Negative Regulation of c-MYC Oncogenic Activity through the Tumor Suppressor PP2A-B56α

By

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List of Abbreviations

APC	Anaphase Promoting Complex
BAD	Bcl-2-Associated Death Promoter
BrdU	Bromo-Deoxyuridine
CIP2A	Cancerous Inhibitor of PP2A
GSK3β	Glycogen Synthase Kinase 3β
LCMT1	Leucine Carboxyl Methyltransferase 1
LT	Large T Antigen
MAPK	Mitogen-Activated Protein Kinase
NFκB	Nuclear Factor kappa B
PP2A	Protein Phosphatase 2A
PP2A A	Structural (A) Subunit of PP2A
PP2A B	Regulatory (B) Subunit of PP2A
PP2A C	Catalytic (C) Subunit of PP2A
PI3K	Phosphoinositide-3-Kinase
pS62	Phosphorylation at Serine 62
pT58	Phosphorylation at Threonine 58

RTK	Receptor Tyrosine Kinase
ST	Small T Antigen
SV40	Simian Virus 40
SCC	Squamous Cell Carcinoma

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Abstract

Reversible protein phosphorylation plays an important role in regulating cellular signaling and deregulation of this mechanism can contribute to many aberrant cellular behaviors, including many cancers. The Protein Phosphatase 2A (PP2A), is one of the major phosphatases in the cell that has a tumor suppressor function through its regulation of c-MYC and other key signaling pathways. PP2A is a critical negative regulator of c-MYC through its ability to dephosphorylate Serine62. The c-MYC oncoprotein is stabilized and activated by phosphorylation at Serine 62 (phosphoS62-MYC) in many cancers. How PP2A is deregulated in cancer remains an ongoing research topic. The overall objective of this thesis is to test the hypothesis that PP2A function is required for normal cell homeostasis and its activity is suppressed in cancers by overexpression of cellular oncoproteins and targeting these inhibitors can reactivate PP2A and therefore modulate downstream signaling such as c-MYC.

In chapter 2, I show that two endogenous inhibitors of PP2A, the SET oncoprotein and CIP2A, are overexpressed in breast cancer and inhibition of either SET or CIP2A reduces the tumorigenic potential of breast cancer cell lines both *in vitro* and *in vivo*. Treatment of breast cancer cells with OP449, a novel SET antagonist, decreases the tumorigenic potential of breast cancer cells and induces apoptosis. I show that this is, at least in part, due to decreased phosphoS62-MYC and

reduced c-MYC activity and target gene expression. Taken together these results show that PP2A activity is deregulated in breast cancer by SET or CIP2A and reactivation of PP2A, either through inhibiting SET or potentially CIP2A, can be a novel anti-tumor strategy to post-transnationally target c-MYC in breast cancer.

In chapter 3, I characterize a mouse model for B56α deletion. The PP2A holoenzyme has 3 subunits: a catalytic subunit, a structural subunit, and a variable regulatory (B) subunit, which directs PP2A to specific targets. Because loss of the regulatory subunit B56α was shown to be required for human cell transformation and that B56α negatively regulates c-MYC, we generated a novel mouse model of B56α knockout. I found that although the knockout of the B56α gene was in the whole body, the primary phenotypic effects of B56α loss were spontaneous skin lesion formation and increased immune cell infiltrations in the spleen and liver. I show that phosphoS62-MYC and its target gene Cdk4 are increased in these skin lesions. Because B56α negatively regulates many oncogenes such as c-MYC and β–catenin, our model suggests that B56α is important for homeostasis of cells and B56α loss could lead to increased activity of these oncogenes and subsequently aberrant cell proliferation.

