increased c-Myc protein stability contributing to its overexpression in breast cancer.

c-Myc protein stability is regulated by a signaling pathway that controls phosphorylation events on the two conserved amino acids: Serine 62 and Threonine 58. Phosphorylation of the two amino acids has opposite roles on c-Myc protein stability. Phosphorylation of Serine 62 stabilizes while phosphorylation of Threonine 58 destabilizes c-Myc and this requires prior phosphorylation at Serine 62. Here I show that c-Myc protein stability is increased in breast cancer cell lines and is associated with increased phosphorylation at Serine 62 and decreased phosphorylation at Threonine 58. Moreover, this shift of phosphorylation of Serine 62 and Threonine 58 levels also occurs in primary human breast cancer. Thus increased c-Myc protein stability is a new mechanism of c-Myc overexpression in human breast cancer.

To test the underlying mechanisms of increased c-Myc protein stability, I have focused on two proteins, the tumor suppressor Axin1 and the oncoprotein HER2. Axin1 is a scaffold protein that coordinates a destruction complex for c-Myc. My study show that deregulation of Axin1, including decreased total level of

Abstract

Axin1 and a preferred expression of an Axin1 splice variant, Axin1v2, correlates with deregulation of c-Myc degradation in breast cancer. Axin1v2, unlike the other Axin1 splice variant, Axin1v1, is not able to promote c-Myc dephosphorylation at S62, nor is it able to inhibit c-Myc activity. Knocking down of Axin1 in non-transformed cells increases, while increasing Axin1 expression in cancer cell lines inhibits c-Myc's oncogenic activity. Thus, these results identify deregulation of Axin1 as a new mechanism of deregulating c-Myc in human breast cancer.

To explore other mechanisms that lead to deregulation of c-Myc, I examined c-Myc regulation by the Tyrosine kinase receptor HER2, which is overexpressed in 25-30% of human breast cancer. Activation of HER2 can lead to activation of the two key pathways that control phosphorylation of c-Myc at Serine 62, Ras-MAPK and PI(3)K-Akt. Here I show that activation of HER2 increases c-Myc protein stability, phosphorylation at Serine 62 and DNA binding at c-Myc target gene promoters, while inhibiting HER2 does the opposite. Thus, overexpression of HER2 can be another way of deregulating c-Myc in human breast cancer.

Taken together, these results provide mechanistic insight into how c-Myc protein stability can be deregulated in human breast cancer. Understanding whether and how deregulation of c-Myc protein stability occurs in human cancer is critical for developing Myc-targeted therapies.

Chapter One :

Introduction

c-Myc Background

c-Myc was originally identified as a cellular homolog of v-Myc (Vennstrom et al. 1982). v-Myc is the transforming sequence of the MC29 avian tumor virus that causes myelocytomatosis (MYC) in chickens. The c-Myc proto-oncoprotein has been intensively studied for the past 30 years and it is now generally accepted that c-Myc is crucial in cell fate decisions such as proliferation, differentiation and apoptosis (Meyer and Penn 2008). Deregulation of c-Myc leads to tumorigenesis in mouse models and is reported in almost all human cancers (Pelengaris et al. 2002).

1. c-Myc is a transcription factor.

c-Myc is the principal member of a non-redundant family of transcription factors that also includes N-Myc, L-Myc, S-Myc and B-Myc. Structurally, c-Myc contains several domains that are common to other transcription factors. The amino-terminus of c-Myc is a transactivation domain (TAD) that is critical for most biologic activities of c-Myc. Within this domain are two conserved regions termed c-Myc box I and II (MBI and MBII). The carboxy-terminus of c-Myc is a basic-helix-loop-helix-Leucine zipper (bHLHZ) domain that mediates sequence-specific DNA recognition of E-Box motifs and oligomerization with its partner protein, also a bHLHZ protein, Max (Fig.1.1) (Landschulz et al. 1988; Davis et al. 1990; Kato et al. 1990; Luscher and Eisenman 1990; Blackwood and Eisenman 1991).

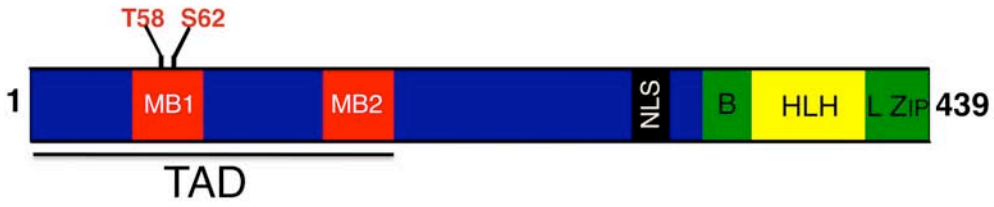

Figure 1.1: Diagram of the c-Myc protein structure (adapted from Hugh Arnold thesis).

The c-Myc protein has several domains typical of transcription factors. These include a transactivation domain (TAD), nuclear localization signal (NLS), basic region (B), helix-loop-helix (HLH), and leucine zipper domain (LZip). In the c-Myc TAD domain, there are two highly conserved regions named myc box 1 (MB1) and myc box 2 (MB2). The two highly conserved phosphorylation sites that are critical for the regulation of c-Myc protein stability, Threonine 58 (T58) and Serine 62 (S62) are also highlighted in MB1.

c-Myc activates transcription as part of a heteromeric complex with Max (Blackwood and Eisenman 1991; Blackwood et al. 1992a; Blackwood et al. 1992b; Kretzner et al. 1992). Myc-Max dimers recruit multiple coactivators and protein complexes to E-box elements with the consensus core sequence CACGTG. Recruited cofactors include those that are involved in histone acetylation such as Transformation/transcription domain-associated protein (TRRAP) and its associated histone acetyltransferases (HATs) as well as ATPase/helicases that contribute to opening chromatin structure for gene activation (Fig.1.2) (McMahon et al. 1998; McMahon et al. 2000; Wood et al. 2000; Frank et al. 2003).

Myc-Max dimers can also repress transcription, although the mechanisms are not well understood. Myc-Max can repress gene expression through tethering

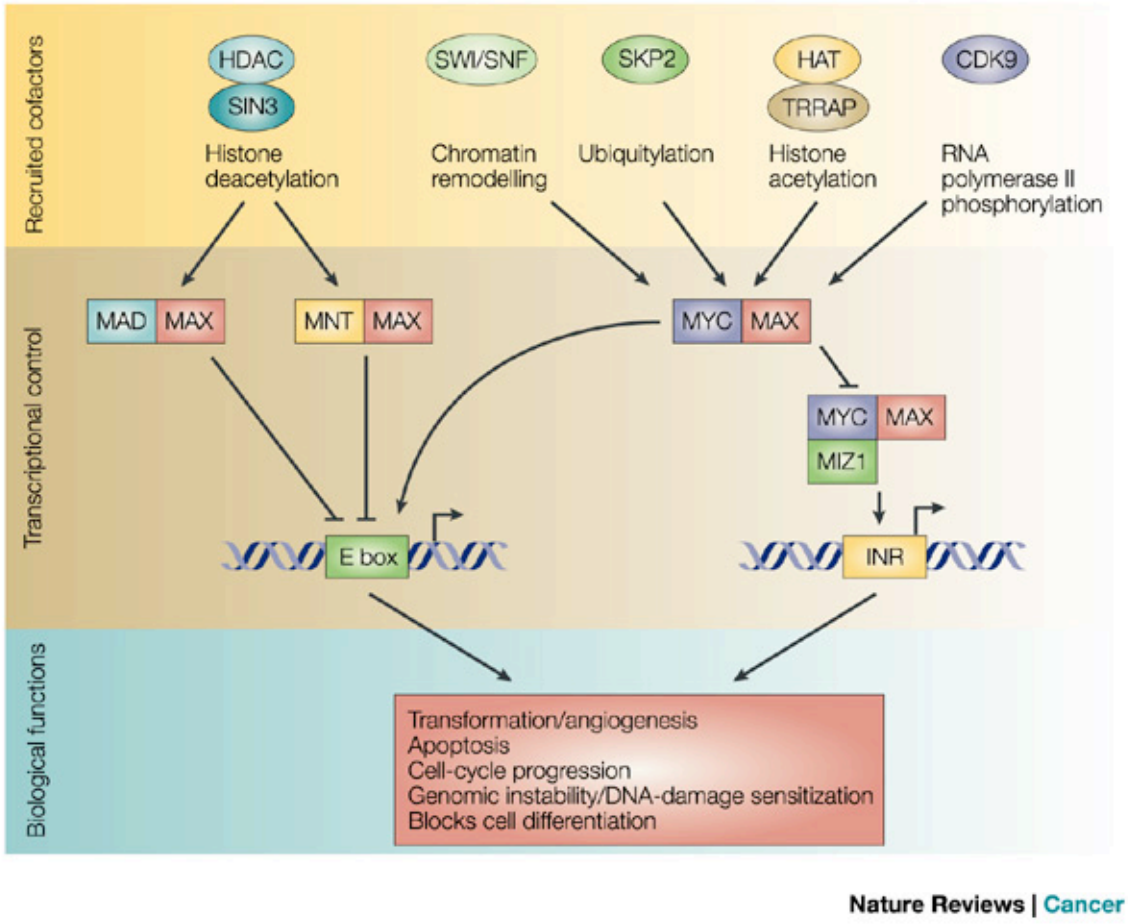

Figure 1.2: The Myc-Max network (Patel et al. 2004).

c-Myc is a basic, helix-loop-helix protein that binds DNA with its partner Max and activates or represses transcription through recruiting cofactor complexes. Recruited cofactors include proteins that are involved in chromatin remodeling, ubiquitylation and histone acetylation. For example, Myc-Max can recruit various histone acetyltransferase (HAT) complexes that share the common TRRAP subunit and therefore activate transcription of genes with E-Box element. Alternatively, Myc and Max interact with Miz1 at certain promoters to repress Miz1 transactivation through the INR element, a DNA element where transcription begins. Besides Myc, Max also dimerizes with Mad and Mnt family proteins and recruits a scaffolding protein called SIN3, which in turn recruits HDACs to repress transcription of genes with E-box element. Together, c-Myc and its partners regulate many biological processes that are important for normal cells and deregulation of these biological processes can lead to cell transformation and tumorigenesis.

with Miz-1, which inhibits gene transactivation by Miz-1(Staller et al. 2001; Herold et al. 2002; Wanzel et al. 2003; Wu et al. 2003). Interestingly, instead of binding to the E-Box elements, the Myc-Max-Miz1 complex binds to initiator (INR) elements where transcription begins. In addition to forming heterodimers with c-Myc, Max can form homodimers and can also heterodimerize with proteins from the Mad and Mnt families that can repress transcription. Like c-Myc, the Mad and Mnt proteins must dimerize with Max to bind the E-box element. However, in contrast to Myc–Max dimers, Mad/Mnt–Max dimers recruit histone deacetylases (HDACs) through the adaptor protein, SIN3, to close chromatin structure and repress transcription (Fig.1.2) (Ayer et al. 1993; Ayer et al. 1995; Hassig et al. 1997; Hurlin et al. 1997).

2. c-Myc regulates many cellular functions.

c-Myc is believed to regulate about 15% of all genes (Patel et al. 2004). Through its extremely large array of target genes, c-Myc is involved in regulating many cellular aspects such as cell cycle progression, cell growth, differentiation, metabolism and apoptosis. One of the key biological functions of c-Myc is its ability to promote cell cycle progression. A rat fibroblast cell line in which both *c-Myc* alleles were ablated shows greatly reduced rates of cell proliferation, accompanied by cell cycle defects in G_1 (Mateyak et al. 1997). c-Myc controls G_1-S transition through activating downstream targets such as cyclin E and its kinase partner, cyclin-dependent kinase 2 (Cdk2) and repressing Cdk inhibitor

p21CIP1(Steiner et al. 1995; Berns et al. 1997; Herold et al. 2002). In addition, c-Myc can increase cell cycle progression and cell growth through its regulation of many genes encoding products involved in ribosome biogenesis and protein translation (van Riggelen et al. 2010). c-Myc's role in cell growth is perhaps best appreciated in studies on its Drosophila orthologue dMyc. *dmyc* null cells in the wing imaginal disc are smaller and dMyc overexpression results in larger cells. Cell cycle distribution is unaffected in both cases (Johnston et al. 1999). c-Myc expression inhibits cellular differentiation and it has been shown to be one of four critical factors to reprogram differentiated cells into pluripotent stem cells (Coppola and Cole 1986; Takahashi and Yamanaka 2006).

c-Myc has a marked role in regulating cellular metabolism. To meet the requirements of increased proliferation, cancer cells often display changes in the metabolism of glutamine and glucose, the two major sources of energy and carbon substrate (Jones and Thompson 2009). Altered glucose metabolism is termed the "Warburg effect", which describes the propensity of cancer cells to use glycolysis over mitochondrial oxidative phosphorylation for glucose-dependent ATP production even in the presence of ample oxygen to fuel mitochondrial respiration (Warburg 1956). c-Myc transcriptionally regulates many genes involved in glycolysis. For example, overexpression of c-Myc can increase aerobic glycolysis through increasing transcription of lactate dehydrogenase (LDH-A), which converts pyruvate, derived from glucose through glycolysis or other sources, to lactate (Shim et al. 1997; Osthus et al. 2000). Recent studies

showed that c-Myc also regulates the metabolism of glutamine. c-Myc can transcriptionally repress microRNA, miR-23a and miR-23b, resulting in greater expression of their target protein, mitochondrial glutaminase, and leading to upregulation of glutamine catabolism (Gao et al. 2009).

Intriguingly, c-Myc also induces apoptosis (Evan et al. 1992; Schreiber-Agus et al. 1995) through a process known as oncogene-induced apoptosis (Pelengaris et al. 2002). The generally accepted explanation for this is that cell-proliferative and apoptotic pathways are coupled and the induction of cell cycle entry sensitizes the cell to apoptosis. This is probably a fail-safe mechanism for the cell to prevent inappropriate proliferation. However, the apoptotic pathway is suppressed as long as appropriate survival factors deliver anti-apoptotic signals. Given this, the predominant outcome of these contradictory processes will depend on the availability of survival factors. c-Myc induces apoptosis through both p53-dependent and p53-independent mechanisms (reviewed in (Dang et al. 2005; Meyer and Penn 2008)). c-Myc upregulates ARF, which in turn activates p53 to regulate a cohort of target genes involved in apoptosis and growth arrest (Zindy et al. 1998). Inactivation of the ARF-p53 pathway accelerates c-Myc-induced tumorigenesis in mouse models (Eischen et al. 1999; Jacobs et al. 1999; Schmitt et al. 1999; Alt et al. 2003; Finch et al. 2006; Bouchard et al. 2007). c-Myc also triggers apoptosis through activation of pro-apoptotic Bax, although the direct connection between c-Myc and Bax is still not clear (Juin et al. 2002; Dansen et al. 2006). Studies have shown that expression of c-Myc is crucial for

the conformational change that activates Bax, leading to cytochrome *c* release from the mitochondria, and therefore the activation of downstream effector caspases that execute apoptosis (Soucie et al. 2001; Annis et al. 2005). c-Myc also upregulates the pro-apoptotic BIM protein (Egle et al. 2004). Myc mutants that are evident both in Burkitt's lymphoma and in *v-myc* isolates are unable to upregulate expression of *BIM* and have reduced apoptotic potential. Mice expressing these mutants succumb earlier to lymphoma (Hemann et al. 2005).

3. c-Myc expression is regulated at multiple levels.

A variety of studies have suggested that tight regulation of c-Myc expression is essential for normal cell function. Whereas homozygous deletion of the c-Myc gene in mice results in embryonic lethality (Charron et al. 1992; Davis et al. 1993), overexpression of c-Myc in animal models often results in tumorigenesis in many target tissues (Felsher and Bishop 1999a; Pelengaris et al. 1999; D'Cruz et al. 2001). These tumors are clonal and often dependent on continuous high expression of c-Myc such that withdrawal of c-Myc expression leads to tumor regression (Felsher and Bishop 1999a; Pelengaris et al. 1999; D'Cruz et al. 2001), highlighting the importance of understanding the mechanisms that lead to c-Myc overexpression or deregulation in human cancer. Cells have evolved a variety of mechanisms to control c-Myc expression at multiple levels including transcription, mRNA stability, protein translation and post-translational modification (reviewed in (Meyer and Penn 2008)).

Deregulation of one or several of these mechanisms can contribute to its high expression in human cancer.

Increased transcription of the c-Myc gene in human cancer can be due to chromosomal translocation or gene amplification. Activation of human *c-MYC* as a result of chromosomal translocations is common in hematopoietic tumors. Chromosomal translocations, as in the case of Burkitt's lymphoma, activate *c-MYC* transcription by juxtaposing it adjacent to the actively transcribed immunoglobulin gene in B cells (Dalla-Favera et al. 1982; Neel et al. 1982; Taub et al. 1982). This chromosomal translocation was modeled by J. Adams and colleagues in the *Eμ-myc* mouse, which develops a clonal lymphoma in the B-cell compartment (Adams et al. 1985). In contrast to chromosomal translocations in hematopoietic cancers, activation of the *MYC* genes by amplification is commonly detected in solid human tumors. Gene amplification of *MYC* is reported in neuroblastoma, small cell lung cancer, colorectal cancer and breast cancer (Dang et al. 1999). In addition, recent genome wide association studies have found that multiple genetic variants on chromosome 8q24, the region that contains the *c-MYC* locus, are significantly associated with increased susceptibility to prostate, breast and colorectal cancer (Easton et al. 2007). Several studies have shown that multiple enhancer elements are present within this region and regulate transcription of *c-MYC* (Sotelo et al. 2010; Wasserman et al. 2010).

c-MYC mRNA is rapidly turned over in normal cells. The AU-rich 3' untranslated region (UTR) of *c-MYC* plays a negative role in controlling its mRNA stability and protein synthesis and removal of the 3'UTR results in an elevation of *c-MYC* mRNA (Jones and Cole 1987; Brewer and Ross 1988; Yeilding et al. 1996). The *c-MYC* 3'UTR is the binding sites for several RNA binding proteins such as HuR and microRNAs such as Let-7 (Wong et al. 2011; Sampson et al. 2007; Kim et al. 2009). Binding of HuR and Let-7 directs the RNA miRNA-induced silencing complex (RISC) to *c-MYC* mRNA (Kim et al. 2009). Interestingly, Let-7 is also a repressive target of c-Myc, thus forming a positive feedback loop to enhance c-Myc expression (Chang et al. 2009). Targeting of the *c-MYC* 3'UTR by miRNA also inhibits translation. In addition, c-Myc protein translation is also regulated by its 5'UTR, which contains an internal ribosome entry site (IRES) (Stoneley et al. 2000). IRES sequences can initiate protein translation in a cap-independent manner. Activation of the Phosphoinositide 3-kinase (PI3K)-Akt-mTOR signaling and the human epithelial growth factor-2 (HER2) signaling, which are commonly seen in human cancers, can enhance IRES-mediated translation of c-Myc (Kobayashi et al. 2003; Galmozzi et al. 2004). Aberrant translation of c-Myc has been reported in multiple myeloma and is associated with a point mutation in the 5'UTR (Paulin et al. 1998; Chappell et al. 2000).

c-Myc expression is also tightly regulated through post-translational modifications that affect c-Myc protein stability. c-Myc protein has a short half-life

about 20-30 minutes in normal cells (Sears et al. 1999; Malempati et al. 2006), suggesting that c-Myc is rapidly degraded soon after its synthesis. Studies from our lab and others have identified a post-translational pathway that regulates c-Myc protein stability through interdependent phosphorylation events on two conserved amino acids, Serine 62 (S62) and Threonine 58 (T58) (designated the c-Myc T58/S62 degradation pathway, Fig. 1.3) (reviewed in (Sears 2004)). Phosphorylation of the two amino acids has opposite roles on c-Myc protein stability. Phosphorylation of S62 stabilizes while phosphorylation of T58 destabilizes c-Myc and this requires prior phosphorylation at S62. Our previous work has revealed increased c-Myc protein stability associated with altered phosphorylation at S62 and T58 in leukemia (Malempati et al. 2006). Besides the c-Myc T58/S62 degradation pathway, c-Myc protein stability is likely to be regulated by other unknown mechanisms as the c-Myc phosphorylation mutant that can't be phosphorylated at S62, and hence is also not phosphorylated at T58, c-Myc^{S62A}, can also have a short half-life in certain cell types (Sears et al. 1999). In addition, the E3 ligase Skp2 has been reported to target c-Myc for ubiquitinylation and degradation independent of T58 phosphorylation (Kim et al. 2003; von der Lehr et al. 2003). The c-Myc T58/S62 degradation pathway is described in more detail below.

4. The c-Myc T58/S62 degradation pathway.

c-Myc protein levels are tightly regulated with respect to the proliferative status of the cell and the cell cycle. c-Myc protein levels are very low in quiescent cell, but upon serum stimulation and cell cycle entry, c-Myc protein levels accumulate and peak in early G_1 and then quickly decline and are maintained at low levels by late G_1 (Sears 2004). This bell-shaped pattern of c-Myc protein accumulation results from proper control of c-Myc expression at multiple levels as mentioned above. Work from our lab and others have identified a signaling pathway that regulates c-Myc protein degradation through the ubiquitin-proteasome pathway (Fig.1.3). Regulation of c-Myc protein stability through this pathway contributes at least in part to the bell-shaped pattern of c-Myc protein expression upon cell cycle entry.

Proteins involved in this signaling pathway include Ras-activated kinases, the glycogen synthesis kinase 3β (GSK3β), the Pin1 prolyl isomerase, the protein phosphotase 2A (PP2A), the scaffold Axin1 and the E3 ligase F-box protein Fbw7 (Fig.1.3). Ras activation and PI(3)K activation are central components of mitogen signal transduction pathways following mitogen activation of receptor tyrosine kinases (RTKs) (White et al. 1995). Activation of Ras signaling downstream of RTKs enhances c-Myc protein stability in two ways. First, Ras activation leads to activation of the Raf/MEK/ERK kinase cascade, which induces the phosphorylation at S62 by ERK kinase or other mitogen-activated protein (MAP) kinases as well as cyclin-dependent kinases (Hydbring et al. 2010; Lutterbach and Hann 1994; Sears et al. 1999). Simultaneous activation of the

PI3K/Akt pathway by Ras as well as directly through RTKs results in the phosphorylation and inactivation of GSK3β, the kinase that phosphorylates c-Myc at T58 (Cross et al. 1995; Sears et al. 2000). These two RTK signaling pathways thus keep c-Myc singly phosphorylated at S62 in early G_1 and transiently stabilize c-Myc protein (Fig.1.3). In late G_1, Ras and PI3K activity subside, relieving the inhibition on GSK3β activity, allowing GSK3β to phosphorylate c-Myc at T58 (Sears et al. 2000). Importantly, GSK3β is a processive Serine/Threonine kinase that primes off of a phosphorylation at Serine or Threonine four amino acids upstream of its target Serine/Threonine (Chu et al. 1996).

Following phosphorylation at T58, the doubly phosphorylated c-Myc binds to the prolyl-isomerase Pin1, which converts Proline 63 of c-Myc from *cis*- to *trans*-conformation. Pin1 specifically binds phosphorylated Serine/Threonine-Proline protein motifs and catalyzes the *cis/trans* isomerization of the peptidyl prolyl bond (Lu et al. 2006). In *trans*-conformation, the conformation sensitive protein phosphotase PP2A binds to and dephosphorylates S62. PP2A is a heterotrimeric holoenzyme comprised of two common components, the structural (A) and catalytic (C) and a third variable regulatory (B) subunit. Previous research in the Sears lab has shown that B56α is the B family member that is specific for targeting PP2A holoenzyme to c-Myc and dephosphorylating c-Myc at

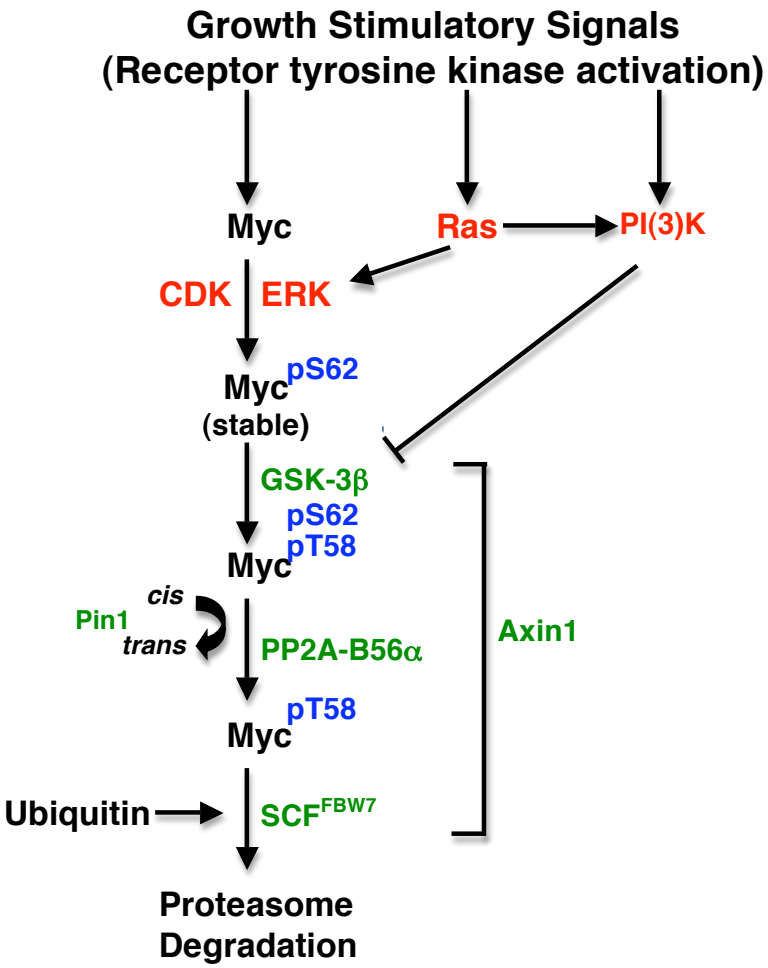

Figure 1.3: Schematic showing the c-Myc T58/S62 degradation pathway (Sears, 2004; Arnold et al, 2009).
In response to cell stimulatory signals, receptor tyrosine kinases located on the cell membrane are activated, resulting the activation of Ras and PI(3)K. Ras can increase phosphorylation of c-Myc at S62 by ERK or CDK. Simultaneous activation of PI(3)K inhibits GSK3β phosphorylation of c-Myc at T58. In later G_1, Ras and PI(3)K activity subsides and the inhibition on GSK3β is relieved. GSK3β then phosphorylates c-Myc at T58, followed by binding of Pin1, which isomerizes P63 in c-Myc from *cis*- to *trans*-conformation. With P63 in *trans*-conformation, c-Myc is dephosphorylated at S62 by the PP2A-B56α phosphatase. c-Myc is then recognized by the E3 ligase SCF^{FBW7}, which multi-ubiquitinates c-Myc and marks it for degradation via the 26S proteasome. The scaffold protein Axin1 promotes c-Myc degradation by coordinating a c-Myc destruction complex that contains GSK3β, PP2Ac and possibly other proteins involved in regulating c-Myc stability. Proteins that increase c-Myc protein stability are shown in red and those that promote c-Myc degradation are shown in green. The two phosphorylation sites, T58 and S62, are shown in blue.

S62 (Arnold and Sears 2006). Thus, without T58 phosphorylation, the Pin1/PP2A steps are inhibited and the mutation of T58 to Alanine (c-Myc^{T58A}) results in high constitutive S62 phosphorylation and no phosphorylation at position 58. This mutant is therefore more stable and studies have shown that it has increased oncogenic potential (Wang et al. 2011; Yeh et al. 2004). In contrast, wild-type c-Myc, once phosphorylated at T58 and dephosphorylated at S62, is recognized by the SCF^{Fbw7} ubiquitin machinery, which multi-ubiquitinylates c-Myc and marks it for degradation by the 26S proteasome (Welcker et al. 2004). Recently, work form our lab established that this signaling pathway is coordinated by the scaffold protein Axin1, which promotes formation of a c-Myc destruction complex that contains GSK3β, Pin1, PP2A and possibly other proteins involved in c-Myc degradation (Arnold et al. 2009). Regulation of c-Myc protein stability by Axin1 is described in detail in the "Axin1 introduction" in Chapter 1.2. Together, 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.

5. Phosphorylation at S62 and T58 regulates c-Myc function.

Based on the c-Myc T58/S62 degradation pathway, proper phosphorylation at S62 and T58 is critical for regulated degradation of c-Myc, which is likely to impact Myc function. The following observations support the important nature of these phosphorylation sites for Myc function. The T58 and S62 phosphorylation sites are conserved across species and c-Myc family

members (Luscher and Eisenman 1990). Phosphorylation at these sites accounts for the majority of induced phosphorylation upon serum stimulation (Lutterbach and Hann 1994). T58 is mutated in all v-Myc proteins and restoration of the wild-type Threonine severely inhibits the transformation properties of v-Myc (Papas and Lautenberger 1985). Mutations at or around T58 that prevent its phosphorylation represent hot spots for *MYC* mutations in Burkitt's lymphoma (Bhatia et al. 1993).

The importance of T58 and S62 phosphorylation in Myc function is further highlighted through studies comparing the function of c-Myc^{WT} with the two c-Myc phosphorylation mutants, c-Myc^{T58A} and c-Myc^{S62A}. The c-Myc^{T58A} mutant has constitutively high phosphorylation at S62 and no phosphorylation at T58 while the c-Myc^{S62A} has no phosphorylation at both sites due to their hierarchical nature. Studies have shown that the c-Myc^{T58A} mutant but not the c-Myc^{S62A} mutant or the c-Myc^{WT} could replace the known requirement for SV40 small T antigen, a specific inhibitor of PP2A, in the transformation of primary human fibroblasts in soft agar assays and xenograft experiments (Yeh et al. 2004). Several studies in mouse models have also shown that c-Myc^{T58A} is more oncogenic than c-Myc^{WT}, even when they are expressed at similar levels. For example, studies form Scott Lowe's lab have shown that hematopoietic stem cells (HSCs) expressing c-Myc^{T58A} transplanted into mice result in early onset aggressive B cell lymphomas with suppressed apoptosis compared to mice transplanted with HSCs expressing wild-type c-Myc (Hemann et al. 2005). Our

recent published study also shows that when expressed at near physiological level, c-Myc^{T58A} but not c-Myc^{WT}, induces mammary gland tumors in transgenic mice (Wang et al. 2011). Taken together, these experiments suggest that mutation at T58, which results in enhanced S62 phosphorylation, qualitatively affects c-Myc function.

6. Relevance of the c-Myc T58/S62 degradation pathway to human cancer.

Since c-Myc protein stability and function is highly regulated through the complicated, yet elegant c-Myc T58/S62 degradation pathway, it is important to know if this signaling pathway is relevant to human cancer. So far, mutations in c-Myc are only found in Burkitt's lymphoma and AIDS-associated lymphoma, and the majority of human cancers have wild-type c-Myc. Could c-Myc be stabilized through alterations in the regulators in the c-Myc T58/S62 degradation pathway? Starting from the top of the pathway, activated Ras signaling and PI(3)K signaling have been reported in many human cancers (Barbacid 1987; Berenjeno and Vanhaesebroeck 2009). Activation of Ras and PI(3)K might increase phosphorylation at S62 and inhibit phosphorylation at T58, thus stabilizing c-Myc. GSK3β phosphorylates c-Myc at T58 and can be regarded as a tumor suppressor here. However, the role of GSK3β on tumorigenesis remains controversial (Luo 2009). Some studies have found that GSK3β expression and/or activity is decreased in skin cancer, while others found overexpression of GSK3β in colon, ovarian and pancreatic cancers (Luo 2009). Pin1 is currently

regarded as an oncoprotein and its overexpression has been reported in many human cancers (Lu et al. 2006). From this, the involvement of Pin1 in c-Myc degradation seems counterintuitive. However, recent unpublished work from Sears lab has shown that Pin1 has a dual role in regulating c-Myc, providing a novel mechanism underlying Pin1's oncogenic activity. Specifically, Pin1 can enhance c-Myc transcriptional activity despite its negative role in c-Myc protein stability and the net result of Pin1 activity on c-Myc is to increase its oncogenic activity. Mutations in the rest of the proteins in the c-Myc T58/S62 pathway such as PP2A, Fbw7 and the scaffold protein Axin1 have also been reported in human cancer (Ruediger et al. 2001; Yeh et al. 2004; Salahshor and Woodgett 2005; Koh et al. 2006). In summary, alterations in regulators of c-Myc protein stability in the c-Myc T58/S62 pathway are found in many human cancers and these alterations might disrupt c-Myc degradation through the signaling pathway. Thus, while the complicated c-Myc T58/S62 degradation pathway provides a way of rapidly and efficiently modulating c-Myc protein expression in response to internal and external signals in normal cells, it might also provide a platform for deregulating c-Myc in cancer cells.

7. c-Myc and breast cancer

Breast cancer was identified as the second leading cause of death in women cancer patients in 2010 according to "Breast cancer facts & figures 2009-2010" released by American Cancer Society. c-Myc is a transcription factor that

regulates cell proliferation, apoptosis and differentiation, all are critical for mammary gland development and their deregulation leads to breast tumorigenesis (Jamerson et al. 2004). The importance of c-Myc in breast cancer is supported by studies from mouse models and human patient samples.

Studies in transgenic mouse models link c-Myc to mammary tumorigenesis. Overexpression of c-Myc under the control of mammary relevant promoters can drive mammary gland tumorigenesis in many reported transgenic mouse models (Jamerson et al. 2004). These mice showed extended mammary tumor latencies, suggesting that additional lesions are required for mammary tumorigenesis. Indeed, when these c-Myc transgenic mice were crossed with transgenic mice that express Ras, HER2, the transforming growth factor α (TGFα), or the pro-survival protein Bcl2, significant synergy in mammary tumorigenesis associated with reduced apoptosis in tumor cells was observed in the resulting c-Myc bi-transgenic mice (Sinn et al. 1987; Cardiff et al. 1991; Amundadottir et al. 1995; Jager et al. 1997), suggesting that c-Myc-induced apoptosis needs to be overcome to support tumorigenesis. As mentioned above, our recently published work has shown that while expression of c-Myc^{WT} at near physiological level is not tumorigenic, expression of c-Myc^{T58A} induced mammary gland tumors and similar to those c-Myc bi-transgenic mice, the c-Myc^{T58A} expressing mammary tumor also showed decreased apoptosis (Wang et al. 2011), suggesting that lesions that affect phosphorylation at T58 and S62 could contribute to tumorigenesis by suppressing apoptosis.

Deregulation of c-Myc in human breast cancer is prominent. Studies have shown that overexpression of c-Myc protein is found in approximately 50 to 100% of human breast cancer depending on the study (Agnantis et al. 1992; Nesbit et al. 1999), while only approximately 16% of human breast cancers have c-Myc gene amplification and 22% have increased *c-MYC* mRNA expression (Bieche et al. 1999; Blancato et al. 2004; Blancato et al. 2006). Unlike Burkitt's lymphoma, translocations involving the *MYC* locus have not been reported in breast cancer thus far. Instead, the chromosome region 8q24, where the c-Myc gene is localized, has been identified as 1 of the 3 most common regions of genomic amplification in breast cancer (Tirkkonen et al. 1998; Teixeira et al. 2001; Kleivi et al. 2002). In addition, the c-Myc promoter is a target of the Wnt signaling pathway, Notch pathway, Ras/Raf/MAPK, NF-κB pathway, and TGF-β pathway (Hynes and Stoelzle 2009). These pathways are all critical in breast cancer and activation of these pathways might lead to increased *c-MYC* mRNA. However, the obvious uncoupling between c-Myc protein and mRNA levels, given that only 22% of tumors with elevated c-Myc protein have elevated *c-MYC* mRNA as mentioned above, argues for other underappreciated mechanisms that might contribute to c-Myc overexpression in breast cancer. Thus, in Chapter 2 we examine c-Myc protein stability and the underlying mechanisms that lead to its increased expression in human breast cancer.

Axin1 background

Axin1 is a multi-domain scaffold protein that regulates many signaling pathways including Wnt/β-catenin, TGFβ, p53, SAPK/JNK and recently Myc signaling (Rui et al. 2004; Salahshor and Woodgett 2005; Li et al. 2007; Lin and Li 2007; Arnold et al. 2009; Li et al. 2009). It was originally identified as the product of the mouse gene called “fused” (renamed Axin1), mutations of which have pleiotropic developmental effects including the formation of axial duplications in homozygous embryos (Perry et al. 1995). Wnt signaling is important for dorsal-ventral determination during development in vertebrates, thus Axin1 was subsequently linked to Wnt signaling. Axin1’s role in Wnt signaling has been under heavy study since then and is perhaps the best characterized among Axin1 functions.

1. Axin1 is a negative regulator of Wnt signaling.

The Wnt pathway that is relevant to this discussion is the canonical Wnt/β-catenin pathway, which regulates the stability of the transcriptional co-activator β-catenin (Fig.1.4). Axin1 is the pivotal player in this Wnt signaling. It is required for constitutive degradation of β-catenin. In the absence of Wnt stimulation, β-catenin is actively degraded by the so-called Axin1 destruction complex. Within the complex, Axin1 and the tumor suppressor protein APC (the adenomatous polyposis coli gene product) form a scaffold that facilitates β-catenin phosphorylation by GSK3β

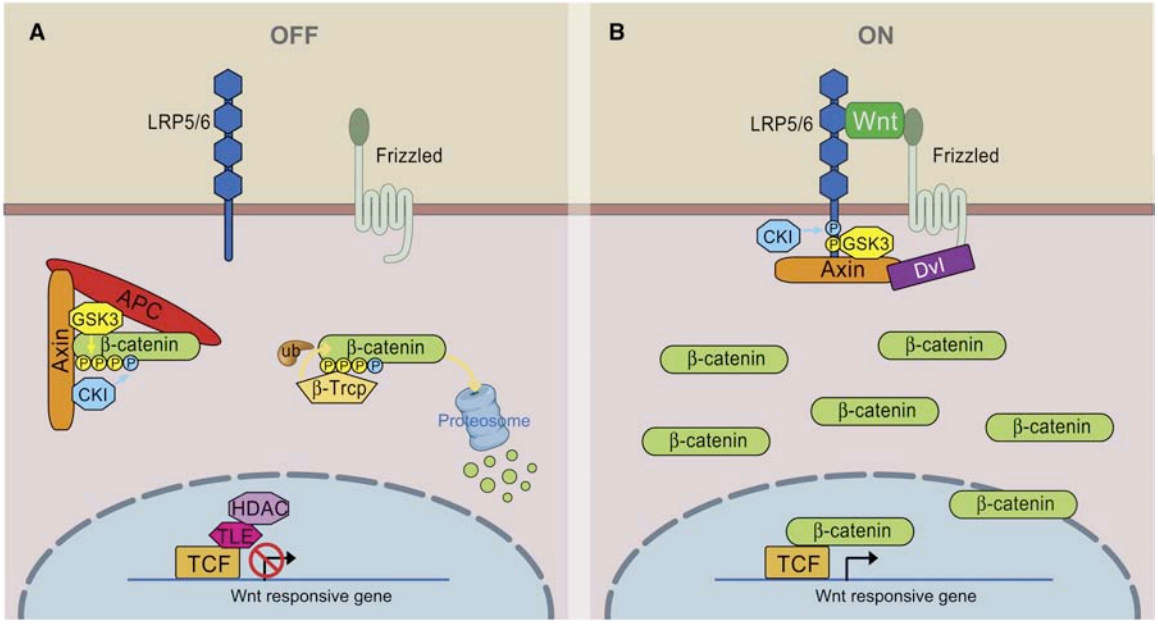

Figure 1.4: The Wnt/ β-Catenin Signaling (MacDonald et al. 2009).

(A) In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin1, APC, GSK3β, and CK1, and is phosphorylated by CK1 (blue) and subsequently by GSK3β (yellow). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteosomal degradation. Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC). (B) In the presence of Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation and Axin1 recruitment. This disrupts Axin1-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a coactivator for TCF to activate Wnt-responsive genes.

and Casein Kinase-1(CK1) α. Phosphorylated β-catenin is subsequently recognized and degraded by the ubiquitin/proteasome pathway, keeping cytosolic β-catenin at a low level (Fig.1.4A).

Wnt binding to cell surface receptors Frizzled (Fz) induces its interaction with the Wnt co-receptors, low-density lipoprotein receptor related protein 5/6 (LRP5/6), and the subsequent rapid phosphorylation of LRP5/6 by CK1γ and

GSK3β at the PPPSPxS (P, Proline; S, Serine or Threonine, x, a variable residue) motifs (Davidson et al. 2005; Zeng et al. 2005). The phosphorylated PPPSPXS motifs provide docking sites for the Axin1-GSK3β complex, thereby recruiting Axin1 to LRP5/6 upon Wnt stimulation (Mao et al. 2001; Tamai et al. 2004; Davidson et al. 2005; Zeng et al. 2005). Dishevelled (Dvl/Dsh), a cytoplasmic scaffold protein that binds to Fz, seems to be important for initiating LRP5/6 phosphorylation by GSK3β as Dvl also interacts with Axin1 (Wong et al. 2003; Bilic et al. 2007; Zeng et al. 2008). The recruited GSK3β can further phosphorylate LRP5/6, leading to the recruitment of more Axin1 to the membrane (MacDonald et al. 2008; Wolf et al. 2008). As a result, Axin1 is no longer available for β-catenin phosphorylation, thereby leading to β-catenin stabilization (Fig. 1.4B). Thus Axin1 and GSK3β seem to have a dual role in the Wnt signaling. In the absence of Wnt signaling, Axin1-GSk3β interaction promotes β-catenin phosphorylation and degradation. In the presence of Wnt, Axin1 recruits GSK3β to Wnt co-receptors LRP5/6 and activates these co-receptors, leading to activation of Wnt signaling (Fig. 1.4B). Accumulated β-catenin enters the nucleus and binds to the TCF/LEF family of DNA-binding factors for activation of gene transcription, including an Axin1 homologue, Axin2 (Liu and He 2010; Barker and Clevers 2006). Unlike Axin1, which is ubiquitously and constitutively expressed, Axin2 is reported to have tissue-specific and β-catenin inducible expression (Zeng et al, 1997). Interestingly, Axin2 has been shown to have similar functions

as Axin1 in regulating β-catenin degradation, thus forming a negative feedback to further increase Wnt signaling (Leung et al, 2002; Lustig et al, 2002).

2. Axin1 binds to GSK3β and PP2A.

Axin1 is a multi-domain protein and binds to a great number of proteins, including well-documented components of the Wnt signaling pathways such as the Wnt co-receptor LRP5/6, Dishevelled or Dvl, APC, GSK3β, β-Catenin, casein kinases, and PP2A (Fig. 1.5). Among these binding proteins, GSK3β and PP2A are of particular interest as they are also key regulators of c-Myc protein stability, and thus their interactions with Axin1 and their functions in Wnt signaling are discussed here.

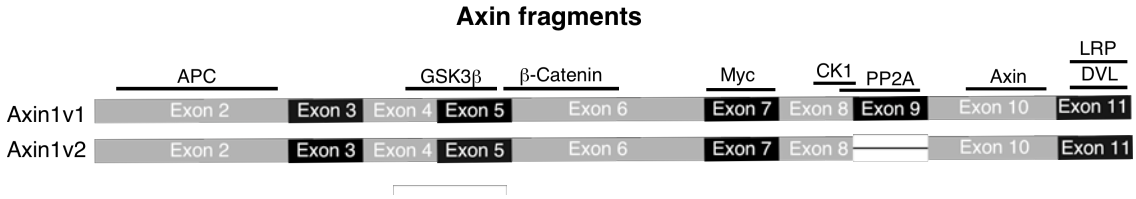

Figure 1.5: Axin1 is a multi-domain scaffold protein (modified from (Salahshor and Woodgett 2005).

The scaffold protein Axin1 has 10 coding exons (exon1 is non-coding) and multiple binding domains for proteins such as adenomatous polyposis coli (APC), GSK3β, β-catenin, Myc, CK1, PP2A, DVL, LRP and Axin1. There are two naturally occurring splice variants of Axin1 with exon 9 being removed in the second version.

Soon after the discovery that Axin1 is an inhibitor of Wnt signaling, several groups reported that Axin1 interacts with GSK-3β, an instrumental enzyme that phosphorylates ß-catenin, an integral step for β-catenin degradation and

downregulation (Ikeda et al. 1998; Kishida et al. 1998; Sakanaka et al. 1998). GSK3β binds to Axin1 in the region of exon 4 and 5, adjacent to the β-catenin binding site (Axin1 region exon 6). GSK-3 and β-catenin can bind simultaneously to Axin1, forming a ternary complex. This complex formation enhances phosphorylation of β-catenin by GSK-3β and β-catenin degradation (Ikeda et al. 1998; Sakanaka et al. 1998; Yamamoto et al. 1998). In addition, Axin1 itself is also a substrate of GSK3β. Several possible GSK-3β phosphorylation sites were found in Axin1. Phosphorylation at these sites by GSK-3β increases Axin1 protein stability (Yamamoto et al. 1999).

The role of PP2A in Wnt signaling is still confusing and controversial. Both positive and negative roles of PP2A have been reported. PP2A is a heterotrimeric enzyme comprising a conserved catalytic subunit (C), a structural subunit (A), and a variable regulatory (B) subunit. 26 different B subunits have been described. The C subunit of PP2A interacts with Axin1 (exon 8-10) (Hsu et al. 1999). The regulatory B56 subunit of PP2A interacts with APC in the Axin1-GSK3β-β-catenin complex (Ikeda et al. 2000). PP2A can promote dephosphorylation of both APC and Axin1 (Willert et al. 1999b; Ikeda et al. 2000). Increasing expression of B56 decreases the abundance of β-catenin, thereby reducing β-catenin signaling (Seeling et al. 1999; Li et al. 2001). While these results support a negative role of PP2A in Wnt signaling, some other reports suggest that the C subunit of PP2A exerts a positive role in Wnt signaling (Ratcliffe et al. 2000). One possible explanation for these seemly controversial

observations is that PP2A regulates multiple levels of Wnt signaling and activates or inhibits Wnt signaling depending on the timing of action and the composition of PP2A.

3. Axin1 promotes formation of a destruction complex for c-Myc.

In light of the fact that GSK3β and PP2A are involved in both the Wnt signaling pathway and the c-Myc degradation pathway, one would speculate that Axin1, which binds both GSK3β and PP2A, might also serve as a scaffold protein for c-Myc degradation. In theory, organizing some of the proteins involved in regulating c-Myc turnover into a complex by a scaffold protein would benefit the high level of regulation and rapid turnover of c-Myc protein.

Our recent research by Hugh Arnold has shown that Axin1 indeed facilitates the formation of a c-Myc degradation complex containing GSK3β, Pin1, and PP2A-B56α (Arnold et al. 2009) (Fig. 1.6). Specifically, he showed that Axin1 and c-Myc associate, which can be detected at endogenous levels along with endogenous GSK3β, PP2A-B56α, and Pin1. Overexpression of Axin1 increases c-Myc association with its regulators and knocking down Axin1 does the opposite, suggesting that alterations in Axin1 expression can globally affect c-Myc interaction with its regulators. Consistently, knocking down Axin1 increases c-Myc levels associated with a decrease in T58 phosphorylation and an increase in S62 phosphorylation and c-Myc protein stability. Ectopically expressed Axin1 increases the ubiquitination and degradation of c-Myc, reduces endogenous c-

Myc protein levels independent of effects on c-*MYC* mRNA expression, and reduces c-Myc-dependent transcription. All of these data suggest that Axin1 promotes the formation of a degradation complex for c-Myc. Mapping experiments showed that exon 7 of Axin1 is critical for at least ectopic c-Myc recruitment (Fig. 1.5). Moreover, binding sites for GSK3β and PP2A facilitate c-Myc association with Axin1, suggesting that the formation of the Axin1-mediated degradation complex for c-Myc is a coordinated process. Given that Axin2 is structurally similar to Axin1, it is likely that Axin2 can also regulate c-Myc degradation, however, we have not observed that Axin2 is upregulated with c-Myc overexpression as it is with β-catenin activation.

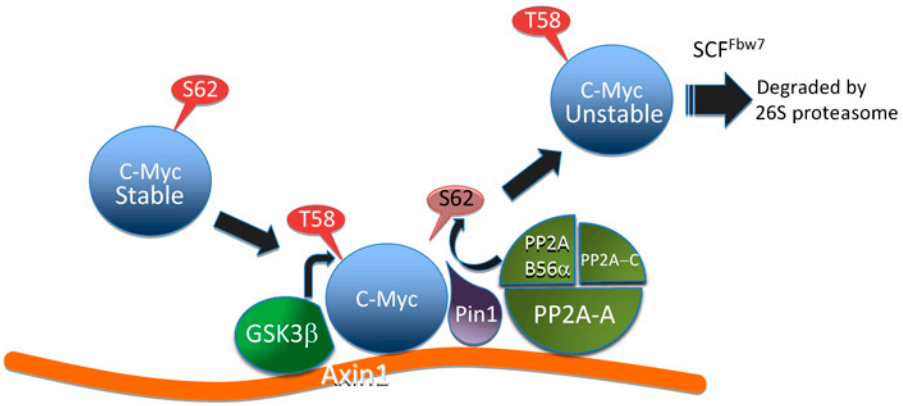

Figure 1.6. Model showing sequential steps in c-Myc degradation and the role of Axin1 as a scaffold in this process (Arnold et al. 2009).

Initial phosphorylation of S62 allows the association of c-Myc with Axin1. Axin1-associated GSK3β can then phosphorylate T58. Pin1 recognizes phosphorylated T58 and isomerizes Proline 63 from *cis* to *trans*. Axin1-associated PP2A-B56α can then dephosphorylate S62, promoting pT58-c-Myc recognition by Fbw7 and subsequent proteasomal degradation.

Phosphorylation at the two conserved sites of c-Myc, S62 and T58, are key events in the c-Myc degradation pathway. They are also important for Axin1's regulation of c-Myc. While Axin1 can regulate phosphorylation at the two sites by coordinating c-Myc's interaction with GSK3β and PP2A, these phosphorylations also affect c-Myc's interaction with Axin1. S62 phosphorylation is required for Axin1 association with c-Myc as the c-Myc^{S62A} mutant is not able to interact with Axin1. In addition, both the c-Myc^{T58A} and c-Myc^{S62D} (S62 to Aspartic acid) mutants have higher affinity for Axin1, likely due to a substrate trapping mechanism since these mutants are insensitive to PP2A and, at least in the case of c-Myc^{T58A}, Axin1-mediated degradation. While acute expression of Axin1 decreases c-Myc^{WT} protein level, this is not seen with either the c-Myc^{T58A} or c-Myc^{S62A} mutant. Thus proper phosphorylation of c-Myc is important for its regulated degradation through the c-Myc degradation pathway that is coordinated by Axin1.

4. Regulation of Axin1.

So far, Axin1 has been shown to regulate several important signaling pathways such as Wnt/β-catenin, Myc, p53, TGFβ and SAPK/JNK. Studies have shown that Axin1 is a concentration-limiting component for the assembly of the Axin1-GSK3β-β-catenin destruction complex and Axin1 abundance has profound influence on Wnt/β-catenin signaling (Lee et al. 2003). This might also be true for

Axin1's regulation of other signaling pathways. Thus, it is important to know how Axin1 is regulated.

Regulation of Axin1 can be through phosphorylation, protein stability and intracellular translocation. Phosphorylation can affect Axin1's interaction with other proteins. Wnt signaling induces dephosphorylation of Axin1 (Jho et al. 1999; Willert et al. 1999a; Yamamoto et al. 1999). The dephosphorylated Axin1 binds ß-catenin less efficiently than the phosphorylated form. Phosphorylation can also affect Axin1's protein stability. Phosphorylation of Axin1 by GSK-3ß increases Axin1 protein stability (Yamamoto et al. 1999; Gao et al. 2002) and dephosphorylation of Axin1 by protein phosphatase1 (PP1) leads to Axin1 destabilization (Luo et al. 2007). The endocytic adaptor disabled-2 (Dab-2) can block PP1 interaction with Axin1, inhibit Axin1 dephosphorylation and thereby stabilize its expression (Jiang et al. 2009). In addition to phosphorylation, Axin1 protein stability is also regulated by poly-ADP-ribosylation (PARsylation). PARsylation of Axin1 promotes its ubiquitination and degradation by a poly (ADP-ribose)-directed E3 ligase, RNF146, through the proteasome system (Zhang et al. 2011; Huang et al. 2009). Two recently reported small molecular inhibitors of Wnt signaling, XAV939 and IWR-1, can stabilize Axin1 protein by inhibiting Tankyrase-mediated Axin1 PARsylation (Chen et al. 2009; Huang et al. 2009).

Intracellular translocation provides another way of regulating Axin1 function. Wnt signaling causes a striking relocation of Drosophila Axin1 from the cytoplasm to the plasma membrane (Cliffe et al. 2003). Membrane bound Axin1

is sufficient for Wnt signaling in the Drosophila embryo (Tolwinski 2009). The mammalian membrane receptor LRP5/6 can also recruit Axin1 to the membrane where Axin1 is somehow degraded (Tolwinski et al. 2003). As a consequence, ß-catenin is no longer bound by Axin1, resulting in nuclear signaling by ß-catenin. These findings suggest that regulation of Wnt signaling is through destruction of the negative regulator Axin1, underlining the importance of proper Axin1 function in the Wnt/β-catenin signaling pathway.

Axin1 might also be regulated through other mechanisms such as altered RNA splicing. Axin1 has two naturally occurring splice variants, variant1 (Axin1v1) and variant 2 (Axin1v2), conserved between different species. Axin1v2 is a shorter form of Axin1, lacking 36 aa encoded by exon 9 (Fig. 1.5) (Salahshor and Woodgett 2005). The function of the polypeptide region encoded by exon 9 is as yet not clear. Exon 9 is located close to the Myc binding domain and is part of the PP2A binding domain (Fig. 1.5) (Hsu et al. 1999; Arnold et al. 2009). If the two variants have different functions as my data suggests (Chapter 2), switching their expression is another way to regulate Axin1.

5. Deregulation of Axin1 in cancer

As Axin1 has the ability to downregulate ß-catenin protein levels and has been found mutated in a number of solid cancer types, it is regarded as a tumor suppressor. Besides its prominent role in regulating β-catenin concentration, Axin1 also regulates several other signaling pathways relevant to cancer

including TGFβ, SAPK/JNK, p53 and Myc. While not well characterized, it is likely that Axin1's regulation of these other pathways contributes to its tumor suppressor function.

Deregulation of Axin1 due to point mutations, deletions, loss of heterozygosity (LOH), and reduced expression at the mRNA or protein levels has been found in many cancers. Single somatic point mutations located within the APC-binding domain of Axin1 have been found in sporadic medullablastoma cases (Baeza et al. 2003). In hepatocellular carcinoma (HCC), one study has shown that three of four HCC cell lines and 5 of 87 primary HCCs they examined have *AXIN1* mutations (Satoh et al. 2000). Adenovirus-mediated expression of wild-type Axin1 induces apoptosis in these cells, suggesting that Axin1 may be an effective therapeutic molecule for suppressing growth of hepatocellular cancers. Another study has shown *AXIN1* mutations in 7 of 73 HCCs and two of 27 hepatoblastomas (Taniguchi et al. 2002). In addition, they have also found LOH at the *AXIN1* locus in four of five HCCs with *AXIN1* mutations, supporting a tumor suppressor function of this gene (Taniguchi et al. 2002). LOH at the *AXIN1* locus has also been found in 6.3% of glioblastomas (Nikuseva Martic et al. 2010). In ovarian endometrioid adenocarcinoma, one cancer cell line with β-catenin accumulation was found to have an *AXIN1* mutation (Wu et al. 2001). In oesophageal squamous cell carcinoma (SCC), several mutations and polymorphisms have been reported in tumours and cell lines, and a correlation

between reduced *AXIN1* expression and tumour progression has been suggested (Nakajima et al. 2003).

While most of these examples of Axin1 deregulations have been linked to its function in β-catenin degradation, work in our lab has shown that cancer cell lines with deletion, mutation, or compromised Axin1 function have impaired c-Myc regulation (Arnold et al. 2009). Specifically, loss of Axin1 expression in the SNU475 HCC cell line is associated with loss of T58 phosphorylation and elevated S62 phosphorylation, a phosphorylation ratio that favors increased stability (Malempati et al, 2006). Additionally, an in-frame deletion of *AXIN1* disrupting most of its GSK3β-binding domain was found in a leukemia cell line that has increased c-Myc protein stability and lacks Myc-GSK3β association (Malempati et al, 2006). The relationship between Axin1 and c-Myc in breast cancer is further explored in this dissertation research and is presented in Chapter 2. I report in this work that reduced Axin1 expression as well as a switch from splice variant 1 to variant 2 contribute to increased c-Myc protein stability and oncogenic activity in breast cancer.

HER2 background

The human epidermal growth factor receptor-2 (HER2) belongs to a family of transmembrane tyrosine kinase receptors that function to activate intracellular signaling pathways in response to extracellular signals. HER2 was initially described as a carcinogen-induced oncogene in a rat brain tumor model and was found to be homologous to the *v-erbB* (avian erythroblastosis virus) viral oncogene later (Shih et al. 1981; Schechter et al. 1984). Since then, the HER family has grown to four: EGFR, HER2 (also known as p185her2/neu, Neu and erbB2), HER3 (erbB3) and HER4 (erbB4) (Akiyama et al. 1986; Toyoshima et al. 1986; Kraus et al. 1989; Plowman et al. 1993; Manning et al. 2002).

1. HER2 and its family proteins.

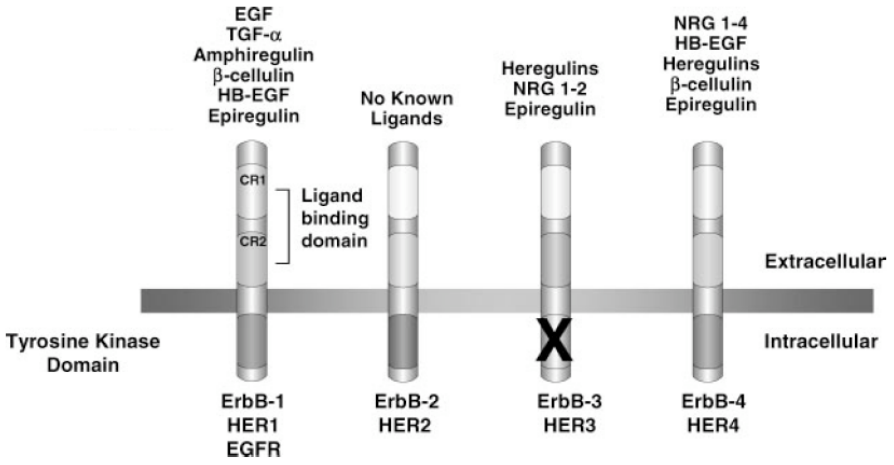

Fig.1.7: Domains of HER2 family proteins (modified from (Rowinsky 2004)).