Chapter One

Introduction

Overview

c-MYC (MYC) is an oncogenic transcription factor that controls many important cellular activities, therefore its expression and activity are tightly regulated in normal cell (Sears, Nuckolls et al. 2000; Meyer and Penn 2008). MYC is overexpressed by various mechanisms such as gene amplification, increased mRNA expression, or increased and stabilized protein expression [reviewed in: (Meyer and Penn 2008)]. Increased MYC protein stability though Serine62-phosphorylation, resulting in a modified form of MYC that is transcriptionally active and oncogenic, has been shown in many cancers (Malempati, Tibbitts et al. 2006; Wang, Cunningham et al. 2011; Zhang, Farrell et al. 2012). To study the mechanisms that lead to MYC stabilization, our lab has shown that increased levels of MYC can result from disruption of the normal MYC protein degradation pathway (Zhang, Farrell et al. 2012). As MYC is a hard-to-drug target, studying its degradation pathway might provide new insights about how to target MYC; therefore this encouraged me to focus on the Protein Phosphatase 2A (PP2A)-B56α, a negative regulator of MYC. PP2A-B56α removes the stabilizing phosphorylation on Serine62 and enhances MYC protein degradation (Arnold and Sears 2006). PP2A-B56α is an important tumor suppressor phosphatase whose inhibition is essential for complete human cell transformation. Despite its important function in cells, it is not clear how PP2A is deregulated in cancer, specifically breast cancer and whether activation of PP2A can be a good

therapeutic strategy for treatment of breast cancers (the focus of chapter 2),. In addition, no mouse model has ever been generated to study PP2A-B56α physiological function (the focus of chapter 3).

The overall objective of this thesis is to test the hypothesis that PP2A-B56α function is required for normal cell homeostasis and its activity is suppressed in cancers by overexpression of cellular oncoproteins, SET and CIP2A, and targeting these inhibitors can reactivate PP2A and therefore modulate downstream signaling such as MYC.

The following background on MYC and PP2A will explain what is known about these proteins and how understanding their functions and relationship will help us better investigate the gaps in the field and design therapies.

MYC Background

Over 30 years ago, an avian acute leukemia virus (MC29), later shown to contain the oncogene v-*myc*, was discovered that had the ability to transform cells and induce myelocytoma. The cellular gene picked up by this virus, designated c-*MYC*, was identified soon after this discovery in normal cells from many species (Wasylishen and Penn 2010). This led to the discovery of many other viral oncogenes that were co-opted cellular genes. Since then, much research has been conducted in order to understand MYC's function in cells.

MYC: Oncogenic Transcription Factor

MYC is an oncogenic transcription factor that regulates many genes involved in numerous cellular functions such as proliferation, growth, apoptosis, and metabolism (Meyer and Penn 2008). MYC contains several domains that are important for its activity. There are conserved regions located at the N-terminus that are called MYC boxes (MBI, II, III, and IV), which are important for its transcriptional activity. The transactivation domain (TAD) contains MBI and MBII that are required for MYC transcriptional and cell transforming activity. Downstream of the MYC boxes, there is a canonical nuclear localization signal (NLS) followed by the C-terminal basic, helix–loop–helix, and Leucine zipper domains (B-HLH-LZ), which mediate MYC dimerization with other HLH-LZ proteins

such as MAX and also binding to DNA (Figure 1.1) (Murre, McCaw et al. 1989; Draeger and Mullen 1994; Ferre-D'Amare, Pognonec et al. 1994; Luscher and Vervoorts 2012; Farrell and Sears 2014).