HER2 family proteins have 3 domains in general, extracellular ligand-binding domain, transmembrane domain and intracellular tyrosine kinase domain. Note that HER2 has no naturally binding ligand and HER3 doesn't have tyrosine kinase activity.

Like most other tyrosine kinase receptors, HER2 and its family proteins have an overall structure of an extracellular ligand-binding domain, a transmembrane domain and an intracellular catalytic tyrosine kinase domain (Fig. 1.7) (Moasser 2007b). The extracellular domain of HER proteins can exist in a closed inactive or an open active conformation. Upon ligand binding, the extracellular domain is induced to the active conformation, leading to homo- or heterodimerization among HER family proteins and consequent auto- and trans-phosphorylation of tyrosine residues in their intracellular domain (Burgess et al. 2003). Phosphorylation of tyrosine residues in turn creates recognition sites for many intracellular signaling intermediates, which link HER family proteins to many downstream transduction cascades (Fig.1.8)(Prenzel et al. 2001; Yarden and Sliwkowski 2001; Barnes and Kumar 2004). Which signaling intermediates are engaged and hence which transduction cascades are activated are determined majorly by the identity of the bound ligand, as there are over 30 ligands in cells, as well as the receptor heterodimer partners (Olayioye et al. 1998; Zaczek et al. 2005). Among the various signaling cascades activated by HER proteins, the Ras-ERK-MAPK pathway is an invariable target of all HER ligands, and the PI(3)K-Akt pathway is downstream of most HER dimers (Yarden and Sliwkowski 2001).

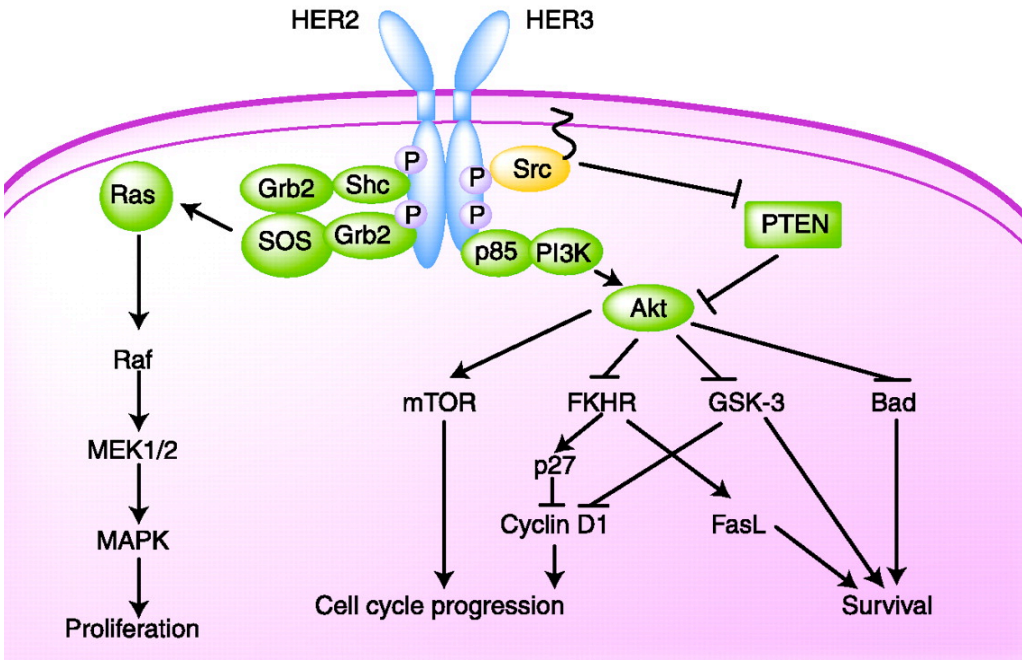

Figure 1.8: The HER2 signaling pathways (Lin and Winer 2007).

Upon ligand binding, HER proteins form homo- or hetero-dimers and activate their intracellular tyrosine kinase. On auto- and trans-phosporylation of the receptor complex, key downstream effectors are recruited. This figure illustrates a HER2-HER3 heterodimer as an example, but HER2 can also form homo- or heterodimers with other members of the HER family. FKHR, forkhead in rhabdomyosarcoma; Grb2, growth factor receptor-bound protein 2; GSK-3, glycogen kinase synthase-3; MAPK, mitogen-activated protein kinase; mTOR, molecular target of rapamycin; PI3K, phospatidyl-inositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SOS, son-of-sevenless guanine nucleotide exchange factor.

Among the HER family proteins, HER2 has the strongest catalytic kinase activity (Tzahar et al. 1996; Graus-Porta et al. 1997). However, unlike other members of the HER family, HER2 lacks ligand-binding activity in its extracellular domain and its activation is dependent on its ligand-bound heterodimeric partners (Sliwkowski 2003). HER2 is also the preferred dimer partner of other family members (Sliwkowski 2003). Theoretically HER2 can form four different types of dimers (with HER1, HER2, HER3, or HER4), but HER2/HER3 heterodimer is the

most abundant one found in human tumors and overexpression of HER2 is usually associated with overexpression of HER3. HER2/HER3 heterodimer is also reported to have the strongest signaling functions among the four HER2-containing dimers (Horan et al. 1995; Wallasch et al. 1995; Tzahar et al. 1996; Britsch et al. 1998; Keely and Barrett 1999; Sliwkowski 2003). Two special properties distinguish HER3 from other HER family members: it doesn't possess intramembrane tyrosine kinase activity; and it contains the most docking sites for p85, the regulatory unit of PI3K (Sierke et al. 1997). These properties allow HER3 to function as a scaffold protein to efficiently trigger the PI(3)K-Akt pathway, which, based on current data, seems to be the most important oncogenic signaling function of the HER2 (Munster et al. 2002) (Zhou et al. 2004; Tokunaga et al. 2006).

2. HER2 signaling pathway regulates many cellular functions involved in cancer development.

HER receptors especially HER2 regulate multiple processes that are important in cancer development, including cell proliferation and apoptosis inhibition. HER receptors exert these functions by modulating activities of their effector pathways such as Ras-ERK-MAPK, PI3K-AKT and Src signaling.

The HER receptor activation plays a significant role in cancer cell proliferation. HER2, the master of the HER network, is particularly important. Activation of HER2 drives cell proliferation majorly by modulating the activity of

p27, an inhibitor of Cyclin E-CDK2, which determines of G1-S transition (Timms et al. 2002). Activated HER2 regulates p27 activity through multiple mechanisms that regulate its localization and proteolysis. Activation of HER2 can increase levels of Cyclin D by activating MAPK and PI3K-AKT signaling pathway (Timms et al. 2002). Cyclin D can compete with cyclin E for p27 binding (Le et al. 2003). Thus, the p27 available for inhibiting cyclin E-CDK2 is reduced, allowing Cyclin E-CDK2 complex to promote cell cycle progression. In addition, activation of HER2 can inhibit p27 activity through recruiting and activating Src kinase (Chu et al. 2007). Src kinase can phosphorylate p27 and promote its proteasome-mediated degradation.

HER2 signaling inhibits apoptosis majorly through its effector PI3K-Akt pathway (Munster et al. 2002). PI3K-Akt is especially important in mediating cell survival as Akt can directly control phosphorylation of a host of proteins that are involved in cell survival. For example, Akt can phosphorylate the apoptosis-inducing protein Bad (Datta et al. 1997). Phosphorylation of Bad creates a binding site for 14-3-3 proteins and prevents Bad from binding to the prosurvival protein Bcl-2 and Bcl-XL, thus releasing them for a cell survival response (Datta et al. 1997). Another important target of Akt is the FOXO family of forkhead transcription factors, which can activate transcription of death ligands such as Fas and Trail and repress transcription of the pro-apoptotic protein Bim. AKT directly phosphorylates FOXO proteins and inhibits their transcriptional activity by sequestering FOXO proteins in the cytoplasm (Zhang et al. 2011).

3. HER2 signaling and breast cancer.

HER2 and its family members are critical in mammary gland tumorigenesis. Hyper-activation of HER2 signaling due to gene amplification, protein overexpression or abnormal transcriptional regulation has been linked with breast cancer prognosis and is also extensively studied as a predictive factor and target for therapy (Ross and Fletcher 1998; Ross and Fletcher 1999; van de Vijver 2001; Ross et al. 2003).

The tumorigenic function of HER2 is irrefutable and is supported by many *in vitro* and *in vivo* studies regarding either overexpression of HER2 or an activated mutant of HER2, NeuT, which was originally identified in a screen for oncogenes using a rat carcinogen-induced tumor model (Gruss et al. 1981). The NeuT mutant has a V664E mutation within the transmembrane domain, which causes dimerization even in the absence of ligand binding and its constitutive activation (Bargmann et al. 1986; Weiner et al. 1989). Expression of the NeuT mutant transforms NIH3T3 cells and mammary epithelial cells *in vitro* and induces mammary gland tumors when expressed in mice (MMTV-NeuT) (Schechter et al. 1984; Muller et al. 1988; Bouchard et al. 1989; Brandt et al. 2001). While these studies of NeuT provide solid evidence of HER2's tumorigenic function, their relevance to human health is challenged as human breast cancers usually have overexpression of HER2 instead of mutation (Moasser 2007b). Several interesting models have been proposed to explain the preference of overexpression over mutational activation in human cancer (Moasser 2007b).

Nevertheless, overexpression of HER2, just like expression of NeuT, also induces proliferation advantage and transformed characteristics in human mammary epithelial cells and induces proliferative and antiapoptotic changes that mimic early stages of epithelial cell transformation in three-dimensional (3D) cell culture models (Muthuswamy et al. 2001; Woods Ignatoski et al. 2003). Similar to transgenic mice expressing NeuT, transgenic mice overexpressing HER2 (MMTV-HER2) in mammary tissues also induce tumor formation (Finkle et al. 2004). Interestingly, the majority of tumors from the MMTV-HER2 mice have acquired deletion mutations within the extracellular juxtamembrane region that promote dimerization and enhanced kinase activity, probably reflecting a growth advantage conferred by these activating mutations. In addition, these tumors frequently have loss of heterozygosity at certain genomic loci that leads to deregulation of genes such as p53, suggesting that additional lesion is required for HER2 driven tumorigenesis (Li et al. 1997; Ritland et al. 1997; Cool and Jolicoeur 1999).

The critical role of HER2 in breast cancer is further supported by studies in human breast cancer. HER2 protein is overexpressed in about 25-30% of breast cancers (Slamon et al. 1989). Gene amplification is considered the main mechanism of HER2 protein overexpression (Slamon et al. 1989; Pauletti et al. 1996). Breast cancers can have up to 25–50 copies of the HER2 gene and up to 40- to 100-fold increase in HER2 protein expression resulting in up to 2 million receptors expressed at the tumor-cell surface (Moasser 2007b). Overexpression

of HER2 seems to be an early step in breast cancer formation as HER2 amplification is seen in nearly half of all *in situ* ductal carcinomas without any evidence of invasive disease. HER2 status is maintained during progression to invasive disease, nodal metastasis and distant metastasis (Liu et al. 1992; Tsuda et al. 2001; Latta et al. 2002; Park et al. 2006).

The clinical success of the HER2-targeted therapy provides strong evidence of the important role that HER2 has in the pathogenesis of breast cancer. Among the many antibodies and small molecular inhibitors that have been developed so far targeting HER2, the most successful ones are trastuzumab (Herceptin) and Lapatinib (Tyverb). Trastuzumab is a humanized monoclonal antibody against the extracellular domain of HER2 (Moasser 2007a). Binding of trastuzumab to HER2 prevents the formation of HER2-containing dimers and hence prevents HER2 activation (Moasser 2007a). Trastuzumab has demonstrated efficacy in HER2-overexpressing metastatic and early-stage breast cancers (Spigel and Burstein 2002). Another example of HER2-targeted therapy is Lapatinib, an orally active, small molecule dual inhibitor of EGFR and HER2 tyrosine kinases that was approved in the US in 2007 for clinical use (Geyer et al. 2006). Lapatinib interrupts signal transduction from the HER2 and EGFR receptors by competing with ATP for the intracellular ATP-binding domain of these RTKs (Nelson and Dolder 2006).

4. HER2 and c-Myc

The c-Myc transcription factor regulates cell proliferation, differentiation and apoptosis. HER2 also regulates cell proliferation and apoptosis inhibition by initiating multiple mitogenic signaling pathways. Both HER2 and c-Myc are overexpressed in human breast cancer and play crucial roles in mammary gland tumorigenesis in transgenic mouse models. HER2 and c-Myc share common pathways such as Ras-MAPK and PI3K-AKT, with HER2 upstream and c-Myc downstream of these pathways, raising the possibility that HER2 and c-Myc are functionally connected.

Several data support a functional connection between HER2 and c-Myc. Firstly, studies showed that *c-MYC* amplification was associated with *HER2* amplification in breast cancer and gastric cancer and coamplification of *HER2* and *c-MYC* was associated with worse outcome than with either amplification alone (Perez et al. ; Park et al. 2005a; Mitsui et al. 2007). Secondly, although c-Myc and HER2 each are powerful oncoproteins, transgenic mice expressing either HER2 or c-Myc alone have long tumor latency (Wang et al. 2011; Cardiff et al. 1991). However, overexpression of both HER2 and c-Myc together greatly accelerated the mammary gland tumor development ((Cardiff et al. 1991) and Sears lab unpublished data), similar to the synergy of Ras and c-Myc in tumorigenesis (Compere et al. 1989). Thirdly, studies showed that c-Myc was required for HER2-driven proliferative signals in breast cancer cell lines and long-term downregulation of HER2 led to decreased c-Myc expression (Neve et al.

2000). Finally, a clinical study investigating trastuzumab's affect in HER2-positive breast cancer patients showed that patients with *c-MYC* amplification had achieved a much bigger recurrence rate than those without *c-MYC* amplification (Kim et al. 2005). This result is unexpected since patients with *c-MYC* amplified tumors normally have poor prognosis. However, it is not completely surprising given that c-Myc's oncogenic activity is coupled with its ability to trigger apoptosis. It is thus proposed by the authors of the study that activation of HER2 selectively suppresses the apoptosis activity of c-Myc, allowing c-Myc to stimulate cell proliferation and cancer development. When HER2 activity is inhibited by trastuzumab, c-Myc regains its ability to trigger apoptosis. Taken together, these data strongly suggest a collaborative action of c-Myc and HER2 during tumorigenesis. However, the molecular mechanisms responsible for these collaborative effects are poorly understood.

As described in the "Myc introduction" part, increases in S62 phosphorylation have been shown to associate with increased oncogenic and reduced apoptotic activities of c-Myc. Given that (1) S62 phosphorylation is enhanced by Ras signaling which is downstream of HER2; (2) HER2 can inhibit apoptosis through PI3K-Akt pathway which can in turn inhibit c-Myc phosphorylation at T58 by GSK3β, we hypothesized that HER2 can regulate c-Myc protein stability and oncogenic function through modifying c-Myc phosphorylation at S62 and T58. This hypothesis is tested and described in Chapter 3 "HER2 enhances c-Myc protein stability".

Chapter Two :

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

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Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

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Abstract

High expression of the oncoprotein c-Myc has been linked to poor outcome in human tumors. While c-Myc gene amplification and translocations have been observed, this can only explain c-Myc overexpression in a subset of human tumors. c-Myc expression is in part controlled by its protein stability, which can be regulated by phosphorylation at Threonine 58 (T58) and Serine 62 (S62). We now report that c-Myc protein stability is increased in a number of breast cancer cell lines and this correlates with increased phosphorylation at S62 and decreased phosphorylation at T58. Moreover, we find this same shift in phosphorylation in primary breast cancers. The signalling cascade that controls phosphorylation at T58 and S62 is coordinated by the scaffold protein Axin1. We therefore examined Axin1 in breast cancer and report for the first time both decreased *AXIN1* expression and a shift in the ratio of expression of two naturally occurring *AXIN1* splice variants. We demonstrate that this contributes to increased c-Myc protein stability, altered phosphorylation at S62 and T58, and increased oncogenic activity of c-Myc in breast cancer. Thus our results reveal both an important mode of c-Myc activation in human breast cancer and a novel mechanism contributing to c-Myc deregulation involving new insight into inactivation of the Axin1 tumor suppressor in breast cancer.

Key words: c-Myc, Axin1, splice variant, breast cancer, protein stability, phosphorylation

Introduction

The c-Myc oncoprotein is a pleiotropic transcription factor involved in controlling many cellular functions, including cell proliferation, cell growth, and cell differentiation, as well as pathways that regulate genome stability and cell death (Felsher and Bishop 1999b; Mai et al. 1999; Yin et al. 1999; Prochownik and Li 2007; Meyer and Penn 2008). High levels of c-Myc expression occur in a wide variety of human tumors, and animal models demonstrate c-Myc-induced tumorigenesis in many tissues (Nesbit et al. 1999; Pelengaris et al. 1999; D'Cruz et al. 2001). These tumors are often dependent on continued high expression of c-Myc and withdrawal of c-Myc can induce tumor regression (Felsher and Bishop 1999a; D'Cruz et al. 2001), highlighting the importance of understanding how c-Myc expression is regulated. In breast cancer, c-Myc protein is reported to be overexpressed in approximately 50 to 100% of breast tumors depending on the study, while only approximately 16% show *c-Myc* gene amplification and 22% show increased mRNA expression (Agnantis et al. 1992; Bieche et al. 1999; Nesbit et al. 1999; Blancato et al. 2004; Blancato et al. 2006). Mechanisms for high c-Myc expression in human breast tumors lacking gene amplification or elevated mRNA expression have not been reported.

Cells have evolved an elegant signalling pathway to help regulate turnover of c-Myc so that c-Myc protein levels are kept low when not needed (Sears 2004; Meyer and Penn 2008). In this pathway, sequential and interdependent phosphorylation events on c-Myc at Serine 62 (S62) and Threonine 58 (T58)

influence c-Myc stability. Initial phosphorylation of S62 by ERK or CDK kinases in response to mitogen signalling transiently increases c-Myc stability while subsequent phosphorylation of T58 by GSK3β triggers dephosphorylation of S62 by protein phosphatase 2A-B56α (PP2A-B56α), ubiquitination by the SCF-Fbw7 E3 ligase, and proteasomal degradation (Welcker et al. 2004; Yeh et al. 2004). Burkitt's lymphoma-derived c-Myc mutations usually occur at or around T58, generally resulting in loss of T58 phosphorylation, elevated S62 phosphorylation, and increased c-Myc protein stability. These c-Myc mutants have increased oncogenic activity compared to wild-type c-Myc when expressed at similar levels, suggesting the importance of the two phosphorylation events in regulating c-Myc oncogenic activity (Yeh et al. 2004; Hemann et al. 2005; Thibodeaux et al. 2009; Wang et al. 2011). However, mutations in c-Myc have not been found in any epithelial cancer. We have been exploring whether wild-type c-Myc can be stabilized in cancer. Here we report that increased stability of wild-type c-Myc is a prominent mechanism for c-Myc overexpression in breast cancer and that this is associated with a change in the ratio of pS62 and pT58 to more closely match mutant c-Myc that has increased oncogenic activity. Exploration into the mechanism behind the altered ratio of S62 and T58 phosphorylation in breast cancer suggests a prominent role for deregulation of the Axin1 tumor suppressor.

Axin1 is a multi-domain scaffold protein that coordinates several different protein complexes involved in regulating Wnt, TGFβ, SAPK/JNK, and p53 signalling (Zeng et al. 1997; Zhang et al. 1999; Rui et al. 2004; Liu et al. 2006;

Arnold et al. 2009). Recently, we found that Axin1 promotes c-Myc degradation and decreases levels of c-Myc S62 phosphorylation by coordinating the formation of a c-Myc destruction complex that includes GSK3β, PP2A, and other proteins involved in degrading c-Myc (Arnold et al. 2009). The *AXIN1* gene expresses two naturally occurring splice variants, variant 1(v1) and variant 2 (v2). *AXIN1V1* encodes an 862 amino acid (aa) protein, while the protein encoded by *AXIN1V2* lacks the 36 aa from exon 9. Whether Axin1v2 functions differently from Axin1v1 has not been reported. Here we show that decreased expression of total *AXIN1* and differential expression of *AXIN1V1* and *AXIN1V2* contribute to increased c-Myc protein stability, altered phosphorylation at S62 and T58, and increased c-Myc oncogenic activity in human breast cancer.

Results

c-Myc protein stability is increased in breast cancer cell lines and is associated with altered phosphorylation at S62 and T58.

To study mechanisms that underlie elevated c-Myc expression in breast cancer we initially focused on five breast cancer cell lines: MCF7, MDA231, SKBR3, LY2, and MDA453, and compared them to MCF10A cells, a non-transformed human mammary epithelial cell line. Relative to MCF10A cells, all five breast cancer cell lines showed increased c-Myc protein expression, while *c-MYC* mRNA was only modestly elevated in two of the cell lines: SKBR3 and LY2 (Suppl. Fig. 2.1A-B). We analyzed the turnover rate of c-Myc protein in these cell lines plus three additional breast cancer cell lines and an additional control cell line, human mammary epithelial cells immortalized with hTERT (hMEC-hTERT). c-Myc half-life was significantly longer in all eight breast cancer cell lines, ranging from 34-90 minutes, compared with c-Myc in the controls, which ranged from 16-20 minutes, consistent with a variety of other non-transformed proliferating cell types (Sears et al. 1999; Malempati et al. 2006) (Fig.2.1 A-C, Suppl. Fig.2.1C-D). These results indicate that increased c-Myc half-life may be an important mechanism contributing to increased c-Myc expression in breast cancer.

While translocated *c-MYC* genes in Burkitt's lymphoma can harbor coding sequence mutations involving T58 that lead to mutant c-Myc with increased stability, this has not been reported in any solid cancers. We found no coding mutations in *c-MYC* in the breast cancer cell lines under study. We then

investigated whether dysfunction of the c-Myc degradation pathway involving T58 and S62 phosphorylation could account for the increased c-Myc stability. As part of this pathway, in normal cells c-Myc is dephosphorylated at S62 soon after T58 is phosphorylated, leading to rapid c-Myc turnover and an overall relatively low level of pS62 and high level of pT58 (Sears 2004; Malempati et al. 2006). In contrast, deregulation of this degradation pathway leads to an overall high level of pS62 and low level of pT58. We examined phosphorylation at T58 and S62 using phospho-specific antibodies (Malempati et al. 2006; Escamilla-Powers and Sears 2007; Arnold et al. 2009) (Suppl. Fig. 2.2A). When comparing to MCF10A cells, we found that the pS62/total c-Myc ratios were significantly higher in many of the breast cancer cell lines, particularly those with the longer c-Myc half lives (Fig. 2.1D). In contrast, pT58 levels trended lower in many of the breast cancer cell lines when calculated relative to total c-Myc, again correlating with increased c-Myc stability. Together, this data indicates a shift of c-Myc phosphorylation status from high pT58/low pS62 in non-transformed cells to high pS62/low pT58 in breast cancer cells with increased c-Myc stability. These results are consistent with our previous observations in leukemia cell lines (Malempati et al. 2006).

c-Myc has altered pS62 and pT58 levels in primary human breast tumors

To study whether this shift of pS62 and pT58 levels occurs in primary human breast tumors, we first validated the use of our phospho-specific antibodies on formalin-fixed/paraffin-embedded tissues (Suppl. Fig. 2.2B). We

then stained slides of patient matched normal and breast tumor with the pS62- and pT58-specific antibodies. Consistent with our observations in the breast cell lines (Fig. 2.1D), normal mammary epithelial cells showed very low pS62 staining whereas cells of ductal carcinoma *in situ* (DCIS) and invasive adenocarcinoma in the same sample showed high pS62 staining (Fig. 2.2A, red). In contrast, and again consistent with our data in the breast cell lines, serial sections from the same patient samples showed very high pT58 staining in normal mammary gland acini (Fig. 2.2B, red), while matched DCIS and invasive adenocarcinoma showed relatively lower pT58 staining. The same trend was observed in other matched normal and breast tumor samples (Suppl. Fig. 2.3A and B), and can be appreciated when adjacent normal and tumor tissue are under the same microscope field (Suppl. Fig. 2.3C). Quantification of the pS62 and pT58 staining from multiple patient-matched normal and breast tumor samples showed increasing levels of pS62 staining from normal to DCIS and invasive adenocarcinoma (Fig. 2.2C), and a corresponding decrease in pT58 staining (Fig. 2.2D). These results indicate that normal mammary epithelial cells and breast tumor cells express different forms of c-Myc due to different post-translational modifications, and suggest that increased c-Myc protein stability occurs in primary human breast tumors. Moreover, our results indicate that deregulation of the c-Myc T58/S62 degradation pathway is common in primary breast tumors. We later expanded our pS62 staining to a total of 22 cases and we found that 16 of the 22 cases showed an increase in pS62 from normal to

invasive carcinoma. Of interest, all the pS62 negative cases were also negative for Estrogen and Progesterone Receptor (Suppl. Table 2.1, ER-PR-).

Axin1 expression is decreased in breast cancer

Phosphorylation of T58 by GSK3β and dephosphorylation of S62 by PP2A-B56α are important steps in the T58/S62 c-Myc degradation pathway (Sears 2004). We examined expression levels of GSK3β and PP2A-B56α in the breast cancer cell lines relative to the MCF10A cells and did not see any obvious difference. Our recent work identified Axin1 as a scaffold protein that coordinates c-Myc's interaction with GSK3β and PP2A (Arnold et al. 2009). Alterations of Axin1 including *AXIN1* gene mutations and decreased Axin1 expression have been reported in several types of solid tumors (Satoh et al. 2000; Webster et al. 2000; Salahshor and Woodgett 2005), but so far no evidence of these alterations of Axin1 has been reported in breast cancer. We analyzed *AXIN1* mRNA expression in primary breast cancer and adjacent matched normal breast tissue (Fig. 3.3A). Out of the 9 sample pairs with sufficient cDNA, 7 breast cancer samples showed decreased *AXIN1* mRNA levels compared to their adjacent normal tissues. Analysis of *AXIN1* mRNA and protein expression in the five breast cancer cell lines relative to MCF10A cells showed a reduction in Axin1 expression only in the MDA231 cells (Fig. 3.3B and C).

Given the low expression of Axin1 in the MDA231 cells, we examined whether increasing Axin1 expression would affect c-Myc protein levels using a

newly published small chemical compound, IWR-1, that can increase Axin1 protein stability (Chen et al. 2009). IWR-1 treatment of MDA231 cells for 24 hours increased Axin1 protein levels and this corresponded with a decrease in c-Myc (Suppl. Fig. 2.4). However, we also observed a decrease in *c-MYC* mRNA that likely reflects Axin1 regulation of b-catenin, which can transcriptionally activate the *c-MYC* gene. To avoid this complication, we treated MDA231 cells with IWR-1 for 4 hours. At this time, IWR-1 caused a consistent increase in *c-MYC* mRNA (Fig. 2.3D, graph), but a reduction in c-Myc protein and pS62 that correlated with a small increase in Axin1 (Fig. 2.3D, western). The increase in *c-MYC* mRNA here might reflect a relief of c-Myc's negative autoregulation on its own transcription when c-Myc protein levels are decreased (Penn et al. 1990). Nonetheless, these results demonstrate that *c-MYC* mRNA and protein expression are strongly uncoupled upon increasing Axin1 expression. Indeed, IWR-1 treatment decreased c-Myc protein half-life from 43 minutes to 19 minutes in MDA231 cells (Fig. 2.3E). Taken together, these data show that restoring Axin1 expression in tumor cells can promote removal of the stabilizing pS62 and decrease c-Myc stability and expression.

Axin1 regulates c-Myc's oncogenic activity in breast cancer

In order to test whether Axin1 can affect c-Myc's oncogenic activity, we made stable cell lines that express doxycycline-inducible c-Myc and either shRNA against Axin1 or the corresponding empty vector in MCF10A cells.

Knocking down Axin1 expression in these cells increased c-Myc levels, consistent with Axin1's role in regulating c-Myc protein stability (Suppl. Fig. 2.5A, lane 4 vs 2). We then performed soft agar assays with two Axin1 shRNA clones (Fig. 2.4A, western) and two control clones to test if Axin1 loss affects c-Myc's ability to transform MCF10A cells. As expected, control clones produced very few colonies in soft agar and overexpression of c-Myc (+Dox) increased this modestly only in one clone (Fig 2.4A, graph). Knocking down Axin1 alone also modestly increased colony numbers. However, when we knocked down Axin1 in the presence of ectopic c-Myc, the numbers of colonies increased dramatically (Fig. 2.4A). In contrast, Axin1 knockdown did not increase the colony number in cells expressing the Axin1-insensitive c-Myc phospho-mutant, c-Myc^{T58A} (Fig. 2.4B) (Arnold et al. 2009). These results indicate that Axin1 knockdown cooperates with overexpression of c-Myc in mammary epithelial cell transformation dependent on regulatable phosphorylation of c-Myc at S62 and/or T58, suggesting a direct effect on c-Myc phosphorylation underlies the cooperation.

We next examined the effects of increasing Axin1 expression in breast cancer cells besides MDA231. In our previous study characterizing the effects of Axin1 on c-Myc, we showed that the SKBR3 breast cancer cell line had decreased interaction between c-Myc and Axin1, PP2Ac, and GSK3β, compared to the control MCF10A cells (Arnold et al. 2009). Thus, even though we did not observe decreased expression of Axin1 in the SKBR3 cells, this result suggested a dysfunction of the Axin1 scaffold protein in these cells. To test whether we

could overcome this dysfunction by increasing Axin1 expression, we treated SKBR3 cells with IWR-1 for 24 hours. Similar to the results in the MDA231 cells, we observed increased Axin1 and decreased c-Myc protein upon IWR-1 treatment and, in this case, no significant difference in *c-MYC* mRNA expression was seen (Fig. 2.4C). In addition, IWR-1 treatment increased interaction between c-Myc and its regulators Axin1, GSK3β and PP2Ac, indicating an increased scaffold function of Axin1 (Fig. 2.4D). Consistently, we saw decreased c-Myc protein stability in SKBR3 cells upon IWR-1 treatment (Suppl. Fig. 2.5B). Moreover, SKBR3 cells treated with IWR-1 grew slower in proliferation studies (Fig. 2.4E) and formed fewer colonies in soft agar (Fig. 2.4F). Interestingly, short-term treatment with IWR-1 that did not result in decreased c-Myc levels in these cells (Fig. 2.4G, western) still resulted in an inhibition of c-Myc promoter binding at its target genes determined by chromatin immunoprecipitation (ChIP) (Fig. 2.4G). This inhibition could underlie, at least in part, the reduced transformation of these cells upon IWR-1 treatment. Taken together, these data demonstrate that increasing Axin1 expression in breast cancer cells can decrease c-Myc expression, stability, and transactivation activity associated with reduced cell transformation, providing a biological relevance of decreased Axin1 expression in breast cancer.

Breast cancer cells have a switch in *AXIN1* splice variants expression that contributes to c-Myc activation

While many of the primary human breast cancer samples showed decreased Axin1 expression relative to their adjacent normal tissue, only one of the five breast cancer cell lines showed reduced Axin1 expression. Since cells like SKBR3 that have a normal level of Axin1 also have an Axin1 dysfunction (Arnold et al. 2009), we examined whether *AXIN1* could be mutated in these cells as well as the rest of the breast cancer cell lines under study. We sequenced *AXIN1* cDNA and did not find any mutation that would affect the Axin1 coding sequence. Interestingly, the *AXIN1* cDNA sequences that we obtained were all from a naturally occurring splice variant of *AXIN1*, termed *AXIN1V2*, suggesting an enrichment in this variant. *AXIN1*, also known as *AXIN1V1*, encodes an 862 aa protein, while the protein encoded by *AXIN1V2* lacks 36 aa encoded by exon 9 (Fig. 2.5A). The function of this domain is unknown, but the PP2A binding domain has been mapped to this region (Hsu et al. 1999). We previously demonstrated that Axin1v2 has a significantly reduced ability to interact with c-Myc and PP2Ac, suggesting that Axin1v2 has a decreased ability to regulate S62 phosphorylation (Arnold et al. 2009). There is no other published reports on the functional significance of this splice variant.

We examined *AXIN1V1* and *AXIN1V2* expression in the breast cancer cell lines and found that the ratios of *AXIN1V1* vs total *AXIN1* were decreased in the breast cancer cell lines except MDA231 where total *AXIN1* was reduced while the ratios of *AXIN1V2* vs total *AXIN1* levels were increased when compared to their expression in MCF10A cells (Fig. 2.5B). This result is likely to at least

partially explain the reduced association between c-Myc and Axin1 and the increased c-Myc stability that we reported in the SKBR3 cells (Arnold et al. 2009). Since IWR-1 treatment can affect c-Myc in SKBR3 cells (Fig. 2.4) we tested its ability to stabilize Axin1v1 and Axin1v2. Although IWR-1 is reported to interact with the c-terminal part of Axin1 including the region absent in Axin1v2 (Chen et al. 2009), we found that IWR-1 could increase both Axin1v1 and Axin1v2 ectopically expressed (for detection purposes) in SKBR3 cells (Suppl. Fig. 2.6A). Thus, it appears that IWR-1 treatment can recover Axin1 function even in tumor cells with enhanced *AXIN1V2* and reduced *AXIN1V1* expression. In addition, similar alterations in splice variant expression were found in primary human breast tumors, where the ratio of *AXIN1V2* vs *AXIN1V1* was significantly increased compared to their expression ratio in patient matched normal samples in 7 out of 11 patients (Suppl. Fig. 2.6B). Moreover, we also observed an enhanced expression of *axin1v2* vs. *axin1v1* in c-Myc/Neu-induced mouse mammary gland tumors relative to normal mammary gland (Suppl. Fig. 2.6C). More importantly, we found that the switch in *AXIN1V2* vs. *V1* expression correlated with increased pS62-c-Myc in sets of patient matched breast tumor relative to normal, as well as in the c-Myc/Neu driven tumors (Fig. 2.5C and Suppl. Table 2.2). Thus, not only can breast cancer exhibit a reduction in total *AXIN1* levels (Fig. 2.3A), but also in many cases increased *AXIN1V2* vs. *AXIN1V1* expression relative to normal cells. To our knowledge, this is the first report to demonstrate altered expression of these splice variants. Taken together,

these data show that a shift toward enhanced Axin1v2 expression is common in human breast cancer, suggesting a functional significance of this switch for breast oncogenesis.

Since Axin1 facilitates PP2A-mediated dephosphorylation of c-Myc at S62 and Axin1v2 shows decreased interaction with PP2A (Arnold et al. 2009), we tested whether Axin1v2 differs in its ability to promote dephosphorylation of S62. For these experiments we used SNU475 cells, a human hepatocellular carcinoma (HCC) cell line with homozygous deletion of exons 1 and 2 of *AXIN1* and no Axin1 expression. Our previous work showed that SNU475 cells have high pS62-c-Myc levels compared to the HCC cell line HepG2 which has wild type Axin1 (Arnold et al. 2009). Expression of Axin1v1 in SNU475 cells consistently decreased pS62-c-Myc levels as expected, while expression of Axin1v2 did not (Fig. 2.5D). No changes in *c-MYC* mRNA levels were observed with expression of either splice variant. We also infected these cells with adenovirus expressing CMV-driven c-Myc as another way to rule out indirect effects via β-catenin. Similar to the results with endogenous c-Myc, we observed a decrease in pS62-c-Myc in cells expressing Axin1v1 but not in cells expressing Axin1v2 (Suppl. Fig. 2.7A). We did not observe a significant change in total c-Myc in either of these experiments, probably due to other unknown defects downstream of Axin1 in the c-Myc degradation pathway in this cancer cell line (Suppl. Fig. 7A). Besides affecting c-Myc protein stability, c-Myc phosphorylation also affects its oncogenic activity in that the high pS62 form of c-Myc is more

oncogenic than the form of c-Myc lacking pS62 (Hemann et al. 2005; Wang 2010), suggesting an effect of altering this phosphorylation on c-Myc target gene regulation. Indeed, recent studies in other labs and in our lab have demonstrated an important role for pS62 in c-Myc binding to target gene promoters (Benassi et al. 2006; Hydbring et al. 2010) (Suppl. Fig. 2.7B). Thus, we analyzed the effects of the two Axin1 splice variants on c-Myc promoter binding and found that Axin1v1 consistently reduced c-Myc binding at the *NUCLEOLIN* and *E2F2* promoters while Axin1v2 didn't inhibit, but rather significantly increased c-Myc promoter binding, suggesting that Axin1v2 might have a dominant negative role in regulating c-Myc promoter binding (Fig. 2.5E). Consistent with the ChIP results, *NUCLEOLIN* and *E2F2* mRNA levels were decreased with Axin1v1 expression and increased with Axin1v2 expression (Suppl. Fig. 2.7C). From this, it appears that the negative effects of IWR-1 in SKBR3 cells (Fig. 2.4C-G) are likely due to increased expression of Axin1v1, rather than Axin1v2. Together, our results show that breast cancer cells commonly express a splice variant of Axin1 that has lost its ability to negatively regulate c-Myc and that compounds which stabilize Axin1 can overcome this.

Discussion

c-Myc is a well-known oncoprotein that regulates many cellular activities important for tumorigenesis. Several mechanisms have been shown to regulate c-Myc oncogenic activity. These include changes in c-Myc protein level, which is commonly elevated in human cancer (Smith et al. 2006; Murphy et al. 2008), and phosphorylation changes at T58 and S62 (Hemann et al. 2005; Smith et al. 2006; Wang et al. 2011). Importantly, these two mechanisms can be linked in that phosphorylation at T58 and S62 also helps to regulate c-Myc protein stability and expression level. Although it has become clear that T58 and S62 phosphorylation play important roles in c-Myc biology, analysis of c-Myc protein stability and/or T58 and S62 phosphorylation levels has not been reported in most human cancer types. In this study we have examined c-Myc stability and phosphorylation in human breast cancer cell lines as well as patient samples, and we have uncovered a novel mechanism to increase pS62-c-Myc and stability in breast cancer, involving downregulation of the Axin1 tumor suppressor protein and newly identified changes in Axin1 splice variant expression (Fig. 2.5F and Suppl. Fig. 2.8).

Increased stability and altered ratios of T58 and S62 phosphorylation in human breast cancer

Our analysis of c-Myc protein stability and pT58 and pS62 levels in human breast cancer cell lines and primary patient breast tumor samples demonstrates

that stabilization of c-Myc associated with altered S62 and T58 phosphorylation ratios is common in breast cancer. Careful examination of the effects of different c-Myc expression levels in mice has revealed different activities at different expression levels (Murphy et al. 2008). In addition, studies have shown that tumor-derived c-Myc mutants, whose mutations affect the phosphorylation status at S62 and T58, are more tumorigenic than wild-type c-Myc (Hemann et al. 2005). Moreover, we have shown using a unique mouse model that at near physiological levels of expression in the mammary gland, the c-Myc^{T58A} mutant, which lacks T58 phosphorylation and has constitutively high S62 phosphorylation, similar to the phosphorylation pattern we report here in breast cancer, is tumorigenic while deregulated near-physiological levels of wild type c-Myc is not (Wang et al. 2011). Recent reports have demonstrated that pS62 is important for c-Myc binding to a number of trans-activated target genes important for cell proliferation, growth, and survival (Benassi et al. 2006; Hydbring et al. 2010). Thus increased c-Myc protein stability and altered T58/S62 phosphorylation are likely to play important roles in breast tumorigenesis.

Aberrant expression of Axin1 in human breast cancer

Axin1 has been characterized as a tumor suppressor, and multiple mutations have been identified throughout *AXIN1* in a number of different cancers (Salahshor and Woodgett 2005). Overexpression of Axin1 in a transgenic mouse model causes increased apoptosis in the mouse mammary

gland (Hsu et al. 2001), suggesting a tumor suppressor function of Axin1 there. However, thus far, no mutation that affects the Axin1 coding sequence or deregulation of Axin1 expression has been reported in human breast cancer. Our study demonstrates that decreased *AXIN1* expression and altered ratios of two splice variants are common occurrences in both breast cancer cell lines and primary tumor samples. Moreover, we show that this contributes to increased c-Myc protein stability and oncogenic activity in breast cancer. Specifically, knocking down Axin1 cooperates with c-Myc in promoting cell transformation in immortalized cells, and increasing Axin1 levels with a small molecule, IWR-1, in breast cancer cells decreases cell transformation associated with suppressed c-Myc binding to target gene promoters. Thus, our study reveals another mechanism of action for the Axin1 tumor suppressor, where in addition to its other targets such as b-catenin, SAPK/JNK, TGFβ, and p53, Axin1 can suppress cell transformation through inhibition of c-Myc. Further research on how Axin1 coordinately regulates all of its targets will help our understanding of Axin1's role in suppressing breast tumorigenesis.

In breast tumor cells, besides decreased expression of total *AXIN1*, we found that a shift from *AXIN1V1* to *AXIN1V2* was common and is correlated with high pS62. The biological role of these two splice variants has not been reported. We previously found that Axin1v2's interaction with c-Myc and PP2Ac is lower than that of Axin1v1 (Arnold et al. 2009). Here we showed that ectopic Axin1v1 decreased pS62-c-Myc levels in Axin1 null cells, while Axin1v2 did not.

Moreover, while Axin1v1 can decrease c-Myc promoter binding, Axin1v2 does the opposite, suggesting a potential oncogenic side of this splice variant. Clearly, Axin1v2 and Axin1v1 differ significantly in their regulation of c-Myc phosphorylation and activity. It is possible that the two splice variants might also differentially regulate other Axin1 targets such as β-catenin and p53. Thus, increasing expression of Axin1v2 represents another way of deregulating Axin1 to promote tumorigenesis. In addition, Axin1 activity is regulated post-translationally, including phosphorylation, protein stability and subcellular localization, and it would be interesting to know in the future if deregulation through these mechanisms contributes to c-Myc stabilization in cancer. However, it is also clear that downregulation of Axin1 or a switch in splice variant expression did not occur in all of the tumors we examined, and other mechanisms are likely to contribute to altered c-Myc T58/S62 phosphorylation, such as deregulation of GSK3β and PP2A.

Therapeutic and diagnostic implications of our results

c-Myc is overexpressed in many human tumors and turning off c-Myc expression has been shown to be an effective and efficient cancer therapy in mouse models (Felsher and Bishop 1999a; D'Cruz et al. 2001; Soucek et al. 2008). In addition, animal modelling has demonstrated that inhibition of some c-Myc activity can be tolerated by many organs (Soucek et al. 2008). Thus understanding how c-Myc phosphorylation and stability are deregulated in

different cancer types becomes critically important to help design rational therapies. Indeed, we have shown that treatment of breast cancer cells with a small molecule that increases Axin1 expression and suppresses c-Myc activity repressed their oncogenic potential substantially. Furthermore, with the phospho-S62-specific antibody that we have developed for c-Myc, it should be possible to screen human breast tumors for those expressing more stable and oncogenically active c-Myc protein in order to help direct treatment.

Acknowledgements

We thank Dr. Xiaoyan Wang for providing mouse tumor samples and Carl Pelz for help with statistics analysis. This study was supported by the Department of Defense Breast Cancer research program award BC061306 to RCS, the Susan. G. Komen Breast Cancer Foundation awards BCTR0201697 and BCTR0706821 to RCS, and the NIH award 1 R01 CA129040-01 to RCS.

Figure Legends:

Figure 2.1l Increased Myc protein stability associated with increased pS62 and decreased pT58 in breast cancer cell lines. **A**. ^{35}S-Methionine pulse/chase analysis showing the decay of radiolabeled c-Myc. c-Myc half-life was determined as described in Suppl. Methods. **B.** Western analysis of c-Myc decay in cells treated with 10μg/ml cycloheximide (CHX). **C.** Summary of c-Myc half-life in control cell lines and in breast cancer cell lines. Bars represent SD. p-value was calculated using one-tailed Student's t-test. **D.** Western analysis of pS62 and pT58 levels in the indicated cells. Total c-Myc antibody and pS62-Myc antibody were probed on one western blot and detected by two-channel imaging with a LICOR Scanner. pT58 was probed separately. Expression of pS62/total Myc (left Y axis) and pT58/total Myc (right Y axis) was quantified and normalized to MCF10A. Bars represent SD. * $p<0.05$. ** $p<0.01$. *** $p<0.001$.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

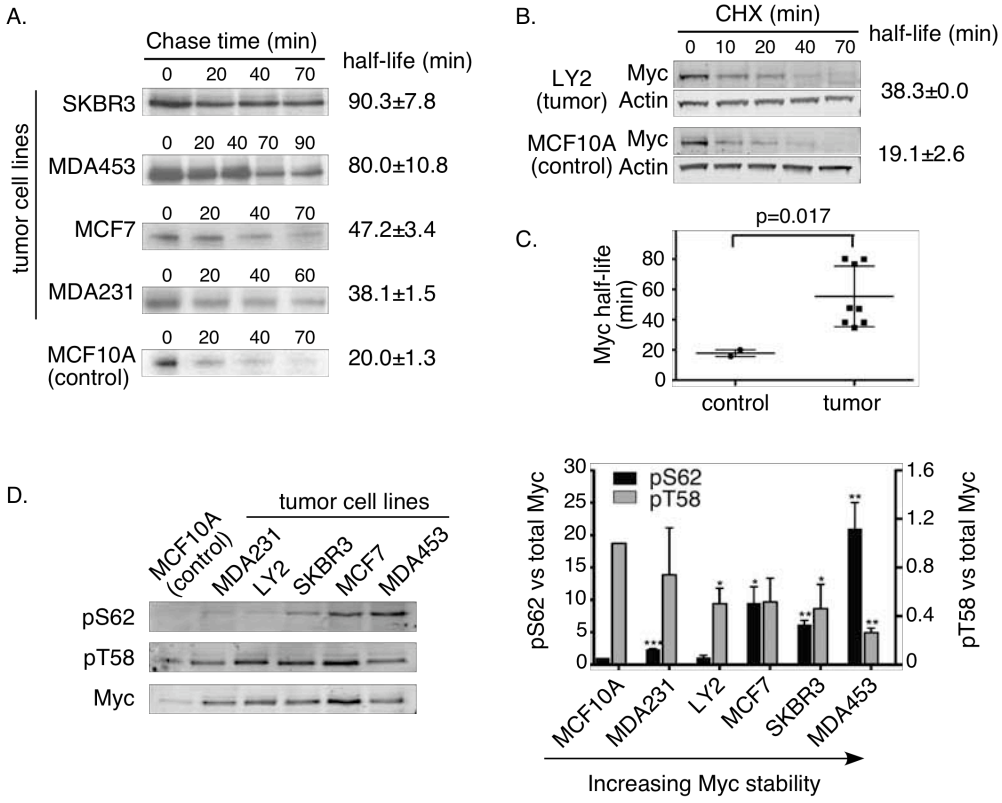


Figure 2.2l Increased pS62 and decreased pT58 levels of Myc in human breast cancer. A. Patient #1 matched sections of tumor and normal tissue were placed on the same slide and simultaneously stained with pS62 antibody (red) and DAPI (blue). Scale bars represent 50 μM. **B**. Serial sections from patient #1 were stained with pT58 antibody (red) and DAPI (blue). Scale bars are 50 μM. **C**. pS62 staining density from 7 patients was quantified as described in Methods and averages ± SD were graphed relative to normal (note patient #4 staining was not attainable in this set). Due to space limit, * indicates $p < 0.001$ between DCIS or Invasive carcinoma and normal samples. **D**. The same as C, except pT58 staining density and inclusive of patient #4. Averages of pT58 levels ± SD were graphed relative to invasive carcinoma or DCIS, which ever was lower.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

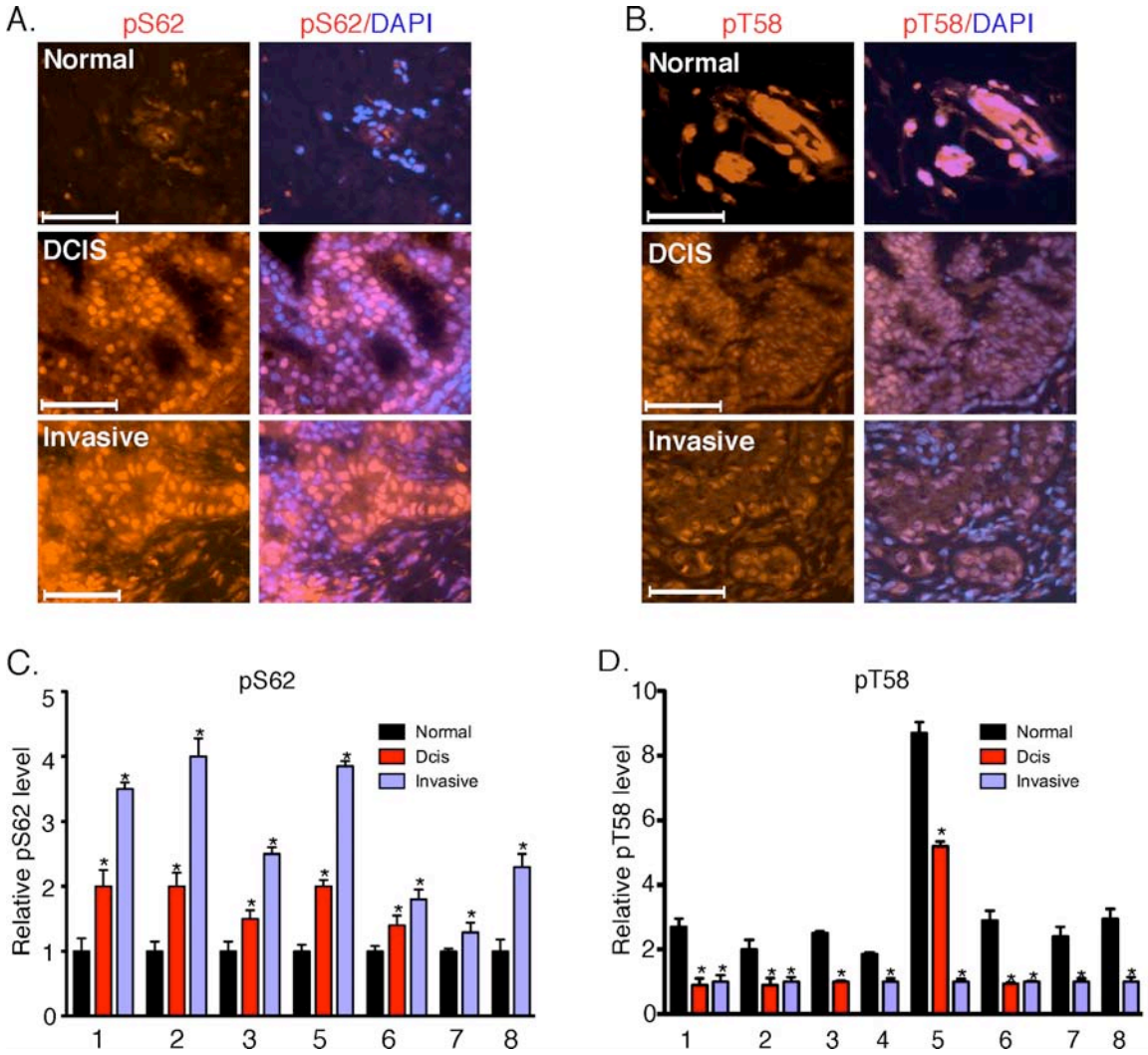


Figure 2.3l Decreased Axin1 in human breast cancer. A. qPCR analysis of *AXIN1* vs *ACTIN* expression tumor samples relative to matched normal samples was graphed in the order of most downregulated *AXIN1.* In this case only, bars represent SD from the triplicate qPCR reactions. Missing sample numbers are due to lack of sufficient cDNA. The matched normal/tumor ratios were log-transformed and a p-value for *AXIN1* downregulation significance was calculated by single-tailed t test, p=0.005. **B.** qPCR analysis of *AXIN1* mRNA levels in human breast cancer cell lines relative to MCF10A cells. **C.** Western analysis of Axin1 protein levels in breast cancer cell lines and control MCF10A cells. **D.** MDA231 cells were treated with 5μM IWR-1 for 4 hours and harvested for qRT-PCR analysis of Myc and western analysis of the indicated proteins. *MYC* mRNA from equal total RNA input was graphed based on changes in Ct and is shown ± SD. **E.** MDA231 cells were starved in 0.1% FBS for 48 hours, treated with DMSO or 10μM IWR-1 for 1 hour and stimulated with 10% FBS for another 3 hours in the presence of DMSO or IWR-1. Protein stability was analyzed as described in figure 1. * $p<0.05$. ** $p<0.01$. *** $p<0.001$.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

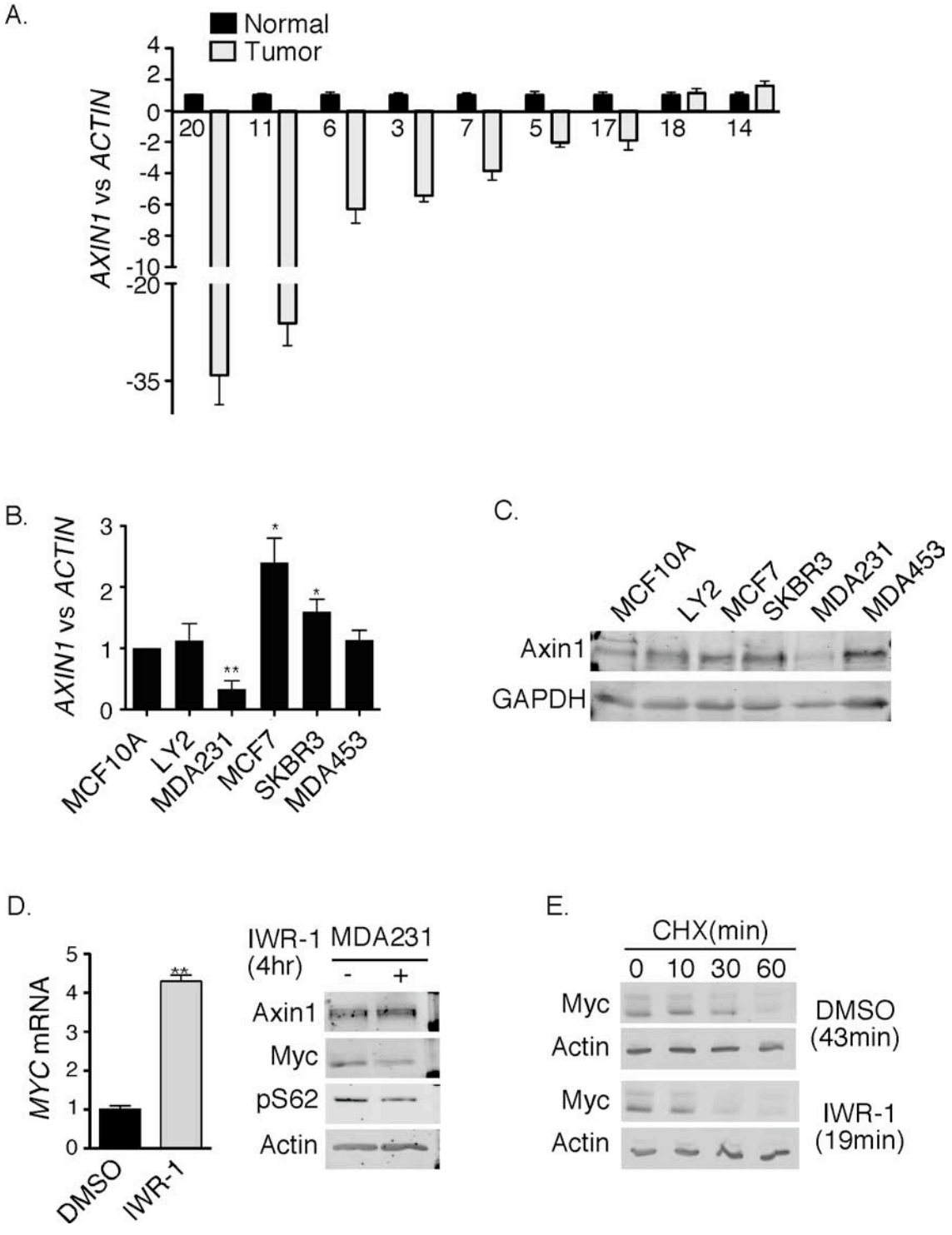


Figure 2.4l Axin1 regulates Myc oncogenic activity in breast cancer. A. The MCF10A stable clones Ctrl#1,2 (empty vector) and shAxin1#4,6 were grown in soft agar for 4 weeks with or without 1μg/mL doxycycline (Dox) and colonies were counted. The degree of Axin1 knockdown in different clones is shown in the western. **B.** Stable clones were infected with adenovirus Ad-Myc^{T58A} for 18 hours. Soft agar assay was done as in A. **C.** SKBR3 cells were treated with 10μM IWR-1 for 24 hours and analyzed for Myc and Axin1 expression by western. *MYC* mRNA expression was analyzed as in Fig. 2.3D. **D.** SKBR3 cells were starved in 0.2% FBS for 24 hours and treated with 5μM IWR-1 or DMSO for another 30 hours. Cells were treated with 10μM MG132 for 4 hours before harvesting for CO-IP with Myc antibody C33. Western analysis of input and IP is shown. **E.** Cell growth curve of SKBR3 cells with or without IWR-1treatment. **F.** Soft agar assay of SKBR3 cells treated with 10μM IWR-1 or DMSO as described in Methods. **G.** SKBR3 cells were treated with 10μM IWR-1 for 4 hours and ChIP was done with N262 antibody followed by qPCR. GAPDH internal primers were used as negative control (Neg. ctrl). Western blot of ChIP inputs is shown on top right corner. * $p<0.05$. ** $p<0.01$. *** $p<0.001$.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