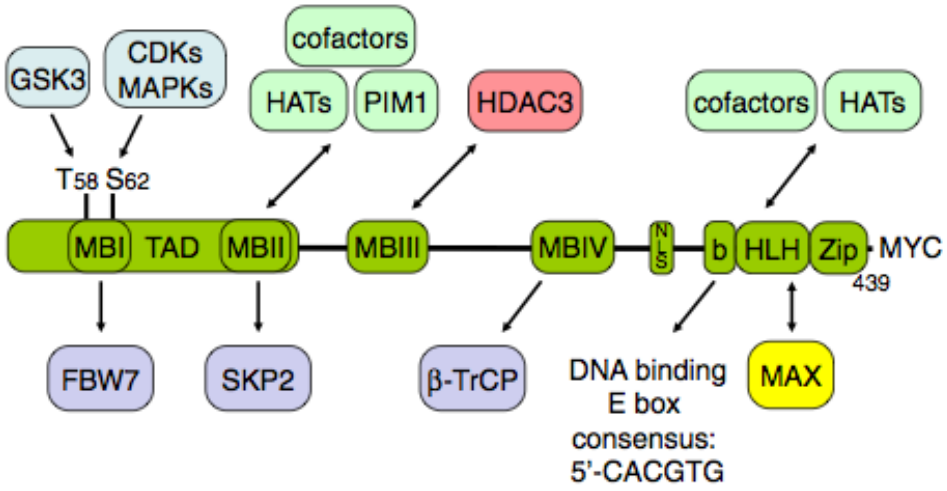

Figure 1.1. Structure of MYC (Luscher and Vervoorts 2012). Several regions within MYC help recruitment of many interacting proteins, co-activators and chromatin modifiers to the promoter of target genes.

By binding to its partner protein MAX, the MYC-MAX heterodimer binds to promoters of many target genes with an E-box sequence (CACGTG) and controls gene transcription through recruitment of multiple cofactors and chromatin modifiers (Figure 1.1) (Luscher and Vervoorts 2012). It is estimated that MYC acts as a global regulator of transcription and controls expression of more than 15% of all genes (Li, Van Calcar et al. 2003; Luscher and Vervoorts 2012). However, newer studies suggest

that MYC may act as an amplifier of transcription by binding to promoters of all active genes (Lin, Loven et al. 2012).

Tight Regulation of MYC Protein

MYC protein expression is tightly regulated in normal resting cells and its stability transiently increases upon stimulation with growth factors (serum in cell culture) or during cell cycle entry (Sears 2004). MYC stability is regulated in part by sequential and reversible phosphorylation at two conserved residues in the MBI, Threonine 58 (T58) and Serine 62 (S62) (Figure 1.2) (Sears, Nuckolls et al. 2000). Phosphorylation at Serine62 (pS62) and Threonine58 (pT58) has opposing effect on MYC stability where pS62 stabilizes and pT58 destabilizes MYC (Figure 1.2) (Sears, Nuckolls et al. 2000). MYC becomes phosphorylated at S62 through the Ras/Raf/MEK/ERK pathway or CDK activation in response to growth signals, and this modification increases its stability as well as its transcriptional and oncogenic activity (Zou, Rudchenko et al. 1997; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004; Hann 2006; Wang, Cunningham et al. 2011). When growth signals cease, GSK3, in a manner dependent upon prior phosphorylation at S62, phosphorylates T58 (Lutterbach and Hann 1994; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). T58 phosphorylation facilitates PP2A-B56α-mediated dephosphorylation of pS62 and recruitment of the E3 ubiquitin

ligase SCF^{Fbw7} to initiate proteasomal degradation of MYC (Welcker, Orian et al. 2004; Arnold and Sears 2008). This process is facilitated by Axin1, which helps nucleate a destruction complex for MYC at target gene promoters (Arnold, Zhang et al. 2009; Farrell, Pelz et al. 2013).