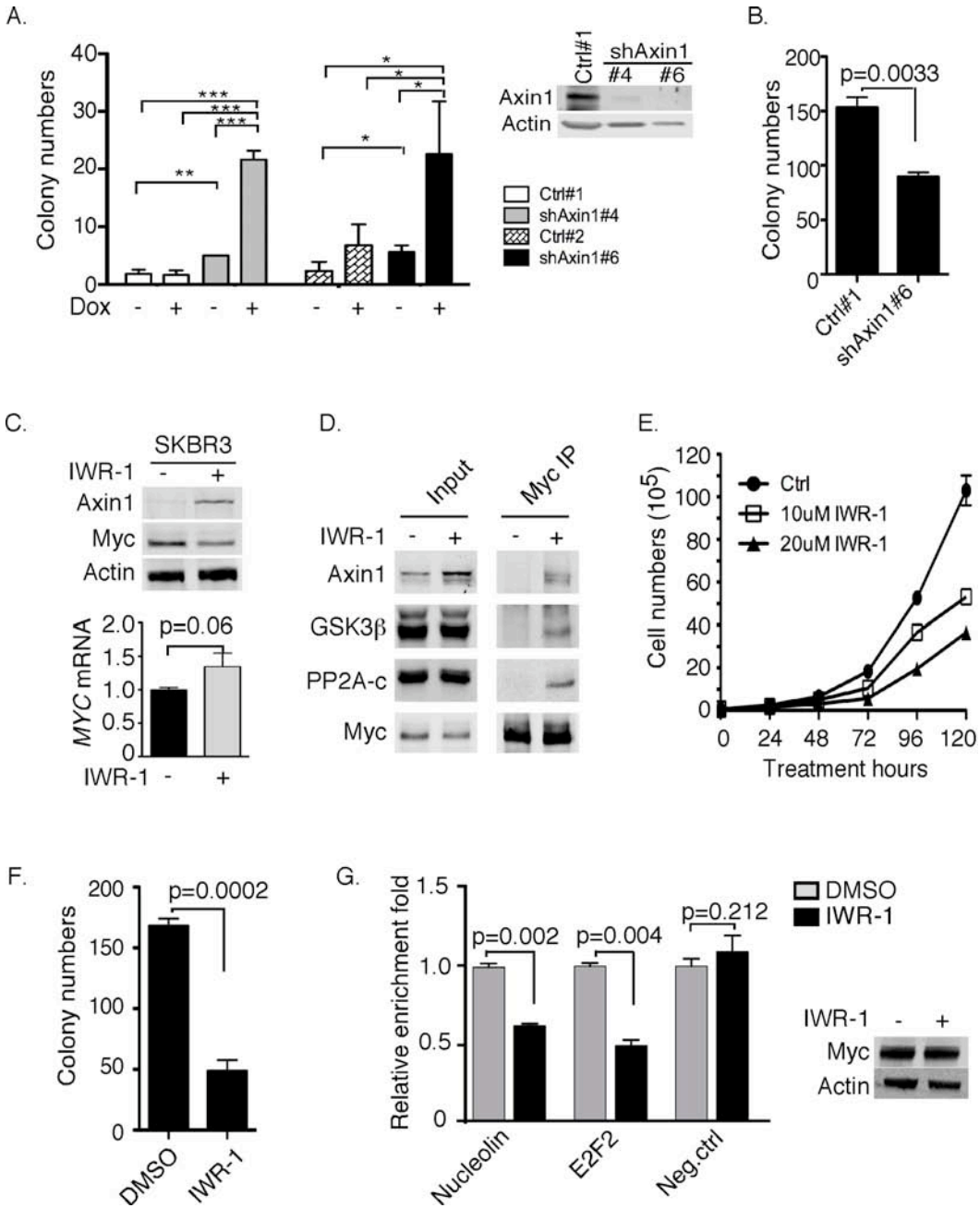
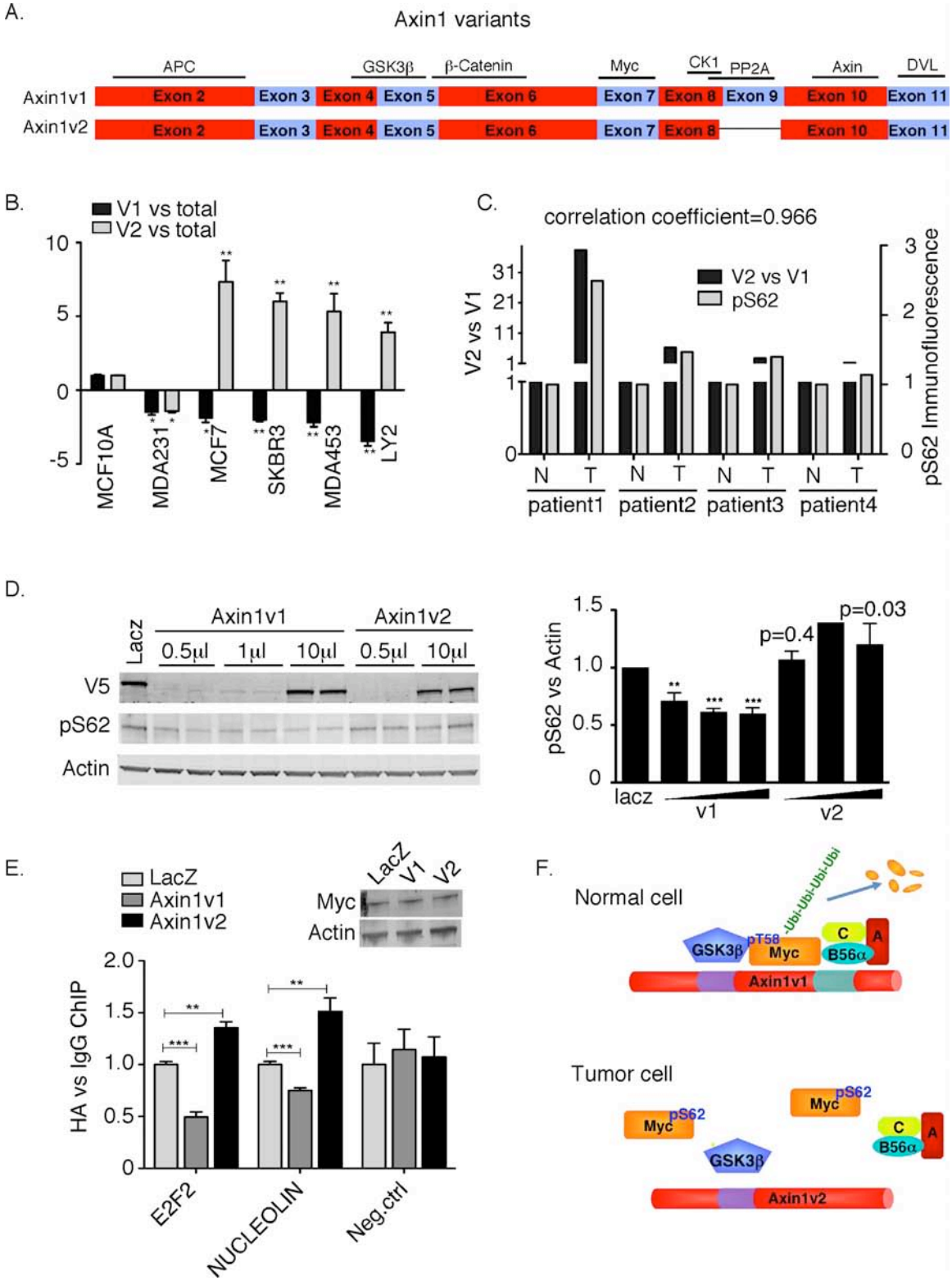


Figure 2.5l Switch from *AXIN1V1* to *AXIN1V2* expression in breast cancer. **A.** Schematic diagram of Axin1v1 and Axin1v2 coding exons modified from (Salahshor and Woodgett 2005). **B.** qRT-PCR analysis of *AXIN1V1* and *AXIN1V2* in breast cancer cell lines. Ratios of *V1* or *V2* vs total *AXIN1* normalized to those of MCF10A were graphed ± SD. **C.** Sets of normal (N) human breast tissue and matched tumor (T) tissue were prepared as snap frozen samples for extracting RNA for qRT-PCR of *V1* and *V2* expression or paraffin-embedded samples for pS62 immunofluorescence. The ratio of *V2* vs *V1* and the pS62 staining intensity in tumor tissue relative to matched normal tissue were graphed. Correlation coefficient was calculated using Excel. **D.** SNU475 cells were infected with lentivirus expressing either V5-tagged LacZ as control or V5-tagged Axin1v1 or v2 protein for 72 hours and analyzed by western. Graph shows pS62-Myc vs Actin levels from multiple experiments. **E.** SNU475 cells were infected as in D for 48 hours and then infected with Adenovirus expressing HA tagged Myc for 18 hours. ChIPs were done with α-HA as described for Fig. 2.4G. Western blot shows ChIP inputs. **F.** A model showing deregulation of Axin1 contributes to stabilization and accumulation of pS62-Myc in breast cancer. In normal cells, Axin1 coordinates a Myc destruction complex including GSK3β, PP2A and other proteins and promotes Myc degradation. In tumor cells, a switch in Axin1 splice variants and/or decreased Axin1 levels leads to disruption of the Myc destruction complex, resulting accumulation of pS62-Myc. See suppl. Fig. 2.8 for a more detail model. * $p<0.05$. ** $p<0.01$. *** $p<0.001$.

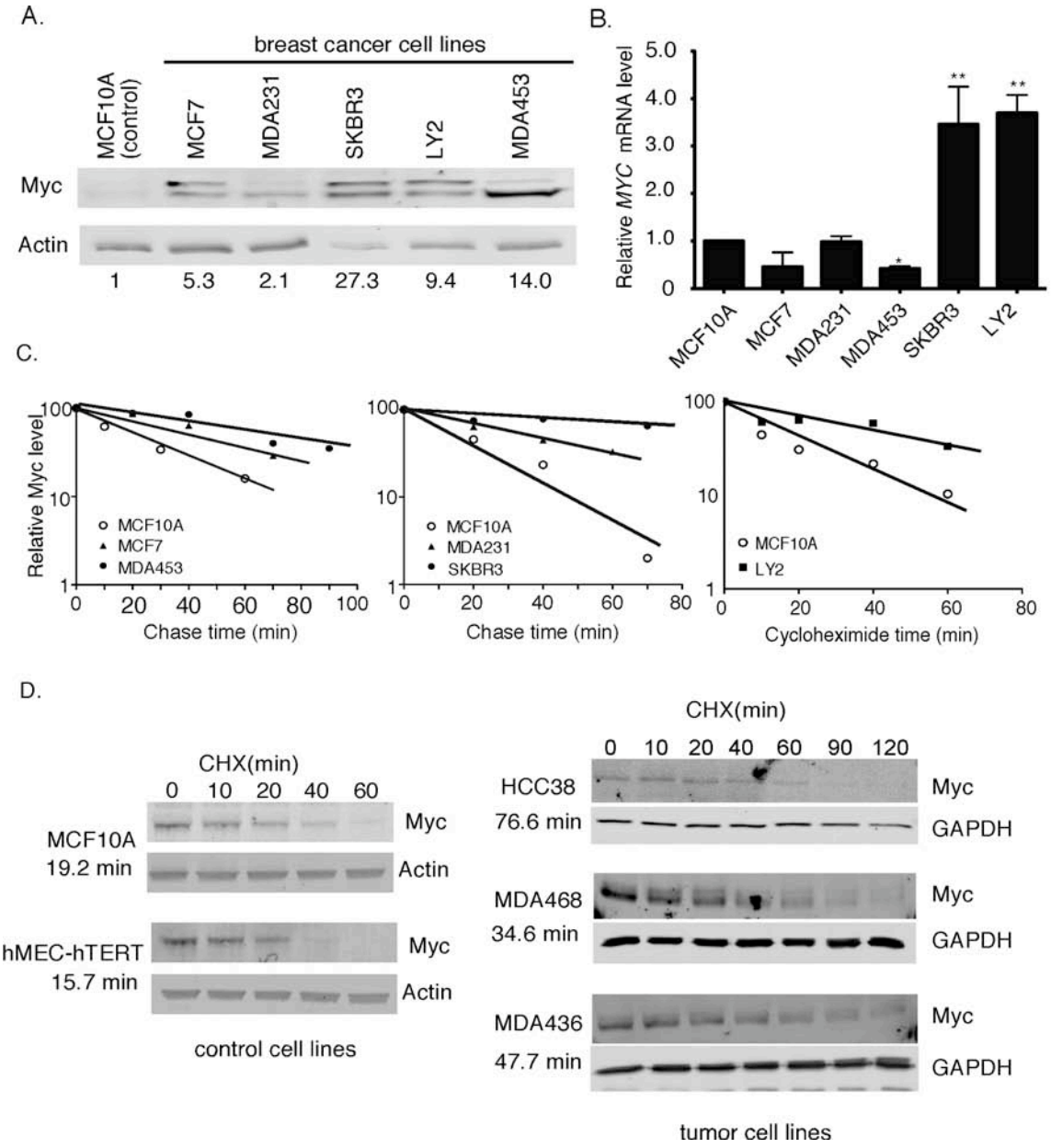
Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression



Supplemental Figure Legends:

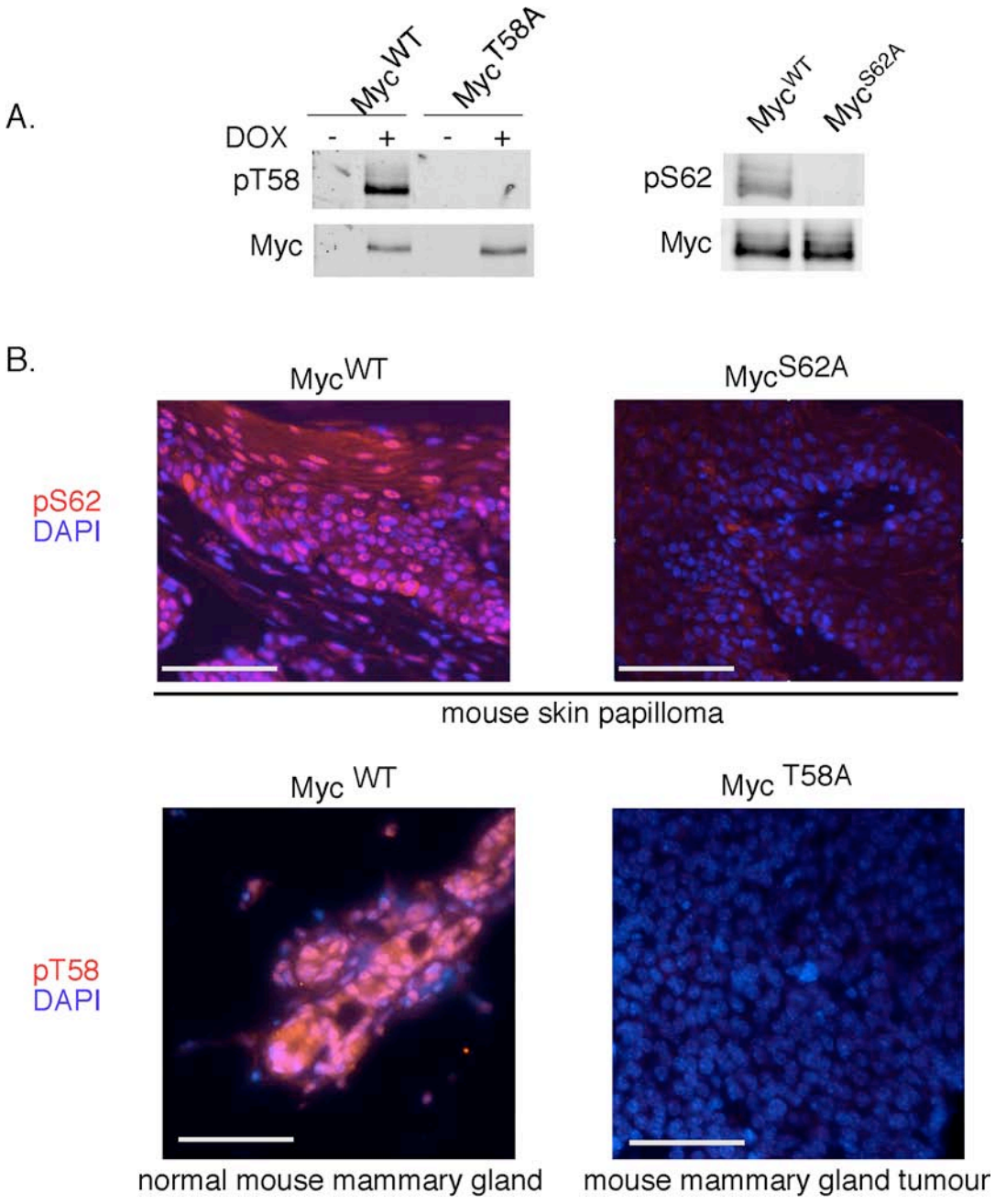
Supplemental Figure 2.1l Increased c-Myc protein stability contributes to c-Myc overexpression in human breast cancer cell lines. A. Western analysis of c-Myc protein expression in human breast cancer cell lines. Representative western blot is shown. Numbers below are the average fold change normalized to b-actin and relative to MCF10A. **B.** Quantitative real time PCR analysis of c-*MYC* mRNA levels in breast cancer cell lines. **p-value <0.01. *p-value<0.05. **C**. The rate of c-Myc degradation for breast cancer and control cell lines is represented in the graph by best-fit exponential lines. c-Myc half-life was calculated from exponential line equations. **D.** c-Myc half-life in control and breast cancer cell lines determined by cycloheximide treatment.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression



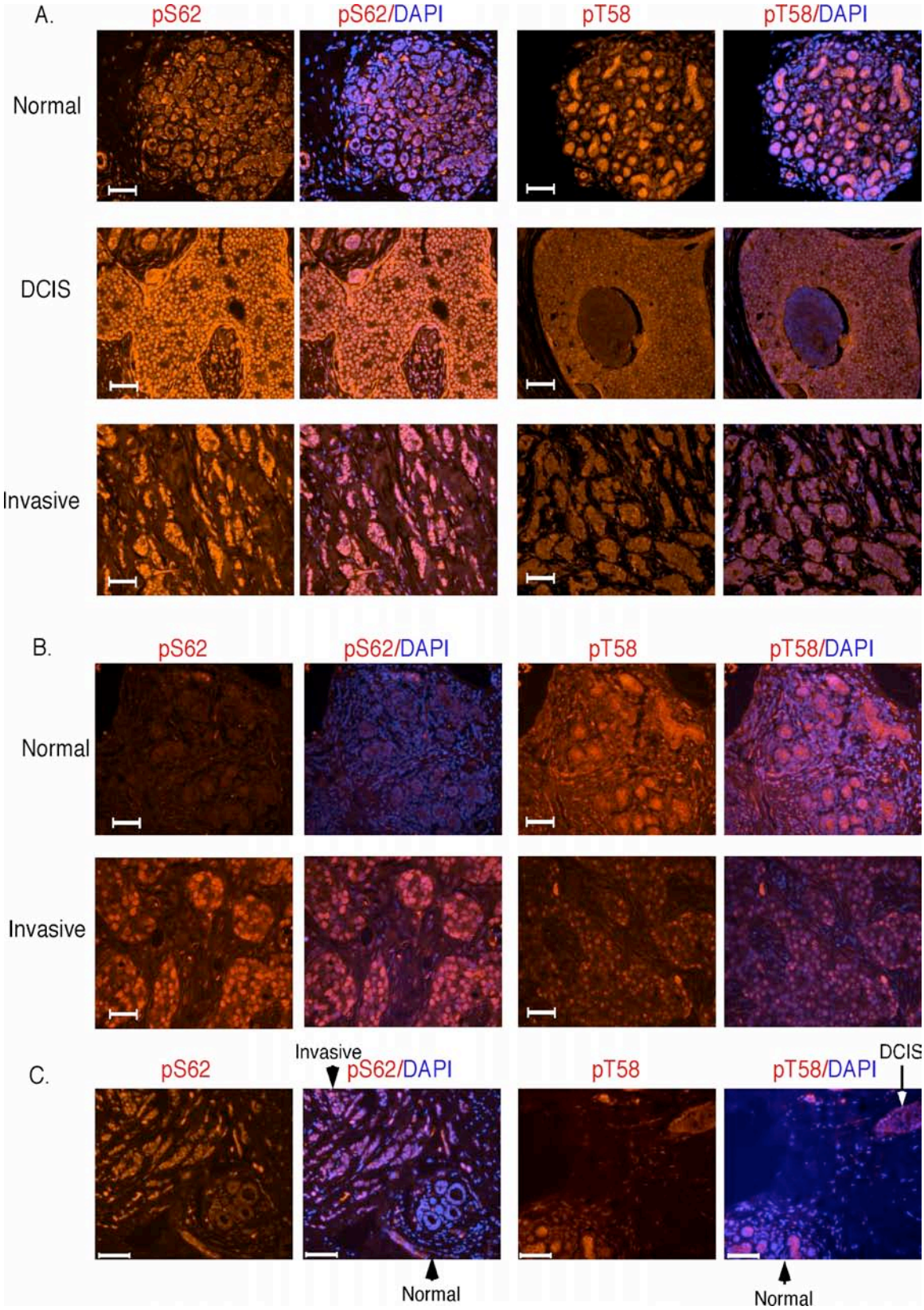
Supplemental Figure 2.2l Characterization of phospho-c-Myc antibodies used in western blot and immunofluorescence staining. A. Western blots showing antibody specificity. Protein lysates from MCF10A cells stably expressing doxycycline-inducible V5-tagged c-Myc^{WT}, c-Myc^{T58A} or c-Myc^{S62A} were analyzed by western blotting with either the V5 antibody for total V5-tagged c-Myc (V5, Invitrogen), the phospho-S62 antibody (E71-161, BioAcademia), or the phospho-T58 antibody (Applied Biological Material) as indicated. Blots were dual-probed with phospho-Myc antibodies and the V5 antibody and imaged using the LICOR imager (see Methods). **B.** The specificity of pT58 and pS62 antibodies for immunofluorescence assays (IF) on formalin-fixed, paraffin-embedded tissue sections. Mouse tissues ectopically expressing either c-Myc^{WT}, c-Myc^{T58A} or c-Myc^{S62A} were formalin fixed, paraffin embedded and analyzed by IF as indicated. Upper panels, mouse skin papilloma sections from our skin cancer model (DMBA/TPA), where expression of c-Myc^{WT} (left panel) or c-Myc^{S62A} (right panel) was driven from the ROSA promoter in response to K5-Cre in epidermal skin cells, were stained with our rabbit polyclonal phospho-S62 antibody (red) (Escamilla-Powers and Sears 2007) and DAPI (blue). Merged image is shown. pS62 staining was positive in c-Myc^{WT}-expressing papilloma, but negative in c-Myc^{S62A}-expressing papilloma. Bottom panels show merged images of phospho-T58 antibody (red) (GenScript) and DAPI (blue) staining of normal mouse mammary gland ectopically expressing c-Myc^{WT} driven by the ROSA promoter in response to WAP-Cre (left panel) and mammary gland tumor induced by

expression of c-Myc^{T58A} driven in the same way (right panel). c-Myc^{WT} in normal cells shows high pT58 levels as expected; c-Myc^{T58A} lacks phosphorylation at T58 and is negative for staining. Note that ectopic expression of c-Myc in these mouse models reduces expression of endogenous c-Myc. Scale bars are 50 μM.

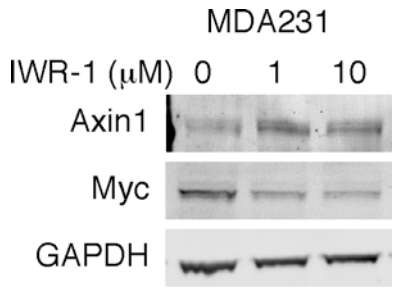


Supplementary Figure 2.3 l Increased pS62 and decreased pT58 levels in human breast tumors compared to patient matched normal breast tissue. **A.** Patient matched sections of tumor and normal tissue were placed on the same slide and stained with phospho-S62 or phospho-T58 specific antibody (red). Nuclei were counterstained with DAPI (blue). Right-hand panel in each column is the merged phospho-Myc/DAPI image. For each antibody, all pictures were taken at the same exposure. Normal acini, regions of DCIS, and invasive adenocarcinoma from patient #6 are shown. **B.** Phospho-S62 and phospho-T58 staining of normal and invasive adenocarcinoma from patient #8 are shown. Immunofluorescence staining was done as in A. **C.** Phospho-S62 and phospho-T58 staining of adjacent normal and tumor cells from patient #6 in the same microscope field are shown. All scale bars represent 50 μM.

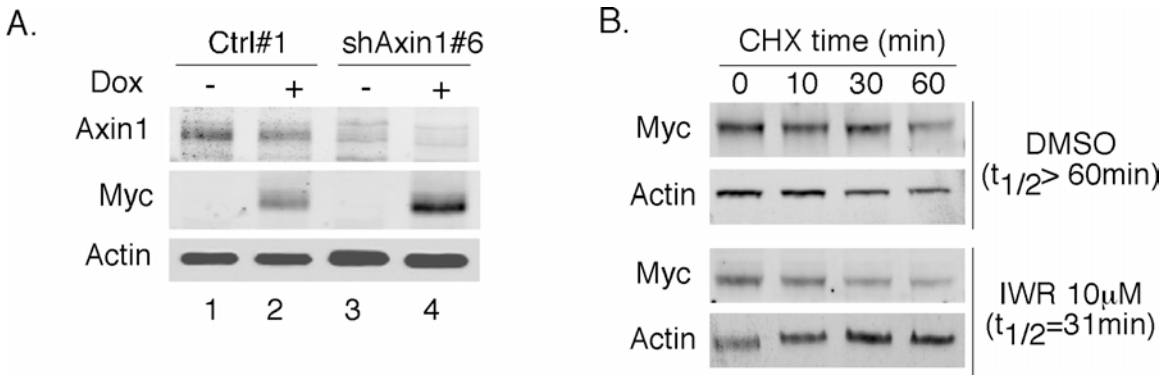
Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression



Supplemental Figure 2.4l IWR-1 decreases c-Myc expression in MDA231 cells. MDA231 cells were treated with IWR-1 at the indicated concentration for 24 hours and Axin1, c-Myc and Actin expression was monitored by western blot.

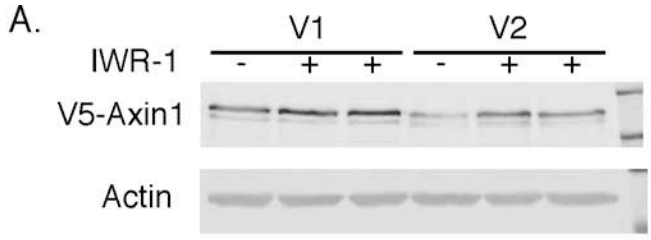


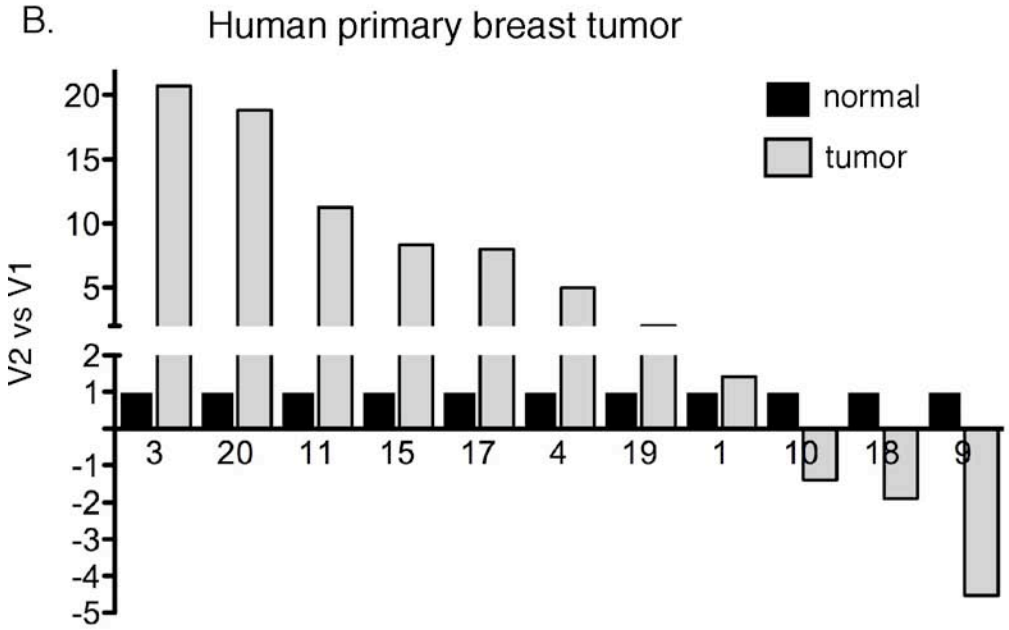
Supplemental Figure 2.5l Axin1 regulates c-Myc protein expression and stability. A. MCF10A cells that express doxycycline-inducible c-Myc were transfected with empty vector (Ctrl) or shRNA against Axin1 and stable clones (Ctrl#1 and shAxin1#6) were selected and analyzed for expression of Axin1 and ectopic Myc with or without 1μg/mL doxicycline (Dox) treatment for 24 hours. **B.** SKBR3 Cells were treated with 10μM IWR1 or DMSO for 4 hours and then treated with cycloheximide (CHX) for the indicated times before harvesting for western analysis. c-Myc half life was determined as in Fig. 2.1B and Suppl. Fig. 2.1D.

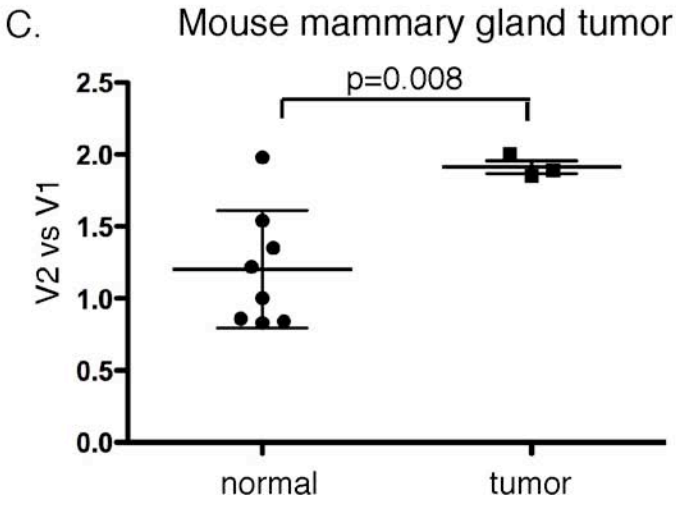


Supplemental Figure 2.6 | Tumor cells have higher ratio of *AXIN1V2* vs *AXIN1V1* than normal cells. A. SKBR3 cells were infected with lentivirus that expresses V5-tagged either Axin1v1 or Axin1v2 for 24 hours and then treated with DMSO or 10 μM IWR1 for another 24 hours. Shown are western blot of V5 antibody for Axin1 expression and loading control Actin. **B.** qRT-PCR analysis of *AXIN1V1 and AXIN1V2* mRNA levels in human breast tumors. The ratio of *V2* vs *V1* in each tumor sample was graphed relative to its matched normal sample. The matched normal/tumor ratios were log-transformed and a p-value for increased ratio was calculated by one-tailed Student's t-test. p=0.015. **C.** *axin1v1* and *axin1v2* expression was analyzed in normal mammary gland tissues by qRT-PCR and in mouse mammary tumors from our Cre-inducible, Rosa-Floxed-Stop (RFS)-Myc^{WT};MMTV or BLG-Cre;NeuNT mice (Wang et al. 2011; Andrechek et al. 2000). Myc^{WT} and Neu synergize in these mice for rapid tumorigenesis. The ratio of *v2* vs *v1* was set to 1 in one of the normal samples and the relative ratios of *v2* vs *v1* in the rest of the samples were calculated and graphed. p-value was calculated using a one-tailed Student's t-test.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression

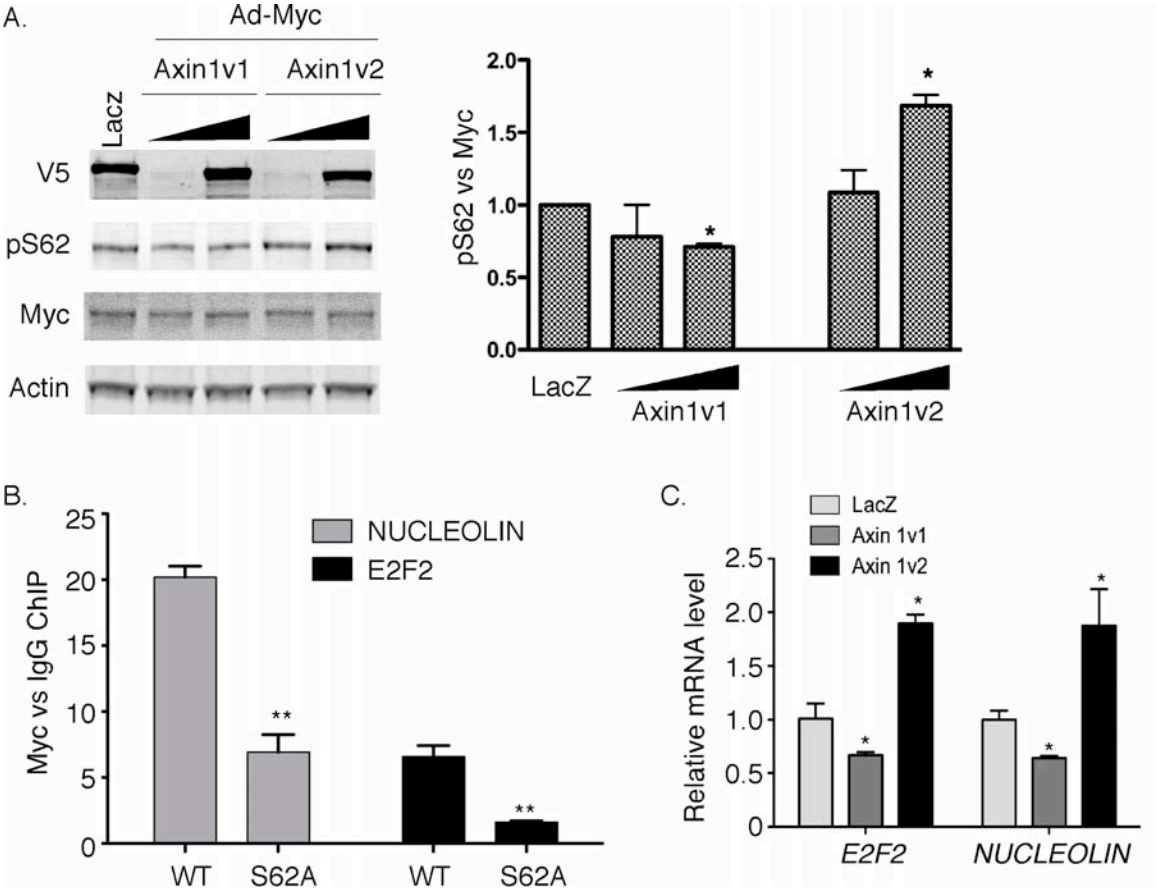




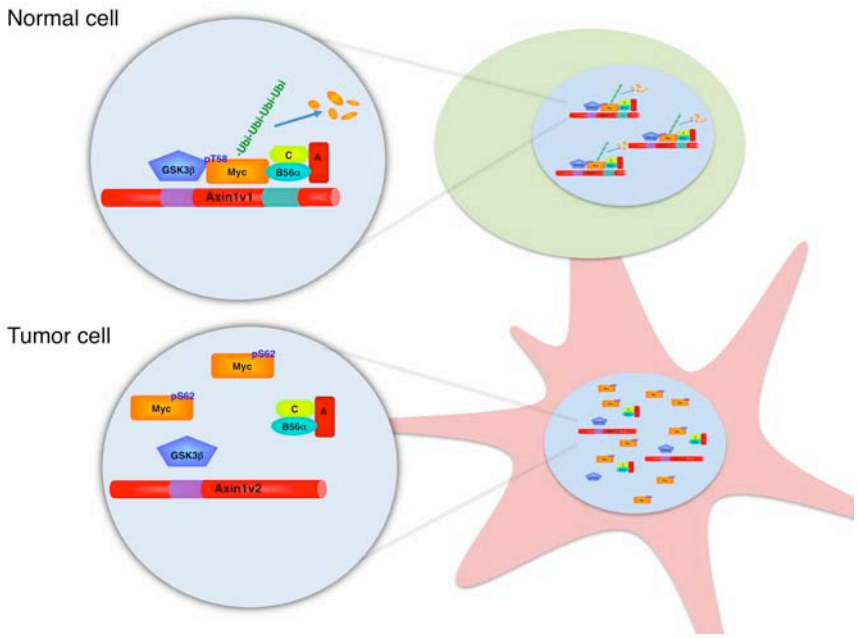


Supplemental Figure 2.7 l Axin1v1 but not Axin1v2 decreases pS62-c-Myc and Myc function. A. SNU475 cells were infected with lentivirus expressing either control LacZ or one of the splice variants of Axin1 for 48 hours followed by infection with Adenovirus expressing HA tagged Myc. Western blot was done to analyze expression of Axin1, pS62, Myc and Actin. pS62 vs Myc levels in Axin1v1 or Axin1v2 expressing cells were normalized to that of LacZ expressing cells and results from two experiments were calculated and graphed. *p-value<0.05. **B.** MCF10A stable cell lines that express doxycycline inducible V5-tagged Myc^{WT} or Myc^{S62A} were treated with doxycycline for 24 hours. ChIP experiment was done with V5 antibody and the amount of V5 bound *NUCLEOLIN* and *E2F2* was normalized to IgG bound ones and graphed. **p-value<0.01. **C.** SNU475 cells were infected as in Fig. 2.5E. *E2F2* and *NUCLEOLIN* mRNA expression were analyzed by qRT-PCR and graphed relative to *GAPDH* ±SD. *p-value<0.05.

Mechanistic insight into c-Myc stabilization in breast cancer involving aberrant Axin1 expression



Supplemental Figure 2.8 I A model showing deregulation of Axin1 and c-Myc protein degradation in cancer cells. In normal cells, c-Myc is recruited to a destruction complex coordinated by the Axin1 scaffold protein containing GSK3β, PP2A and other proteins (not included here). GSK3β phosphorylates c-Myc at T58, which triggers dephosphorylation at S62 by PP2A and its subsequent poly-ubiquitination by the SCF^{Fbw7} E3 ubiquitin ligase that targets it for proteasomal degradation (Welcker et al. 2004). This can occur in the nucleus and Axin1 can be detected at the promoters of Myc target genes (Arnold et al. 2009). In tumor cells, deregulation of Axin1 due to either decreased total Axin1 levels and/or the switch to Axin1v2 and loss of the domain implicated in PP2A binding, leads to disruption of the c-Myc degradation complex. As a result, c-Myc accumulates in the nucleus with high Serine62 phosphorylation.



Supplemental Table 2.1 Summary of human breast ductal invasive carcinomas in this study and their pS62 staining intensity. Hormone receptor (ER/PR) status and Her2 status as well as stage and grade are indicated for the patient samples used in this study. The information was obtained from OHSU pathology in collaboration with OHSU breast pathologist Dr. Megan Troxell. The pS62 immunofluorescence intensity in each tumor and its matched normal tissue was obtained as described in the Methods. The relative pS62 intensity in each tumor was then calculated by normalizing to its matched normal tissue. pS62 intensity was summarized as follows : + (1-1.5); ++(1.5=<2); +++(>2); 0 (no difference); - (0.5-1); --(<0.5). ND, not determined**.**

	ER, PR, Her2	Grade	Stage	pS62 intensity*
1	ND	ND	ND	+++
2	ER+PR+Her2-	II	T3N2	+++
3	ER+PR+Her2-	III	T1N2	+++
4	ER-PR-Her2+	II	T2N1	+++
5	ER+PR+Her2-	III	T1N2	+++
6	ER-PR-Her2-	III	T3N3	+++
7	ER-PR-Her2-	III	T3N3	+++
8	ER+PR+Her2-	I	T1N2	++
9	ER-PR-Her2-	II	T3N3	++
10	ER-PR-Her2+	III	T3N3	++
11	ER+PR+Her2-	I	T1N2	++
12	ER+PR+Her2-	III	T3N2	++
13	ER+PR+Her2-	I	T2N0	++
14	ER-PR-Her2+	II	T3N2	++
15	ER+PR+Her2-	III	T2N0	+
16	ER+PR+Her2-	III	T3N2	+
17	ER-PR-Her2+	II	T3N2	0
18	ER-PR-Her2+	III	T2N3	-
19	ER-PR-Her2-	III	ND	-
20	ER-PR-Her2+	II	T3N2	--
21	ER-PR-Her2-	III	T3N3	-
22	ER-PR-Her2-	III	T4N3	-

* We cannot rule out the possibility that technical issues such as differences in fixation or embedding during sample preparation might affect the immunofluorescence intensity between matched normal and tumor.

Supplemental Table 2.2. Summary of pS62 staining intensity in Myc-Neu driven mouse mammary gland tumors. Three mammary gland tumors were collected from mice that express both Myc and Neu in the mammary gland. Two normal mammary glands were collected from wildtype mice. Normal and tumor tissues were formalin fixed and embedded in paraffin, cut and subject to pS62 staining. Immunofluorescence were done and quantified as in Fig. 2.2. Average pS62 intensity in the two normal mouse mammary glands was set to 1 and relative pS62 intensity in tumors was calculated and summarized as follows: ++(1.5=<2); +++(>2).

Sample	pS62 intensity
Tumor1	++
Tumor2	++
Tumor3	+++
Normal	1

Methods

Cell lines and patient samples: MCF10A, SKBR3, MCF7, MDA231, MDA453, MDA436, MDA468, HCC38, hMEC-hTERT and 293 cell lines were purchased from ATCC (American Type Culture Collection). The LY2 cell line was a gift from Lawrence Berkeley National Laboratory. Cells were grown in the indicated medium containing 1X penicillin/streptomycin. Media used were as following: MCF10A: 45% DMEM, 45% F-12 Hams, 5% horse serum, 2.5mM L-glutamine, 20 ng/mL EGF, 10μg/mL insulin, 500ng/mL hydrocortisone, 100ng/mL cholera toxin; SKBR3, MDA453, MDA468, MDA436 and 293: DMEM with 10% FBS; MCF7: 45% DMEM, 45% F-12 hams and 10% FBS; MDA-MB-231: Iscove's DMEM with 10% FBS; LY2: Modified IMEM with 5% FBS. HCC38: RPMI with 10% FBS. hMEC-hTERT: MEM and F-12 mixture (1:1) supplemented with 1%FBS, 40mg/ml BPE, 12.5μg/ml EGF, 1mg/ml Insulin, 10μg/ml Transferrin, 100mM Phosphorylethanolamine, 100mM Ethanolamine, 1.25μg/ml hydrocortisone, 15nM sodium selente, 50mM ascorbic acid, 1μg/ml cholera toxin and 10nM Triiodothyronine (T3). De-identified patient matched normal and breast cancer samples were obtained from the OHSU Cancer Pathology Shared Resource (IRB #4918 and #2086). cDNA sample used in Figure 2.3A and supplemental Figure 2.6B were obtained from our collaborator Dr. Dexi Chen in Capitol University of Medical Science in China.

Generation of stable cells: MCF10A-TR-Myc cells (MCF10A-Myc) were generated by infecting a 100-mm dish of MCF10A cells with ~10 MOI lentivirus

encoding the tet-repressor, pLenti6/TR (Invitrogen), in 5mL MCF10A modified media (MCF10A media with 5% defined fetal bovine serum (FBS) instead of horse serum) and 6μg/mL polybrene for 12 hours. Media was changed to 10ml modified media for 24 hours. Cells were then split at a 1:10 dilution and maintained in modified media supplemented with 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 V5-tagged Axin1 expressed from Lenti/TO/V5-Dest-Axin1 by transient lentiviral infection. The best suppressing colony was then infected with ~10 MOI lentivirus expressing V5-Myc, pLenti4/TO/V5-Dest-Myc, as described for the tet-repressor infection above. Cells were selected in 5μg/mL Blasticidin and 200 μg/mL Zeocin (Invitrogen) for 10 days until distinct colonies formed. Six colonies were picked, expanded and screened for their ability to only express V5-Myc when treated with 1 μg/mL Doxycycline. The best clone was then used for further experiments and continually maintained in modified media with 5μg/mL Blasticidin and 200μg/mL Zeocin. Stable MCF10A-Myc-shAxin1 or empty vector control cells were generated by transfecting MCF10A-TR-Myc cells with shRNA plasmid expressing shAxin1 (NM_003502.2-612s1c1 and NM_003502.2-2728s1c1, Sigma) or empty vector (Sigma) and selected in modified media with 5mg/ml Blasticidin, 200mg/ml Zeocin and 5mg/ml puromycin for colony growth.

Antibodies: c-Myc N262 (sc-764, Santa Cruz Biotechnology) 1:1000, Axin1 (A0481, Simga), Axin1 (C76H11, Cell Signaling), V5 (R-960-25, Invitrogen) c-Myc

Y69 (ab32072, Abcam) 1:1000, c-Myc C33 (SC-42 AC, Santa Cruz Biotechnology), c-Myc Phospho-T58 (Y011034, Applied Biological Material) (used in western analysis) 1:1000, c-Myc Phospho-Thr58 (A00242, GenScript) (used in immunofluorescence) 1:50, monoclonal c-Myc Phospho-Serine 62 (E71-161, BioAcademia Inc) (western analysis) 1:1000, Generation of the polyclonal c-Myc Serine 62 phospho-specific antibody used in immunofluorescence is described in reference (Escamilla-Powers and Sears 2007) and is used at 1:25 dilution.

***^{35}S*-Methionine pulse/chase experiments** were done as described in reference (Malempati et al. 2006). Briefly, cells were pulse-labeled with ^{35}S-Methionine/Cysteine for 20 minutes followed by chase in medium containing excess unlabeled Methionine and Cysteine. ^{35}S-labeled c-Myc was immunoprecipitated from equal cell counts at each chase time point and visualized by SDS-PAGE autoradiography and quantified by phosphor imager. Representative experiments are shown. The rate of degradation of endogenous c-Myc in each experiment for each breast cancer cell line as well as MCF10A was calculated relative to the starting time point set at 100% and graphed on a semi-log graph. Best-fit exponential lines were drawn with Excel. c-Myc half-life was calculated from exponential line equations and the average half-life ± SD is shown for each cell type. Pulse-chase results shown here are representative of 2–3 independent experiments for each cell line.

Immunofluorescence and quantification of immunofluorescence staining intensity: serial paraffin sections from patients with matched normal and tumor formalin-fixed tissues were incubated with the Phospho-Serine 62 (1:25) or Phospho-Threonine 58 (1:50) c-Myc-specific antibody overnight at 4°C followed by AlexaFluor 594 donkey anti-rabbit IgG and mounted using antifade containing DAPI. Matched normal and tumor sections were placed on the same slide and stained simultaneously or adjacent normal was present in the tumor block and thus on the same section. Images were taken with a Hamamatsu digital camera mounted on a fluorescence microscope and exposure and magnification were not changed within a slide comparing normal and tumor. Immunofluorescence density was analyzed with Openlab 5.5 software. Specifically, representative pictures from the same or adjacent sections were taken of normal acini, DCIS, and invasive carcinoma cells from each patient. Epithelial cell fluorescence was quantified in these pictures using the Measure Density tool. Up to 10 representative Regions of Interest (ROI) (clusters of epithelial cells) were measured and averaged for each condition and graphed ± SD.

Cell proliferation assay were done with 80,000 SKBR3 cells in 60mm dish containing 4mL of media. 18 hours after plating, IWR-1 or DMSO was added to cells at indicated concentrations. Media with compound were changed every other day for 5 days and cells were counted at the indicated times with a hemacytometer.

Soft agar assay: The bottom and top agar layers were 0.8% and 0.35% Nobel Agar, respectively. For each MCF10A clone, $2X10^4$ cells were plated in triplicate in a six-well plate. Culture medium with or with out 1μg/mL doxycycline on top of the agar was changed every 3 to 4 days. 4 weeks after plating, colonies were fixed and stained with 0.005% crystal violet in 50% methanol/50% PBS. Colonies that were in clusters of at least 3 cells in diameter were counted in 10 random microscopic fields. For SKBR3 soft agar assays, $2.5X10^4$ cells were plated and cell culture media with 20mM IWR-1 or DMSO on top of the agar was changed every day. Colonies were visible by day 6 and were counted as described above.

Chromatin Immunoprecipitation Cells were crosslinked with 1% formaldehyde for 10 minutes and lysed in 700ml ChIP lysis buffer (0.1% SDS, 0.5% Triton X-100, 20mM Tris-HCl (pH 8.1), and 150mM NaCl). Cell lysates were sonicated 6X (output = 3.5, 30% duty, 10 pulses) and then cleared by centrifugation. Cell lysates was pre-cleared with protein A beads. Immunoprecipitations were performed with antibody overnight at 4ºC. Immunoprecipitates were washed 6 times with ChIP lysis buffer and twice with 1X TE and eluted from the beads with elution buffer (0.1M $NaHCO_3$ and 1% SDS). Elution products were raised to 0.2M NaCl and incubated at 65ºC overnight. For quantitative ChIP experiments, the internal GAPDH primers were used as a negative control. See supplemental information for antibodies, primers and calculations used in ChIP.

ChIP primers and antibodies: For quantitative ChIP experiments, primers to the promoter regions of c-Myc target genes, as well as internal GAPDH primers were

used to amplify DNA. The internal GAPDH primers were used as a negative control. Quantitative PCR (qPCR) was used to measure signals in 1% of the input material, as well as each immunoprecipitation (IP). Primers used were as following: *NUCLEOLIN* forward TTGCGACGCGTACGAGCTGG, *NUCLEOLIN* reverse ACTCCGACTAGGGCCGATAC, *E2F2* forward TCACCCCTCTGCCATTAAAGG, *E2F2* reverse AGCAGTGTATTCCCCAGGCC. The percentage of input was then calculated for each IP (control IgG and specific) as the IP signal above the input signal using the formula: $100 \times 2^{(\text{input Ct} - \text{IP Ct})}$. Relative level of bound DNA was then graphed as the percent input of the specific IP relative to the percent input of the mock IgG control using GraphPad Prism. Antibodies used in ChIP: Myc (N262), HA-11(AbM), normal rabbit IgG (Santa Cruz Biotechnology), normal mouse IgG (Santa Cruz Biotechnology).

Quantitative RT-PCR: RNA was isolated from breast cancer cell lines using TRIzol reagent (Invitrogen). Isolated RNA was DNase I treated and purified using RNeasy mini kit (Qiagen). cDNAs were made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Quantitative RT-PCR analysis was done using Taqman primers. Primers used in quantitative RT-PCR: c-*MYC* (Hs00905030_m1), *18s* (Hs99999901_s1), total *AXIN1* (Hs00394718_m1), *AXIN1V1* (Hs00394723_m1), *AXIN1V2* (Hs01558063_m1) and *ACTIN* (Hs99999903_m1). Mice: *axin1v1* F: CGTGTCGGACTTGGAACTCT, *axin1v2* F: CCAAGCAGAGGACAAAATCAC, mouse *axin1r4* (used for both V1 and *V2*): AGCTCCCTTCTTGGTTAGC.

Statistics: Standard deviation (SD) was analyzed with Microsoft Excel with results from 3 independent experiments unless otherwise indicated. P-value was analyzed by Student's t-test. Two-tailed method was used unless otherwise indicated.

Generation of transgenic mice: RFS-Myc mice (Wang et al. 2011) were crossed with NeuNT (Andrechek et al. 2000) and MMTV-Cre or BLG-Cre mice (a gift from Owen Sansom in Beatson Institute for cancer research, UK) to get mice that express both Myc and Neu in response to Cre-mediated recombination in the mammary gland. Mammary gland tumors were harvested and frozen for RNA analysis or embedded in paraffin for immunofluorescence staining.

Chapter Three :

HER2 enhances c-Myc protein stability

Abstract:

c-Myc is a transcription factor that regulates genes involved in nearly all aspects of cellular function. Deregulation of c-Myc activity, associated with increased c-Myc protein stability, is seen in the majority of human breast cancer. c-Myc protein stability and activity are regulated by interdependent phosphorylation events on Serine 62 (pS62) and Threonine 58 (pT58) that are controlled by Ras and PI3K/Akt signaling pathways. HER2 receptor tyrosine kinase signaling activates many down stream signaling pathways involved in cell proliferation and cell survival including Ras and PI3K/Akt signaling. HER2 is overexpressed in approximately 25-30% of human breast cancer. Whether HER2 signaling contributes to increased c-Myc protein stability and oncogenic activity in breast cancer is still unknown. Here we report that HER2 increases c-Myc expression by increasing its protein stability and this is associated with increased phosphorylation at S62. We show that this phosphorylation is required for HER2 to increase c-Myc expression. We also show that the HER2/EGFR kinase inhibitor, Lapatinib, decreases c-Myc protein but not mRNA expression and this is associated with decreased pS62 and increased pT58. In addition, we show that HER2 increases c-Myc's DNA binding activity, consistent with its upregulation of pS62. Our study of HER2 regulation on c-Myc expands our understanding of HER2's oncogenic mechanisms, offers a new player that regulates c-Myc protein expression and activity, and provides a new possible way of targeting c-Myc in tumor cells with HER2 overexpression.

Introduction:

c-Myc is a transcription factor that regulates a wide array of genes involved in cellular proliferation, growth, apoptosis, and differentiation (Pelengaris et al. 2002; Meyer and Penn 2008). c-Myc protein level is kept low in normal cells but the majority of human tumors have high levels of c-Myc (Nesbit et al. 1999). Studies using animal models showed that c-Myc overexpression leads to cancer in many targeted tissues and withdrawal of c-Myc in these models often results in spontaneous tumor regression (Felsher and Bishop 1999a; Nesbit et al. 1999; Pelengaris et al. 1999; D'Cruz et al. 2001), suggesting that c-Myc is a good target for new cancer therapies. Indeed, a recent mouse model has demonstrated that Myc inhibition by a dominant-interfering Myc mutant triggers rapid regression of incipient and established lung tumors with mild and tolerated side affects on normal tissues (Soucek et al. 2008). Thus, it is important to understand the mechanisms as well as identify the players involved in regulating c-Myc protein levels and activity.

Overexpression of c-Myc in human tumors occurs through several mechanisms, including gene amplification, increased transcription and increased protein stability (Malempati et al. 2006; Meyer and Penn 2008). An elegant signaling pathway helps to regulate c-Myc protein stability through interdependent and sequential phosphorylation events on two conserved phosphorylation sites, Serine 62 (S62) and Threonine 58 (T58) (Sears 2004). Phosphorylation of these two sites has opposing roles in controlling c-Myc

degradation: S62 phosphorylation stabilizes c-Myc while T58 phosphorylation promotes c-Myc degradation (Sears et al. 2000). In response to stimulatory signals, receptor tyrosine kinases (RTKs) are activated, leading to activation of Ras signaling and PI3K signaling. Activation of Ras transiently stabilizes c-Myc by increasing ERK or cyclin dependent kinase (CDK)-mediated phosphorylation at S62 (Hydbring et al. 2010; Sears et al. 1999; Sears et al. 2000). Concurrently, Glycogen synthase kinase 3β (GSK3β) activity is inhibited by RTK activation of PI(3)K/Akt signaling, and GSK3β-mediated phosphorylation at T58 is inhibited. In later G1 phase, this inhibition is relieved and GSK3β phosphorylates T58. This phosphorylation stimulates c-Myc degradation by recruiting the Pin1 prolyl-isomerase that catalyzes a *cis* to *trans* isomer change at Proline 63. This allows removal of the stabilizing Serine 62 phosphate by the trans-specific protein phosphatase 2A (PP2A) (Yeh et al. 2004). Phosphorylation at T58 also recruits the c-Myc E3 ubiquitin ligase, SCF^{Fbw7}, which then directs c-Myc polyubiquitination and degradation by the 26S proteasome (Welcker et al. 2004; Yada et al. 2004). The scaffold protein Axin1 facilitates c-Myc degradation through this pathway by promoting formation of a destruction complex for c-Myc that includes Pin1, PP2A, GSK3β and possibly other proteins involved in regulating c-Myc protein stability (Arnold et al. 2009).

The human epidermal growth factor receptor-2 (HER2) belongs to a family of transmembrane tyrosine kinase receptors that also includes HER1, -3 and -4 (Moasser 2007a). Activation of HER2 and its family proteins in response to ligand

binding activates many downstream signaling pathways to regulate cellular functions critical for cancer development, including cell proliferation, cell mobility, invasion, and apoptosis inhibition. For example, activation of HER2 can increase cell proliferation by activating the cyclinE-CDK2 complex through the Ras-MAPK signaling (Timms et al. 2002). Activation of HER2 also inhibits apoptosis primarily through the PI3K-Akt signaling as Akt can directly controls phosphorylation of many proteins involved in cell survival (Munster et al. 2002). Many data support the oncogenic function of HER2 in mammary gland. Activation of HER2 transforms mammary epithelial cells in cell culture and induces proliferative and antiapoptotic changes that mimic early stages of epithelial cell transformation in three-dimensional (3D) cell culture model (Muthuswamy et al. 2001). Activation of HER2 also leads to mammary gland tumorigenesis in many transgenic mouse models (Bouchard et al. 1989; Muthuswamy et al. 2001; Finkle et al. 2004).

Both HER2 and c-Myc are overexpressed in human breast cancer, with HER2 in approximately 25-30% and c-Myc in 50-100% of breast cancers (Slamon et al. 1989). Interestingly, studies have shown that *HER2* amplification is associated with *c-Myc* amplification in breast cancer and coamplification of the two genes is associated with worse outcome than with either amplification alone (Perez et al. 2011; Park et al. 2005), indicating a biological link between the two oncogenes in human breast cancer. However the molecular mechanisms underlying the biological link between c-Myc and HER2 is still not clear. Increases in S62 phosphorylation have been shown to associate with increased

oncogenic and reduced apoptotic activities of c-Myc (Yeh et al. 2004; Hemann et al. 2005; Wang et al. 2011). Given that (1) S62 phosphorylation is enhanced by Ras signaling which is downstream of HER2; (2) HER2 can inhibit apoptosis through PI3K-Akt pathway and PI3K-Akt can inhibit c-Myc phosphorylation at T58 by GSK3β, we reasoned that HER2 might modify c-Myc's activity by regulating c-Myc phosphorylation and protein stability. Consistently, we have shown previously that increased c-Myc protein stability occurred in the majority of human breast cancers and this was associated with increased phosphorylation at S62 and decreased phosphorylation at T58 (Zhang et al. 2011). Thus we examined the regulation of HER2 on c-Myc and we report here that HER2 increases c-Myc protein stability, phosphorylation of c-Myc at S62 and oncogenic activity, while Inhibition of HER2 kinase activity in breast cancer cells does the opposite. Given the clinical implication of HER2 in breast cancer, our study of HER2 regulation on c-Myc expands our understanding of HER2's oncogenic mechanisms and provides a new potential way of targeting tumor cells with Myc overexpression.

Results:

HER2 signaling increases c-Myc protein expression.