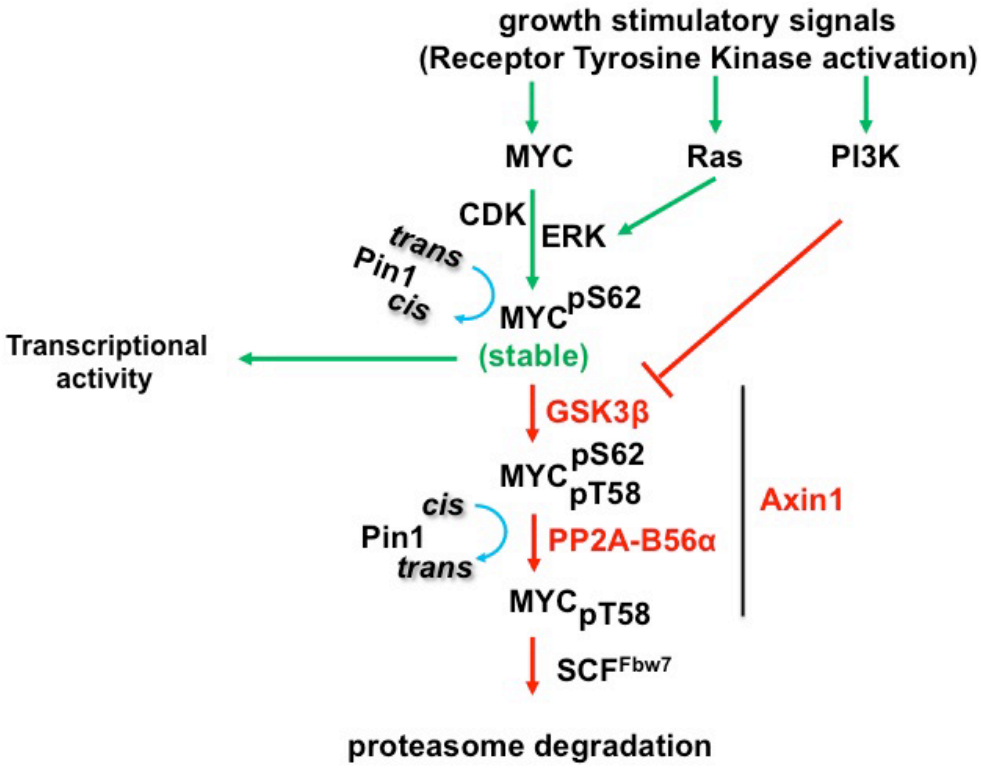

Figure 1.2: pS62/pT58 MYC degradation pathway. Proteins in red stabilize and/or activate MYC. Proteins in green facilitate MYC degradation.

MYC Overexpression in Cancer

High levels of MYC expression have been reported in almost all cancers ranging from hematological malignancies, such as Burkitt's lymphoma (91%) to solid tumors such as neuroendocrine prostate cancer (40%), colon cancer (45%), and breast cancer (45%) (Naidu, Wahab et al. 2002; Frost, Newell et al. 2004; Beltran, Rickman et al. 2011; Georgakopoulos, Tsiambas et al. 2013). MYC overexpression occurs through many mechanisms including insertional mutagenesis, chromosomal translocation, gene amplifications, increased mRNA stability, and increased protein stability (Meyer and Penn 2008; Zhang, Farrell et al. 2012).

MYC in Breast Cancer

Different studies have shown MYC overexpression in breast cancers ranging from 15% to 45% (Deming, Nass et al. 2000; Chrzan, Skokowski et al. 2001; Naidu, Wahab et al. 2002; Blancato, Singh et al. 2004). *MYC* gene amplification has been reported in about 15% of breast tumors, increased mRNA levels in 22% to 35% of tumors, and protein overexpression in about 45% of breast tumors (Deming, Nass et al. 2000; Chrzan, Skokowski et al. 2001; Xu, Chen et al. 2010). Interestingly in molecular subtyping of breast cancer, the MYC-MAX pathway activation was shown to be associated with triple negative Claudin-low tumors

(Heiser, Sadanandam et al. 2012). Other studies have also shown that MYC overexpression occurs in about 50% of basal-like tumors, 38% of normal-like, 11% of luminal, and 9% of Her2 positive tumors (Xu, Chen et al. 2010), suggesting that although MYC might not be a primary pathway but its expression could be deregulated in different tumor subtypes; deregulated MYC potentially contributes to tumor progression and correlates with more aggressive tumors (Deming, Nass et al. 2000).

Our lab has shown that increased protein stability contributes to MYC protein overexpression in breast cancers and that this correlates with high pS62- and low pT58-MYC (Zhang, Farrell et al. 2012). Decreased expression of Axin1 has been suggested as one of the mechanisms for increased MYC stability, contributing to its overexpression in breast cancers (Zhang, Farrell et al. 2012). In chapter 2 of this thesis, I examine the effect of another component of the MYC degradation pathway, PP2A, and how this contributes to MYC overexpression in human breast cancer. In order to study on PP2A, it is important to explain what the PP2A complex is and how it is formed and regulated in cells.

PP2A Background

Reversible protein phosphorylation plays an important role in regulating cellular signaling and deregulation of this mechanism can contribute to many aberrant cellular behaviors, including many cancers (Westermarck and Hahn 2008). It has been demonstrated that many oncogenes and proto-oncogenes are kinases, and that uncontrolled elevated levels of their activity lead to tumorigenic transformation (Janssens, Goris et al. 2005). However, the role of specific phosphatases in these processes and their relevance to human cancer is much less understood.

PP2A is a heterotrimeric Serine-Threonine protein phosphatase that is ubiquitously expressed in eukaryotic cells (Westermarck and Hahn 2008) and mediates 30-50% of cellular S/T protein phosphatase activity (Arnold and Sears 2008). PP2A is involved in regulation of numerous signaling pathways, including stem cell self-renewal, proliferation, differentiation, migration, cell survival, and apoptosis. This chapter will discuss the structure of PP2A, its regulation, and its impact on cell signaling. It will also describe the role of PP2A in transformation and cancer and how this information can contribute to the design of therapies.

PP2A Structure

The term PP2A is used to describe the heterotrimeric holoenzyme that contains three major subunits: a catalytic (C) subunit (36 kDa), a structural (A) subunit (65kDa), and a variable regulatory (B) subunit, which directs the PP2A holoenzyme to specific targets and subcellular locations (Figure 1.3) (Sablina and Hahn 2008; Westermarck and Hahn 2008). In mammals, the A and C subunits are found in two isoforms, α and β. Aα and Aβ share 87% sequence identity, whereas Cα and Cβ are 97% identical (Sablina and Hahn 2008). Four unrelated families of B subunits have been identified to date: B, B′, B″, and B‴ (Zhou, Pham et al. 2003; Sablina and Hahn 2008). The A subunit consists of 15 non-identical huntington-elongation-A subunits-TOR-like (HEAT) repeats which facilitates the protein-protein interactions and binding of other subunits (Arnold and Sears 2008). The B subunits bind to HEAT repeats 1-10, the simian virus 40 small T antigen (SV40 ST) oncoprotein binds repeats 2-8, and as such, can disrupt most B subunit binding, and the C subunits bind to repeats 11-15 (Zhou, Pham et al. 2003). Altogether, it is estimated that over 100 PP2A complexes can exist in cells. Details about these complexes and their activities remain unclear.