We first tested whether HER2 can increase c-Myc protein expression. Since HER2 activation relies on ligand binding, we used a constitutively active mutant of HER2, Neu8142, which has a deletion mutation in its extracellular domain and can form dimers constitutively. Stable cell lines that express Neu8142 or an empty vector in MCF10A cells (named MCF10A-HER2 or MCF10A-ctrl) were made. Western blot showed that there is increased endogenous Myc protein expression in MCF10A-HER2 comparing to MCF10A-ctrl (Fig. 3.1A, western) and this is not associated with an increase in c-*MYC* mRNA in the MCF10A-HER2 cells (Fig. 3.1A, graph). Similar results are seen with c-Myc expressed from an Ad-Myc construct lacking most of the c-*MYC* 5' and 3'UTR (Fig. 3.1B), which harbor sequences that have been reported to affect c-*MYC* mRNA translation and stability. To test if the increased Myc expression is a side effect of long-term oncogenic HER2 expression, we examined endogenous c-Myc protein levels in cell lines that inducibly express wild-type HER2. As shown in Fig. 3.1C, inducible HER2 expression in MCF10A cells also increases ectopic c-Myc levels expressed from the Ad-Myc construct. Taken together, our data demonstrate that HER2 post-translationally increases c-Myc protein levels.

To more conclusively determine if HER2 affects c-Myc protein translation in our system, we did pulse-labeling experiments to examine incorporation of ^{35}S-

Methionine/Cysteine into actively translating c-Myc protein expressed from Ad-Myc. As shown, comparing to MCF10A-ctrl, a slight decrease is consistently seen at 10 minutes of pulse-labeling (Fig. 3.2A). We also performed polysome profile experiments to check the distribution of c-*MYC* mRNA expressed from Ad-Myc in polysomes, monosomes, and as free RNA. mRNA undergoing translation is associated with polysomes. Ribosome fractions from MCF10A-ctrl and MCF10A-HER2 cells infected with Ad-Myc were collected and initially ribosome protein L5 expression was analyzed in these fractions (Fig. 3.2B, western). This demonstrates that MCF10A-HER2 cells have a similar polysome profile as MCF10A-ctrl, suggesting that HER2 doesn't affect the global protein translation. However, analysis of c-*MYC* mRNA percentage in these fractions shows that there is not an increased, but a slightly decreased distribution of c-*MYC* mRNA in the ribosome polysome fractions in MCF10A-HER2 cells comparing to MCF10A-ctrl. Thus, results from both the pulse-labeling experiment and the polysome profile experiment show that HER2 doesn't increase c-Myc protein translation in our system.

HER2 signaling increases c-Myc protein stability.

All of our data so far supports a post-translational mechanism for HER2-mediated increased c-Myc expression. We therefore checked the effect of HER2 on c-Myc protein stability. As shown in Fig. 3.3A, c-Myc half-life is increased from about 20 minutes in MCF10A-ctrl cells to 67 minutes in MCF10A-HER2 cells and

this is associated with increased pS62 vs total c-Myc in MCF10A-HER2 cells (Fig. 3.3B), consistent with our previous finding that increased c-Myc protein stability is associated with increased pS62 (Zhang et al. 2011). Taken together, our results show that increased c-Myc protein stability contributes to high c-Myc expression in HER2 expressing cells.

HER2 increases c-Myc^{WT} and c-Myc^{T58A} but not c-Myc ^{S62A} expression.

To check if HER2 regulates c-Myc protein stability by modulating c-Myc S62 phosphorylation, we infected MCF10A-ctrl or MCF10A-HER2 cells with Adenovirus that express either c-Myc^{WT}, or the phospho-mutant c-Myc^{T58A} or c-Myc^{S62A}. The c-Myc^{T58A} mutant can't be phosphorylated at T58 and has increased protein stability. The c-Myc^{S62A} can't be phosphorylated at both T58 and S62. As shown in Figure 3.3C, HER2 increased both c-Myc^{WT} and c-Myc^{T58A} but not c-Myc^{S62A} expression, similar to the affect of Ras on these c-Myc isoforms (Sears et al. 2000), indicating that HER2 increases c-Myc protein stability through phosphorylation of S62.

HER2 reduces the dependence of c-Myc expression on serum.

In normal cells, in response to serum stimulation, cells enter cell cycle. c-Myc protein levels peak at early G_1 and decrease at late G_1, following a bell-shaped pattern. We tested how HER2 might affect c-Myc protein expression pattern during the G1 phase of the cell cycle. To avoid the complication of altered

c-MYC mRNA expression, we expressed the activated HER2 in our MCF10A-TR-Myc cells that express doxycycline inducible, CMV-driven V5-tagged c-Myc. Cells were starved in 0.1%FBS for 48 hours and then doxycycline was added for 2 hours followed by stimulation with 10% FBS. Expression of c-Myc was examined at the indicated time after adding serum. As showing in Fig. 3.4, in control MCF10A-TR-Myc cells, c-Myc expression peaks at 1.5-hour and then goes down gradually at 3- and 6-hour. However, in HER2 expressing cells, while the basal level of Myc is higher, the peak at 1.5-hour of serum stimulation is dampened and c-Myc expression does not go down till the 6-hour time point. This result suggests that activated HER2 reduces the dependence of c-Myc expression on serum, which might be a common character of c-Myc in cells with defect in its degradation pathway.

Lapatinib decreases c-Myc protein but not mRNA expression in HER2-positive breast cancer cell lines and this is associated with altered pS62 and pT58.

Lapatinib is a small molecular inhibitor of HER2 and EGFR kinase that is currently used in the clinic for treating HER2-positive breast cancer. To test if inhibiting HER2 kinase activity affects c-Myc expression in cancer cells, we treated the HER2-positive breast cancer cell lines SKBR3 and MDA453 with Lapatinib. As shown in Fig. 3.5A, when SKBR3 cells were treated with Lapatinib for 8 hours, a time point where cell survival is not affected yet (data not shown),

c-Myc protein levels were already reduced. Similar results are seen when MDA453 cells are treated with Lapatinib (Fig. 3.5B, western). *c-MYC* mRNA doesn't decrease with Lapatinib treatment (Fig. 3.5B, graph). Thus the reduced expression of c-Myc protein probably reflects a post-translational effect. Consistently, pS62 vs total c-Myc is decreased dramatically while pT58 vs total c-Myc is increased in Lapatinib-treated MDA453 cells (Fig. 3.5C). These data are consistent with the role of HER2 in regulating c-Myc protein stability, suggesting that inhibition of c-Myc expression might be one of the mechanisms underlying Lapatinib's clinical efficacy. Since Lapatinib is an EGFR and HER2 dual-kinase inhibitor, further experiments inhibiting HER2 activity with shRNA against HER2, will help determine if inhibiting HER2 activity is required for Lapatinib-induced inhibition of c-Myc activity. In addition, testing the affect of inhibiting HER2 on c-Myc activity in non-transformed cells such as MCF10A will give information about whether the shift in c-Myc phosphorylation and activity is cancer cell specific.

HER2 affects c-Myc's promoter binding activity.

Recent studies have demonstrated an important role for pS62 in c-Myc binding to target gene promoters (Hydbring et al. 2010; Benassi et al. 2006; Zhang et al. 2011). HER2 signaling increases c-Myc pS62, thus we tested how HER2 affects c-Myc promoter binding with chromatin immunoprecipitation (ChIP) assay followed by qPCR. To avoid serum interference, we starved MCF10A-HER2 and MCF10A-Ctrl cells for 48 hours before ChIP analysis. As shown in

Figure 3.6, we find that in MCF10A-HER2 cells there are increased binding of c-Myc to the promoters of its two trans-activating genes, *E2F2* and *NUCLEOLIN*. This result suggests that HER2 might increase c-Myc's transcriptional activity to collaborate with c-Myc in tumorigenesis.

Discussion:

Several data support a functional connection between the two oncogenes, c-Myc and HER2, that are both overexpressed in human breast cancer. Firstly, although c-Myc and HER2 each are powerful oncoproteins, transgenic mice expressing either HER2 or c-Myc alone in the mammary gland have long tumor latencies (Wang et al. 2011; Cardiff et al. 1991). In contrast, overexpression of both HER2 and c-Myc together greatly accelerates the mammary gland tumor development ((Cardiff et al. 1991) and Sears lab unpublished data), similar to the synergy of Ras and c-Myc in tumorigenesis (Compere et al. 1989). Secondly, studies have shown that c-Myc is required for HER2-driven proliferative signals in breast cancer cell lines and long-term downregulation of HER2 led to decreased c-Myc expression (Neve et al. 2000). HER2 has been reported to enhance 5'UTR-mediated translation of c-*MYC* mRNA through the PI3K/Akt/mTOR pathway in an ovarian carcinoma cell line (Galmozzi et al., 2004). Here we show that HER2 also increases c-Myc protein stability and this is associated with alterations in phosphorylation at S62 and T58 and increased c-Myc DNA binding activity. Thus, these results provide a novel mechanism underlying the collaborative effects between c-Myc and HER2.

The dual tyrosine kinase inhibitor against EGFR and HER2, Lapatinib, has been used in the clinic for treating HER2-positive breast cancer patients. This is probably due to Lapatinib's ability to inhibit HER2 downstream signaling, including Erk1/2 and Akt signaling, as reported in breast cancer cell lines and

xenograft models (Rusnak et al. 2001; Xia et al. 2002). We find that Lapatinib also inhibits c-Myc protein expression in HER2-positive breast cancer cell lines and this is associated with a switch in c-Myc from the high pS62/low pT58 form that we usually find in tumor cells, to the high pT58/low pS62 form found in normal cells. Since high levels of c-Myc and pS62 are critical for c-Myc's oncogenic activity (Wang et al. 2011; Hemann et al. 2005; Murphy et al. 2008) and tumors are usually dependent on high c-Myc expression (Felsher and Bishop 1999a; Nesbit et al. 1999; Pelengaris et al. 1999; D'Cruz et al. 2001), the decreased c-Myc expression as well as the coupled shift in c-Myc phosphorylation caused by Lapatinib treatment might underlie its clinical efficacy. Furthermore, this shift in c-Myc phosphorylation upon inactivating HER2 might also explain a study in which treating HER2-positive breast cancer patients with another HER2 inhibitor, Trastuzumab, a humanized antibody to HER2, resulted in those with *c-MYC* amplification recover better than those without (Kim et al, 2005). As we showed in this study, activation of HER2 can increase c-Myc phosphorylation at S62, leading to selective suppression of c-Myc's apoptotic activity and allowing c-Myc to stimulate cell proliferation and cancer development (Wang et al. 2011). When HER2 activity is inhibited by Trastuzumab, c-Myc could no longer be highly phosphorylated at S62, allowing it to regain its ability to trigger apoptosis (Kim et al, 2005). Thus these data suggest that inactivating c-Myc is crucial for clinical efficacy of HER2-targeted therapies. In support, activation of Akt has been reported to be a major mechanism that cancer cells

develop to bypass HER2-targeted therapies (Kataoka et al. 2011; Berns et al. 2004; Eichhorn et al. 2008). Since Akt inhibits GSK3β-medicated phosphorylation of c-Myc at T58 and leads to increased phosphorylation at S62 (Sears, 2004), strategies aimed at decreasing c-Myc phosphorylation at S62 in future could prevent the development of resistance observed in HER2-targeted therapy.

Figure Legends

Figure 3.1l HER2 increases c-Myc protein expression. A. Endogenous c-Myc protein expression (western blot) and mRNA (qRT-PCR, right graph) in MCF10A-HER2 or MCF10A-ctrl cells. **B.** MCF10A-HER2 stable clone #1 and #4 or ctrl (pCDNA clone #4) cells were infected with c-Myc expressing adenovirus for 18 hours and then analyzed for c-Myc, HER2 and GAPDH expression by western (left) and *c-MYC* mRNA levels by qRT-PCR. **C.** MCF10A-TR-HER2 cell line was made to express doxycycline inducible wild type HER2 in MCF10A cells. These cells were then treated with doxycycline or PBS for 24 hours before infected with Ad-Myc for another 18 hours. Shown are expression of HER2, Myc and GAPDH in western blot.

HER2 enhances c-Myc protein stability

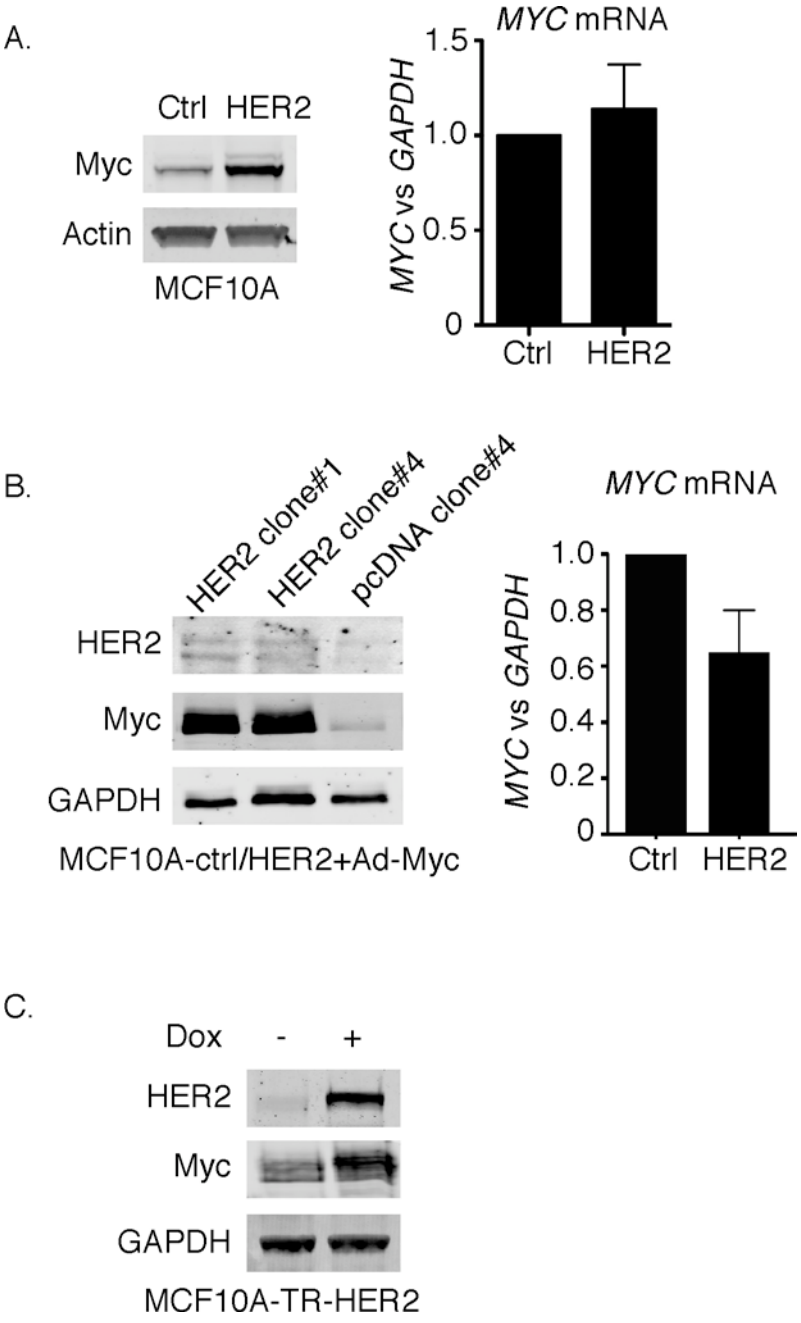
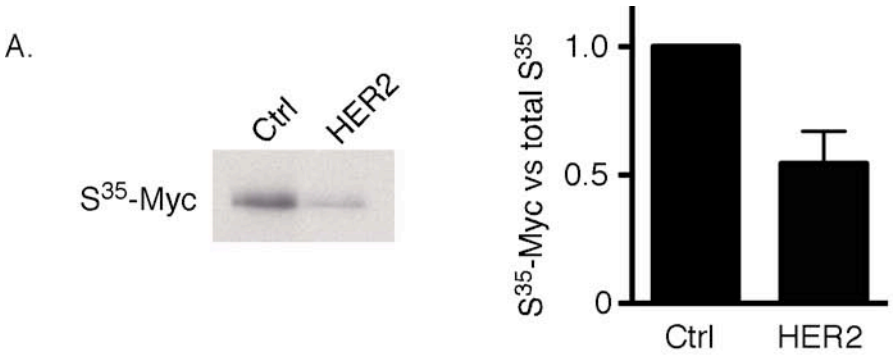
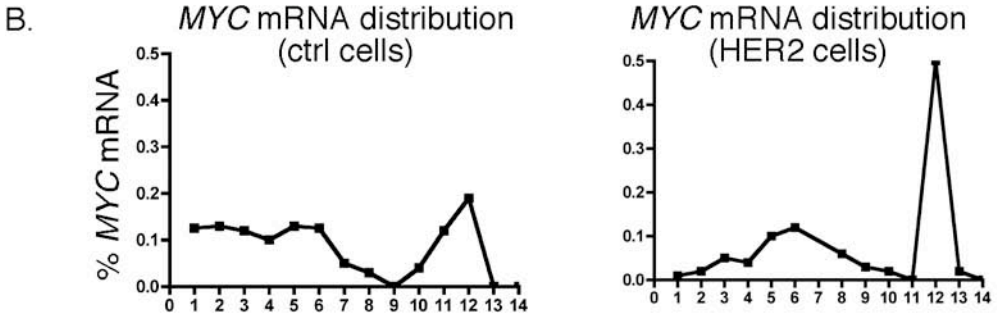


Figure 3.2 | HER2 doesn't increase c-Myc protein translation. A. Analysis of c-Myc protein translation by ^{35}S-methionine/cysteine pulse-labeling experiments. MCF10A-ctrl or MCF10A-HER2 cells were infected with adenovirus expressing HA-tagged c-Myc for 18 hours and then cells were labeled with ^{35}S-Methionine/Cysteine for 10 minutes. Cell lysates were then subject to anti-HA immunoprecipitation and run on western. S^{35}-labeled c-Myc protein was imaged with a phosphorimager. Total S^{35} amount in cell lysates was quantified by a LS6500 scintillation counter. Graph shows ratios of S^{35}-labeled Myc vs total S^{35}. Bars represent SD from two independent experiments. **B.** Analysis of *c-MYC* mRNA distribution in polysomes and monosomes. MCF10A-ctrl or MCF10A-HER2 cells were infected as in A followed by treatment with Cycloheximide for 10 minutes to stop new protein synthesis before harvesting in lysis buffer containing RNase inhibitor. Supernatant of cell lysate were added onto the top of sucrose density gradient and fractionated by ultracentrifuge. 14 fractions were taken for each cell line and extracted for RNA using TRIZOL. qRT-PCR were done to analyze *c-MYC* mRNA level in each fraction and the percentage of *c-MYC* mRNA level in each fraction vs total *c-MYC* mRNA were calculated and plotted in the graph. Levels of ribosome protein L5 from each fraction were analyzed by western and shown on the bottom.


A.
Ctrl
HER2
S35-Myc
S35-Myc vs total S35
1.0
0.5
0
Ctrl
HER2


B.
MYC mRNA distribution
(ctrl cells)
% MYC mRNA
MYC mRNA distribution
(HER2 cells)

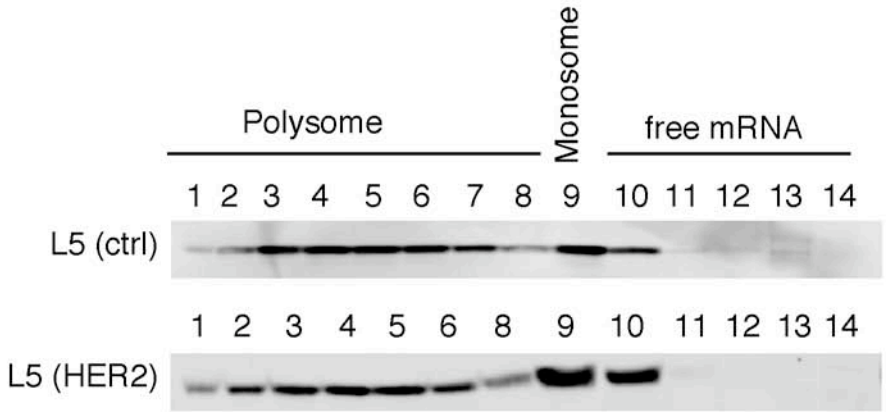

Polysome
Monosome
free mRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
L5 (ctrl)
1 2 3 4 5 6 8 9 10 11 12 13 14
L5 (HER2)

Figure 3.3l HER2 increases c-Myc protein stability dependent on S62. A. MCF10A-ctrl of MCF10A-HER2 cells were infected with Ad-Myc for 18 hours and then treated with cycloheximide for the indicated time. Cells were lysed and run on western and c-Myc protein half-life was determined as described in methods. **B.** MCF10A-TR-Myc cells expressing doxycycline inducible Myc were transfected with pCDNA3 or pCDNA3-HER2 to generate stable cell lines of MCF10A-Myc or MCF10A-Myc-HER2. The two stable cell lines were then treated with doxycycline for 24 hours and expression of ectopic Myc protein levels and pS62 levels were then analyzed by western and quantified using Licor. pS62 vs total c-Myc were shown on the right graph. **C.** MCF10A-ctrl or HER2 cells were infected with equal MOI of adenovirus that expresses either c-Myc^{WT}, c-Myc^{T58A} or c-Myc^{S62A} for 18 hours and c-Myc protein levels were analyzed by western with Myc antibody Y69.

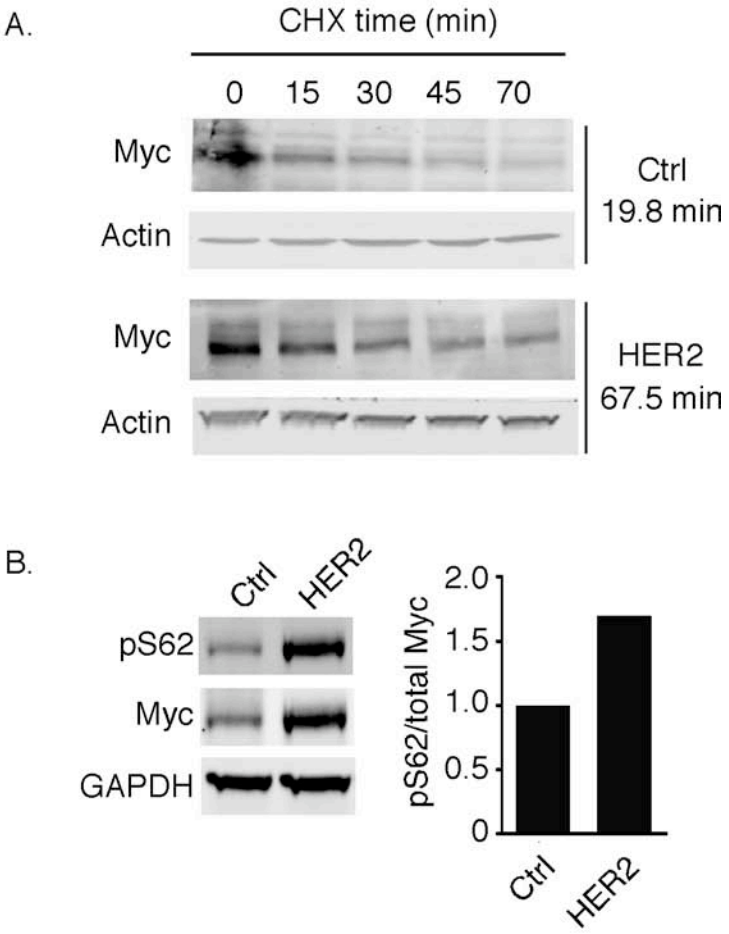

A.
CHX time (min)
0
15
30
45
70
Myc
Actin
Ctrl
19.8 min
Myc
Actin
HER2
67.5 min
B.
Ctrl
HER2
pS62
Myc
GAPDH
pS62/total Myc
2.0
1.5
1.0
0.5
0
Ctrl
HER2
C.
WT
T58A
S62A
ctrl
HER2
ctrl
HER2
ctrl
HER2
Myc
Actin

Figure 3.4l HER2 decreases the dependency of c-Myc expression on serum stimulation. MCF-Myc-HER2 and MCF-Myc cells were starved in 0.1%FBS for 48 hours and then doxycycline was added to induce expression of V5-Myc for 2 hours. Cells were then cultured in normal growth media for the indicated time. Expression of V5-Myc and Actin at each time point was analyzed by western and quantified using Licor. Representative data is shown here. The ratio of Myc vs Actin was calculated, normalized to that of 0 hour time point for each cell line and graphed on the bottom.

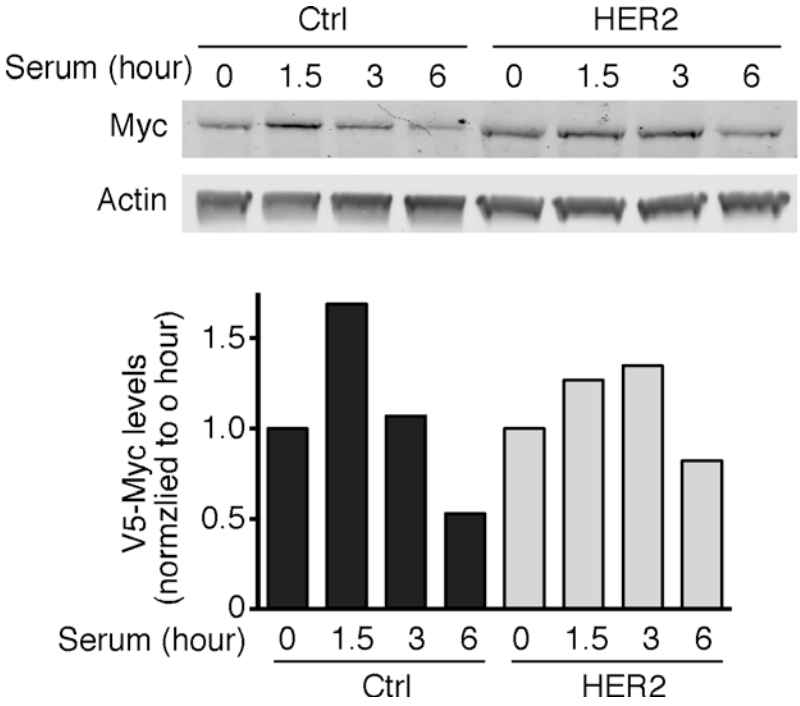


Figure 3.5l Lapatinib decreases c-Myc protein but not mRNA expression in HER2 positive breast cancer cell lines and this is associated with altered pS62 and pT58 vs total Myc ratio. A. SKBR3 cells were treated with DMSO or 1 or 5μM of Lapatinib for 8 hours and analyzed for c-Myc and Actin expression by western. **B.** MDA453 cells were treated with DMSO or 1 or 5μM of Lapatinib for 8 and 24 hours. Left, western blot showing expression of c-Myc and GAPDH protein. Right, graph shows expression of mRNA analyzed by qRT-PCR. **C.** MDA453 cells were treated with DMSO or 5μM of Lapatinib for 8 hours and analyzed for expression of c-Myc, pS62 and pT58 by western. pS62/total c-Myc and pT58/total c-Myc were calculated based on quantification from Licor and graphed on the right.

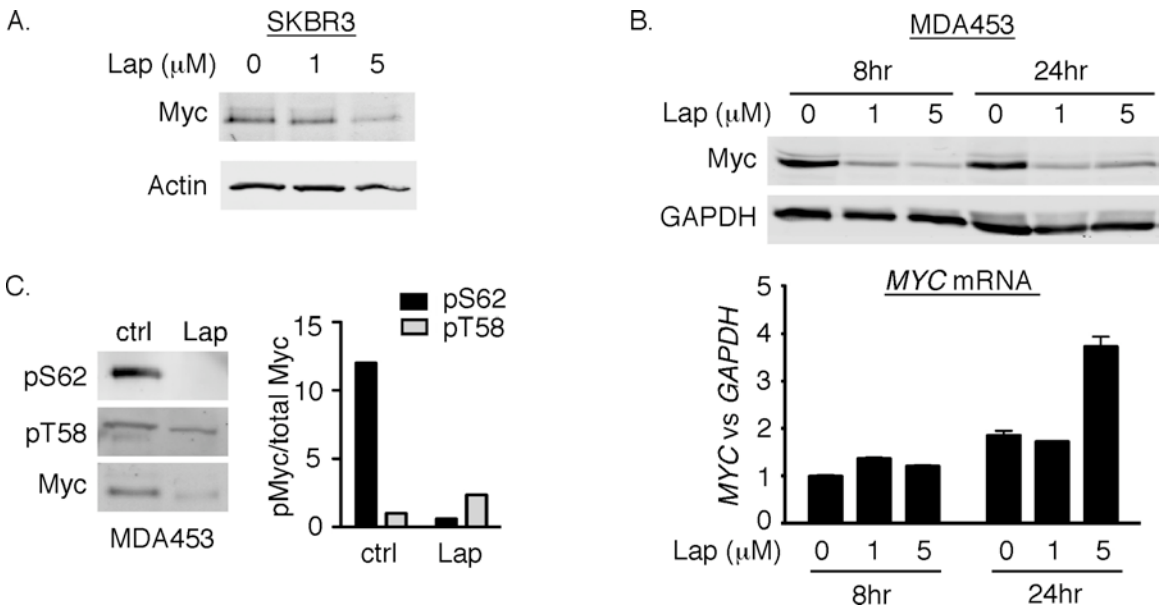
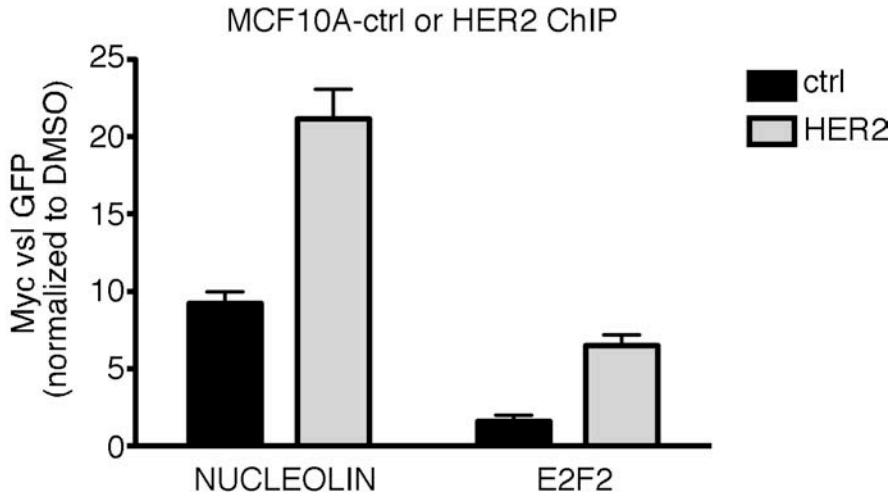


Figure 3.6l HER2 increases c-Myc's promoter binding activity. MCF10A-ctrl and MCF10A-HER2 were starved in 0.1%FBS media for 48 hours and then ChIP experiment was done with either α-GFP or N262 antibody to examine Myc protein binding activity at *NUCLEOLIN* and *E2F2* promoter region. Bars represent SD from 3 experiments.



Methods:

Cell culture: MCF10A stable cell lines were cultured in MCF10A media with 5% defined FBS instead of horse serum. Blasticidin, zeocin or neomycin was added to MCF10A media depending on the selection. SKBR3 and MDA-MB-453 cells were cultured in DMEM plus 10% FBS.

Generation of stable cell lines: MCF10A-ctrl and MCF10A-HER2 stable cells were generated by transfecting MCF10A cells with pcDNA3 or pcDNA3-HER2 (contains the Neu8142 active mutant) plasmid. 24 hours after transfection, cells were split and cultured in Neomycin containing media for about 2 weeks until distinct colonies formed. 6 colonies from each transfection were picked and tested for expression of HER2 and representative colonies were chosen for study. MCF-Myc and MCF-Myc-HER2 cells were generated by transfecting MCF10A-TR-Myc (Zhang et al. 2011) with pcDNA3 or pcDNA3-HER2 plasmid.

Western blot: See reference (Arnold et al. 2009) for western method. Immunoblots were visualized via LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) that can simultaneously detect Fluor 680 and IRDye 800 anti-rabbit and anti-mouse secondary antibodies. Quantification of western blots was done using LI-COR Odyssey Infrared software version 1.2 which is linear over 4 orders of magnitude.

Antibodies: c-Myc N262 (sc-764, Santa Cruz Biotechnology), c-Myc Y69 (ab32072, Abcam) 1:1000, c-Myc Phospho-T58 (Y011034, Applied Biological Material) 1:1000, c-Myc Phospho-Serine 62 (E71-161, BioAcademia Inc) 1:1000.

α-HER2 (Neu H-200, sc-134481, Santa Cruz Biotechnology) 1:1000. α-HA tag (G036, Applied Biological Material). α-GFP (sc-8334, Santa Cruz Biotechnology)

Quantitative RT-PCR: RNA was isolated using TRIzol reagent (Invitrogen). Isolated RNA was DNase I treated and purified using RNeasy mini kit (Qiagen). cDNAs were made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Quantitative RT-PCR analysis was done using Taqman primers with *c-MYC* (Hs00905030_m1) and *GAPDH* (Hs02786624_g1).

Chromatin immunoprecipitation (ChIP): See reference (Zhang et al. 2011) for methods. Primers to the promoter regions of c-Myc target genes, as well as internal GAPDH primers were used to amplify DNA. The internal GAPDH primers were used as a negative control. Quantitative PCR (qPCR) was used to measure signals in 1% of the input material, as well as each immunoprecipitation (IP). Primers used were as following: *NUCLEOLIN* F TTGCGACGCGTACGAGCTGG, NUCLEOLIN R ACTCCGACTAGGGCCGATAC, *E2F2* Forward- TCACCCCTCTGCCATTAAAGG, *E2F2* Reverse- AGCAGTGTATTCCCCAGGCC. The percentage of input was then calculated for each IP (control IgG and specific) as the IP signal above the input signal using the formula: 100 x 2(input Ct – IP Ct). Relative level of bound DNA was then graphed as the percent input of the specific IP relative to the percent input of the mock IgG control using GraphPad Prism.

Chapter Four :

Summary and Discussion

Summary

Overexpression of c-Myc occurs in the majority of human breast cancer. Reported mechanisms such as c-Myc gene amplification and increased transcription can only explain c-Myc overexpression in a subset of human breast cancer (Agnantis et al.1992; Bieche et al. 1999; Nesbit et al. 1999; Blancato et al. 2004; Blancato et al. 2006). In this study, I examined whether increased c-Myc protein stability also contributes to its overexpression in human cancer. I have shown that c-Myc protein half-life is significantly longer in all the analyzed breast cancer cell lines, compared with c-Myc in two non-transforming breast epithelial cell lines. Analysis of protein and mRNA levels revealed that increases in c-Myc protein levels are uncoupled from increases in c-*MYC* mRNA levels in most of these cancer cell lines. Thus increased c-Myc protein stability is a predominant mechanism underlying c-Myc overexpression in human breast cancer.

c-Myc protein stability is regulated by phosphorylation events at Serine 62 (S62) and Threonine 58 (T58) (Sears 2004). Phosphorylation at S62 increases c-Myc protein stability while phosphorylation at T58 triggers c-Myc degradation. I have now shown that increased c-Myc protein stability in breast cancer cell lines is correlated with increased phosphorylation at S62 and decreased phosphorylation at T58. To investigate if increased c-Myc protein stability also occurs in primary human breast cancers, I examined pS62 and pT58 levels by immuno-fluorescence staining using validated phospho-specific antibodies. The

results showed that increased S62 and decreased T58 phosphorylation is also a feature of primary human breast cancers.

The scaffold protein Axin1 coordinates the signaling cascade that controls c-Myc degradation (Arnold et al. 2009). I therefore examined if Axin1 is deregulated in human breast cancer. I found that human primary breast cancers have decreased *AXIN1* mRNA expression. Analysis of human breast cancer cell lines with increased c-Myc protein stability showed that only one of them, MDA231, has decreased total *AXIN1* level. However, all the other cancer cell lines examined have altered ratios the two splice variants, with increased *AXIN1V2* expression relative to *AXIN1V1* compared to control MCF10A cells, a non-transformed breast epithelial cell line. In primary human breast cancers, increased ratio of *AXIN1V2* vs *AXIN1V1* is correlated with increased phosphorylation of c-Myc at S62. Further analyses revealed that Axin1v2, unlike Axin1v1, is not able to decrease c-Myc phosphorylation at S62 nor is it able to inhibit c-Myc binding to target gene promoters. Thus these results suggest that many human breast cancer cells have decreased Axin1 scaffold function, which may contribute to the increased c-Myc protein stability seen in these cancer cells.

To study if Axin1 regulates c-Myc oncogenic activity, I knocked down *AXIN1* in non-transformed MCF10A cells. Knocking down Axin1 cooperates with c-Myc^{WT} but not the c-Myc^{T58A} in transforming MCF10A cells in anchorage independent growth assays, suggesting that Axin1 regulates c-Myc oncogenic activity in a phosphorylation dependent manner. In breast cancer cell lines that

have increased c-Myc protein stability and deregulation of Axin1, increasing Axin1 expression not only decreases c-Myc protein stability, phosphorylation at S62 and c-Myc binding at target gene promoters, but also inhibits anchorage independent cell growth. Thus these results suggest that inactivation of the Axin1 tumor suppressor is an important lesion that can contribute to increased c-Myc oncogenic activity in breast cancer.

HER2 is a tyrosine kinase receptor that is overexpressed in human breast cancer. Studies have shown that HER2 and c-Myc collaborate in tumorigenesis, but the molecular mechanisms of how the two oncogenes collaborate are still not clear. I have found that activation of HER2 increases c-Myc protein expression by increasing c-Myc protein stability, but not protein translation or gene transcription. Increased c-Myc protein stability is associated with increased c-Myc phosphorylation at S62. Activation of HER2 increases c-Myc^{WT} and c-Myc^{T58A} but not c-Myc^{S62A} expression, suggesting that HER2 regulates c-Myc protein stability through the c-Myc T58/S62 degradation pathway. Activation of HER2 also increases c-Myc binding at target gene promoters, indicating increased c-Myc transcriptional activity in HER2 activated cells. Inhibiting HER2 kinase activity in HER2-positive breast cancer cell lines by Lapatinib leads to decreased c-Myc protein but not mRNA levels, associated with a shift in phosphorylation levels of S62 and T58. These results suggest that Lapatinib can inactivate c-Myc in HER2-positive cancer cells, which might be a mechanism underlying the clinical efficacy of Lapatinib.

Taken together, as summarized in Fig. 4.1, I have shown that increased c-Myc protein stability associated with increased pS62 and decreased pT58 is a prominent mechanism of c-Myc overexpression in human breast cancer. Deregulation of proteins in the c-Myc T58/S62 degradation pathway, such as overexpression of HER2 and decreased scaffold function of Axin1, can lead to increased c-Myc expression and oncogenic activity in cancer. Thus my results reveal both an important mode of c-Myc activation and critical insight into mechanisms of c-Myc deregulation in human breast cancer.

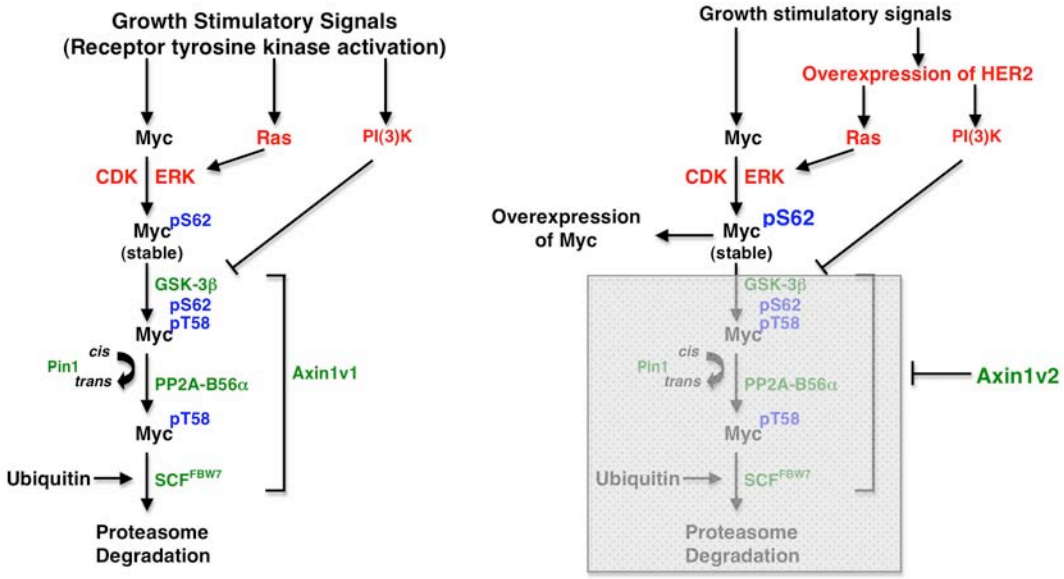


Fig.4.1: c-Myc degradation is blocked in breast cancer cells.

A. In normal cells, c-Myc protein is degraded through the c-Myc T58/S62 degradation pathway soon after its synthesis. See Fig. 1.3 for detail information.
B. In breast cancer cells, the c-Myc T58/S62 degradation pathway is blocked due to deregulation of proteins regulating phosphorylation at S62 and T58. For example, overexpression of HER2 can increase c-Myc protein stability through activation of Ras and PI(3)K signaling. c-Myc degradation can also be disrupted by deregulation of Axin1, including decreased total level of Axin1 and a preferred expression of Axin1v2, a splice variant of Axin1 that can't promote c-Myc degradation.

Discussion

c-Myc protein is overexpressed in 50-100% of human breast cancer (Agnantis et al. 1992; Nesbit et al. 1999). Prior to this research it had not been determined if increased c-Myc protein stability could contribute to c-Myc overexpression in human breast cancer. In this thesis, I have shown that deregulation of c-Myc protein degradation occurs in most of the human breast cancer cell lines examined and is associated with increased pS62 and decreased pT58. I have also shown that human primary breast cancer has increased staining of c-Myc pS62 and decreased staining of pT58 compared with their matched normal breast tissue, suggesting that deregulation of c-Myc protein degradation is a prominent feature of human breast cancer. To identify the molecular mechanisms leading to deregulation of c-Myc, I have focused my research in this thesis on two proteins, the oncoprotein HER2 and the tumor suppressor Axin1.

1. Activation of HER2 increases c-Myc protein stability.

Both HER2 and c-Myc are overexpressed in human breast cancers. Results from many studies indicate that c-Myc and HER2 signaling experience cross-talk during carcinogenesis (described in the discussion of Chapter 3); however, the molecular details of how the two oncogenes collaborate with each other remain poorly elucidated. I have shown that activation of HER2 increases c-Myc protein stability, associated with increases in pS62 and decreases in pT58,

and increases c-Myc binding to target gene promoters, thus providing a novel mechanism for c-Myc and HER2 collaboration in tumorigenesis.

My initial studies indicated that activation of HER2 can increase c-Myc pS62, thus I examined primary human breast cancers for a correlation between HER2 status and pS62 intensity. Suppl. Table-2.1 summarizes the 22 cases of human primary breast cancer samples that I analyzed for pS62 intensity by immunofluorescence staining. As shown, 16 of the 22 cases have increased pS62 intensity in tumors (including ductal carcinoma *in situ* and invasive carcinoma) when compared with their matched normal tissue. However, I failed to find any correlation between HER2 status and pS62 intensity in these samples. HER2-positive cases have an equal distribution between pS62 positive and negative groups. Since we only have 6 HER2-postive cases, future studies are needed to expand analysis to more samples in order to make a conclusion. However, it is possible that overexpression of HER2 is not sufficient for increasing pS62 *in vivo*. One possibly is that HER2 status as scaled by HER2 staining intensity may not reflect HER2 activation status. In fact, others have shown that only 12-58% of HER2-positive cancers have activated HER2 as determined by phosphorylation of Tyrosine 1248 of HER2 (DiGiovanna et al. 1996; Thor et al. 2000; DiGiovanna et al. 2002). Thus detection of activated HER2 protein might be a better marker to examine breast carcinomas for correlation studies with c-Myc pS62 intensity.

2. Correlation between increased c-Myc Serine 62 phosphorylation and ER/PR status in breast cancer.

When analyzing pS62 and HER2 status in primary human breast tumor samples, one interesting observation is that, as shown in Table-2.1, all the pS62 negative cases are also negative for estrogen receptor and progesterone receptor (ER-PR-) and all the ER+PR+ cases are also positive for pS62. This result is unexpected and unbiased since we started the experiment for testing correlation between HER2 status and pS62 intensity as HER2 can increase c-Myc protein stability. Since in the cases under this study, ER status is always the same as PR status, it is not possible to determine if pS62 is correlating with both ER and PR status or just one of them. However, many studies have supported a biological link between c-Myc and ER. First, c-Myc expression is regulated by ER-α. ER-α can increase both c-Myc transcription as well as pS62 and pT58 controlled c-Myc protein stability (Rodrik et al. 2006). Second, c-Myc is a downstream effector of ER-α and is required for estrogen induced breast cancer cell proliferation. Knocking down c-Myc inhibits estrogen-induced cell proliferation in a manner similar to that of antiestrogens (Dubik et al. 1987; Watson et al. 1991; Carroll et al. 2002; Park et al. 2005). Third, gene expression analysis of estrogen-treated or c-Myc-induced breast cancer cells showed that half the estrogen regulated genes are also regulated by c-Myc, indicating that a significant component of estrogen-induced mitogenesis is mediated by c-Myc (Musgrove et al. 2008). Together, these data suggest that ER-α is a regulator of

c-Myc protein stability and transcriptional activity and c-Myc is an important target of ER-α. Based on the pS62 analysis of primary human breast cancers, I conclude that high levels of c-Myc pS62 is an important feature of ER+ and/or PR+ breast cancer and might be important for tumorigenesis of ER+ and/or PR+ breast cancer. In addition, these results also suggest that activation of c-Myc in the absence of ER/PR, which is observed in 6 of the 12 ER-PR- cases and 3 of the 6 triple negative cases, might mimic ER/PR signaling. Supporting this idea, a meta-analysis of gene expression from 5 large microarray data sets relative to ER-α status revealed that increased transcriptional activity of Myc is a characteristic of triple negative breast cancers; namely, it mimics a large part of an estrogen response in the absence of ER-α, suggesting a mechanism by which these cancers achieve estrogen independence (Alles et al. 2009).

3. Preferred expression of the *AXIN1* splice variant, *AXIN1V2*, in human breast cancer.

As described in Chapter 2, I have shown that deregulation of c-Myc protein stability is associated with increased pS62 and decreased pT58 in breast cancer and deregulation of *AXIN1* contributes to deregulation of c-Myc. Two mechanisms of Axin1 deregulation are involved, decreased total *AXIN1* level and a preferred expression of *AXIN1V2*, a naturally occurred splice variant of *AXIN1V1*. Although both mechanisms of Axin1 deregulation are found in breast cancer, the latter one shows a better correlation with increased pS62 in breast

cancers as supported by analysis in human breast matched normal and tumor samples as well as analysis in c-Myc-driven mouse mammary gland tumors. Thus the aberrant splicing of *AXIN1* seems to be a more relevant mechanism underlying deregulation of c-Myc in breast cancer.

One crucial mechanism regulating pre-mRNA splicing is the coupling between transcription and splicing, a concept suggested by the findings that RNA processing occurs on RNA polymerase II, that promoter structure contributes to splice site selection, and that modifications in transcription elongation rate affect splicing decisions (Cramer et al. 1997; Roberts et al. 1998; Cramer et al. 1999; Hirose and Manley 2000; Kadener et al. 2001). Both estrogen and progesterone receptors have been reported to regulate pre-mRNA splicing by coupling target gene transcription and splicing (Auboeuf et al. 2002; Dowhan et al. 2005). As discussed above, our analysis on primary breast cancer samples shows that all ER+PR+ cases are positive for c-Myc pS62 staining, which is in turn correlated with deregulation of *AXIN1* splicing. Thus ER and/or PR might regulate the ratio of *AXIN1V1* and *AXIN1V2* by coupling *AXIN1* pre-mRNA splicing with *AXIN1* transcription and it would be important in the future to test if *AXIN1* is a transcription target of ER and /or PR. Regulating *AXIN1* pre-mRNA splicing might be one reason why ER and/or PR status of human breast cancer correlates with high levels of pS62 c-Myc.

Activation of HER2 might also regulate *AXIN1* pre-mRNA splicing. My preliminary data showed that comparing to control MCF10A cells, the ratio of

AXIN1V2 vs *AXIN1V1* was higher in MCF10A cells with activated HER2 (data not shown). Akt signaling, which is a major downstream effector pathway of HER2, has been shown to alter genome-wide estrogen receptor binding and impact estrogen signaling in breast cancer (Bhat-Nakshatri et al. 2008), probably because Akt can phosphorylate ER-α, which can in turn affect ER-α transcriptional activity (Campbell et al. 2001). Thus HER2 via Akt signaling might also regulate *AXIN1* pre-mRNA splicing through regulating ER-α transcriptional activity as suggested by the authors of the study (Bhat-Nakshatri et al. 2008). Activation of HER2 might also regulate *AXIN1* pre-mRNA splicing through Ras signaling (Yea et al. 2008). Like Akt, Ras activated MAPK can also phosphorylate ER-α and thus might also affect ER-α-regulated pre-mRNA splicing (Kato et al. 1995).

4. Axin1v2 is functionally different from Axin1v1.

Axin1v2 is a naturally occurring splice variant of Axin1v1, where Axin1v2 does not include exon 9. This splice form is conserved between different species, suggesting a conserved function. However, no study on whether the two splice variants have differential regulation on Axin1 substrates or not has been reported yet. Here I have shown that ectopic expression of Axin1v1 in the *AXIN1*-null cell line SNU574, inhibits both c-Myc phosphorylation at S62 and c-Myc binding to target gene promoters, but ectopic expression of Axin1v2 does not. This suggests that exon 9 is necessary for Axin1's inhibitory regulation of c-Myc.

The mechanisms underlying the functional differences between Axin1v1 and Axin1v2 are not clear. Exon 9 of Axin1v1 is part of the PP2A interaction domain (Hsu et al. 1999)(Fig. 1.5). Our previous study showed that Axin1v2 has a decreased interaction with PP2A, GSK3β and c-Myc when compared with Axin1v1 (Arnold et al. 2009). This decrease or loss of scaffold function in Axin1v2 might be one of the mechanisms for Axin1v2's failure to inhibit c-Myc. Exon 9 also contains a predicted CK1 phosphorylation site (Salahshor and Woodgett 2005). Phosphorylation of Axin1 by CK1 promotes Axin1-GSK3β interaction and leads to downstream inhibition of β-catenin signaling (Luo et al. 2007). Thus lacking a CK1 phosphorylation site in Axin1v2 might explain the decreased interaction between Axin1v2 and GSK3β we reported previously (Arnold et al. 2009). The disrupted interaction between Axin1v2 and GSK3β might lead to activation of c-Myc through two mechanisms, increasing c-Myc protein stability and increasing c-Myc access to chromatin due to constitutive activation of β-catenin, as β-catenin plays an important role in chromatin remodeling (Mosimann et al. 2009).

Exon 9 of Axin1v1 is positioned between two potential Axin1 nuclear export signals (Fig.1. 5). Interestingly, it has been shown that staining for Axin1 is seen in the cytoplasm of normal epithelial cells, whereas it is located in the nucleus in tumors (Salahshor and Woodgett 2005). Since antibodies can not differentiate between Axin1v1 and Axin1v2 and our data shows that Axin1v2 is the predominant form in tumors, it is possible Axin1v2 is predominantly localized

in the nucleus. Studies have shown that Axin1 interacts with β–catenin in the nucleus and induces β-catenin's cytoplasmic shifting (Cong and Varmus 2004; Wiechens et al. 2004; Krieghoff et al. 2006). If this is also true for Axin1's regulation of c-Myc, Axin1v2 will not have the ability to inhibit c-Myc function by relocating c-Myc to the cytoplasm.

5. Crosstalk between HER2 signaling, ER/PR signaling and Axin1.

In this study, I have shown that both deregulation of Axin1 and activation of HER2 contribute to deregulation of c-Myc in breast cancer cells. I have also shown that pS62 intensity is correlated with ER/PR status in human breast cancer, indicating that ER/PR signaling may be another mechanism contributing to increased c-Myc protein stability and oncogenic activity. One interesting question is whether these mechanisms are connected with each other. As discussed above, activation of ER and/or PR may lead to altered transcription and subsequent alteration of transcription-coupled *AXIN1* pre-mRNA splicing. Activation of HER2 may affect ER and/or PR regulated *AXIN1* pre-mRNA splicing by increasing Akt or MAPK-mediated phosphorylation of ER and/or PR. Additionally, HER2 signaling and deregulation of Axin1 may also be connected through GSK3β, which is downstream of HER2 signaling and also increases Axin1 protein stability (Yamamoto et al. 1999). HER2 may, through activation of PI(3)K-Akt signaling, inhibit GSK3β activity and thus decrease Axin1 protein stability. On the other hand, Axin1 has also been shown to inhibit Ras signaling

pathway by promoting degradation of Ras and Axin1-promoted Ras degradation is inhibited by the non-degradable β-catenin mutant (Jeon et al. 2007). Similar results were reported in the thesis of Hugh Arnold, a previous graduate student from the Sears lab. He found that overexpression of Axin1 decreases protein expression of both c-Myc and Ras and expression of c-Myc^{T58A} inhibits Axin1-promoted Ras degradation. Detailed molecular mechanisms of how Axin1 promotes Ras protein degradation are still not clear; however, proper degradation of other Axin1 substrates is required, presumably because non-degradable mutant substrates can sequester Axin1 as in the case of c-Myc^{T58A} and Axin1 is reported to be limiting (Arnold et al. 2009).

In summary, I propose a model where activation of HER2, ER/PR and deregulation of Axin1 collaborate to increase c-Myc protein stability and oncogenic activity (Fig. 4.2). HER2 signaling and ER/PR signaling may inhibit Axin1 function through deregulating *AXIN1* pre-mRNA splicing to increase expression of the *AXIN1V2*, an *AXIN1* variant that is not able to inhibit c-Myc. In addition, HER2 signaling may decrease Axin1 protein stability through inhibiting GSK3β. Reciprocally, Axin1 may inhibit HER2 signaling by promoting Ras protein degradation. I propose that a feedback loop occurs between HER2 signaling and Axin1, where deregulation of either HER2 or Axin1 will lead to deregulation of the other. Thus, while normal cells have developed a complicated regulation system to ensure c-Myc protein stays at low levels, disturbance at one of the regulation steps in tumor cells might lead to a cascade of deregulations at other steps in the

pathway increasing the potential of having increased c-Myc protein stability and oncogenic activity in mammary tumor cells.

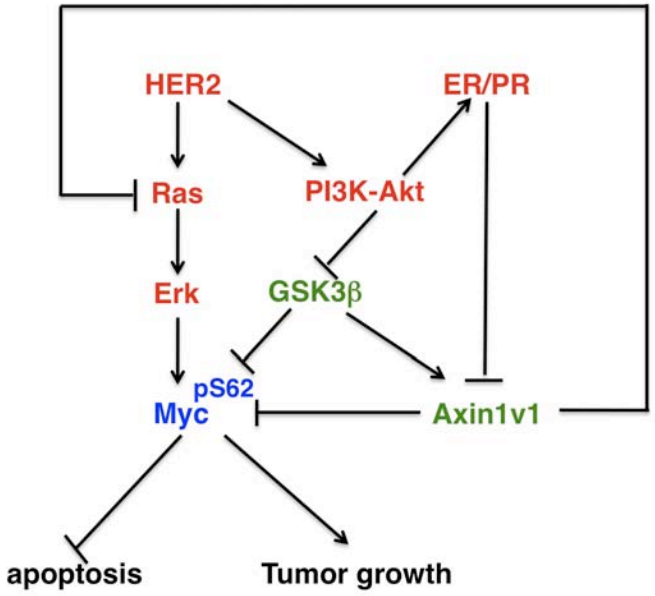


Fig.4.2: A proposed model showing crosstalk between mechanisms that deregulate c-Myc protein stability and oncogenic activity in breast cancer.

Both activation of HER2 and deregulation of Axin1 can lead to increased c-Myc pS62. Activation of HER2 can deregulate c-Myc through activation of ERK and inhibition of GSK3β. Deregulation of Axin1 due to decreased Axin1 level and preferred expression of Axin1v2 also leads to increased c-Myc pS62 and c-Myc activation. In addition, ER/PR signaling may be another mechanism regulating c-Myc as ER/PR status correlates with c-Myc pS62 intensity in human breast cancer. Activation of HER2 and ER/PR and deregulation of Axin1 may crosstalk. ER/PR may regulate *AXIN1* pre-mRNA splicing and thus increase c-Myc pS62. Activation of HER2 may alter *AXIN1* pre-mRNA splicing through Akt-mediated phosphorylation of ER and promote Axin1 degradation through inhibiting GSK3β. Deregulation of Axin1 may inhibit Axin1-promoted Ras degradation, thus increase Ras signaling. Increases in c-Myc pS62 may then overcome c-Myc's intrinsic apoptotic activity (Wang et al. 2011) and promote tumor growth.

Appendix

Contribution to Figures:

Figure 2.1: Bryan Laraway did pulse-chase for MDA453 (two repeats) and MCF10A (1time) in panel A.

Figure 2.3: cDNA samples used in panel A and in supplemental figure 6A are gifts from Dr. Dexi Chen in Capital University of Medical Sciences in China.

Figure 2.4: Charley Scanlan did soft agar experiments in panel A and F. Amy Farrell did soft agar experiments in panel B and ChIP experiments in panel G. Colin Daniel did CO-IP experiments in panel D.

Figure 2.5: Karyn Taylor performed lentivirus infection in panel D. Amy Farrell did ChIP experiments in panel E. Colin Daniel designed the model shown in panel F.

Suppmental figure 2.1: Mahnaz Janghorban did experiments for testing c-Myc half-life in HCC36, MDA468 and MDA436.

Supplemental figure 2.2: Amy Farrell did immunofluorescence staining with pS62 antibody in panel B. Xiaoyan Wang provided mouse tissue slides in panel B.

Supplemental figure 2.5: Arun Kumar Krishnamoorthy did western analysis in panel A.

Supplemental figure 2.6: Xiaoyan Wang provided frozen tissue for RNA analysis in panel C.

Supplemental figure 2.7: Amy Farrell did ChIP experiments in panel B.

Supplemental figure 2.8: Colin Daniel designed the model.

Supplemental table 2.1: Charley Scanlan did immunofluorescent staining for some of the cases.

Supplemental table 2.2: Xiaoyan Wang provided mouse tissue slides for immunofluorescence staining.

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