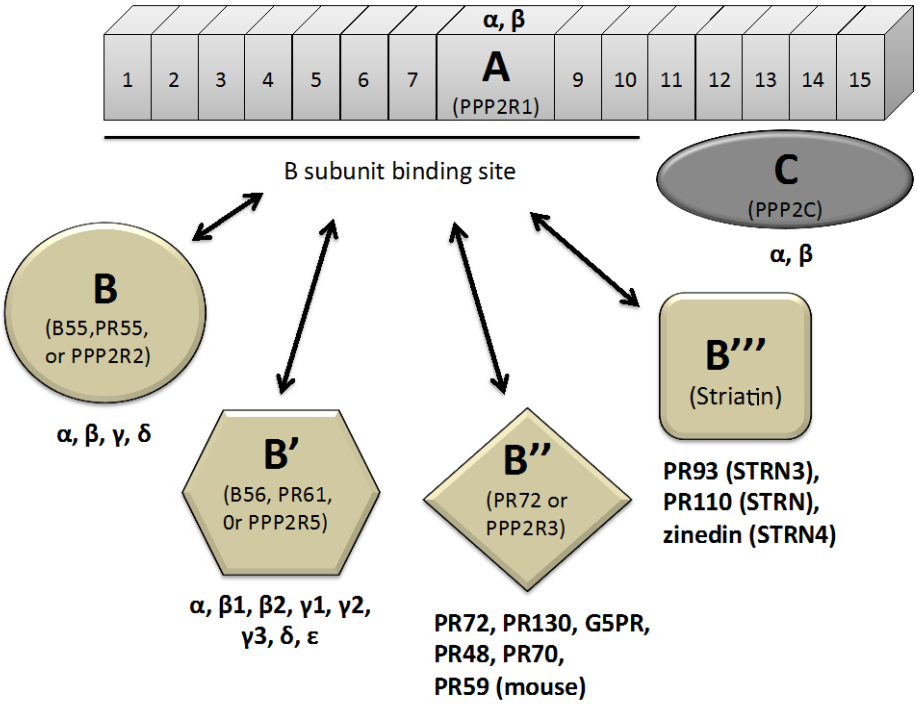

Figure 1.3: The PP2A heterotrimeric holoenzyme. Over 100 PP2A complexes can form in cell. The B subunits are responsible to direct the PP2A holoenzyme to a specific target and location.

The structural A/C complex

The structural A subunits are encoded by two distinct genes *PPP2R2A* (Aα) *and PPP2R1B* (Aβ), with 87% identical sequences (Zhou, Pham et al. 2003; Sablina and Hahn 2008). They are both ubiquitously expressed, although most PP2A complexes contain Aα. Both isoforms have unique functions, as Aβ cannot substitute for the Aα loss in mice (Ruediger, Ruiz et al. 2011). Also Aα overexpression is not able to reverse the transformation induced by Aβ suppression suggesting that Aα and Aβ

induce transformation through distinct mechanisms (Sablina, Chen et al. 2007). The main difference between these two isoforms is in their ability to interact with different B and C subunits and viral oncogenes (Zhou, Pham et al. 2003). Aα is reported to have stronger interaction with most of the subunits, and although both isoforms bind polyoma virus middle t antigen, only the Aα binds to the SV40 small t antigen (Zhou, Pham et al. 2003). All of this could underlie differential roles for the two A subunits in cell transformation. In addition, Aα is highly expressed in almost all tissues whereas Aβ is very low in all tissues except those of the testes (Zhou, Pham et al. 2003). Aα expression was shown to be high and relatively constant across cancer cell lines, whereas Aβ levels varied (Zhou, Pham et al. 2003).

The C subunit is one of the most conserved proteins during evolution (Lechward, Awotunde et al. 2001). Similar to the A subunit, two genes encode the C subunit: *PPP2CA* (Cα) and *PPP2CB* (Cβ), and they are 97% identical (Lechward, Awotunde et al. 2001; Sablina and Hahn 2008). Cα expression is about 10-folds higher than Cβ due to its stronger promoter (Janssens and Goris 2001) and Cβ can not compensate for Cα as Cα knockout in mice is embryonically lethal (Gotz, Probst et al. 1998). The C subunit levels are tightly controlled at the translational and post-translational levels as well as through interaction with the B and other regulatory subunits, to maintain the constant levels of PP2A (Baharians and Schonthal 1998; Janssens and Goris 2001). While ectopic expression

of the C subunit is extremely difficult and only 20% increased expression has been achieved (Baharians and Schonthal 1998), loss of the C subunit results in apoptosis (Gotz, Probst et al. 1998; Strack, Cribbs et al. 2004). This highlights the importance of proper PP2A regulation. Other regulatory mechanisms will be discussed below.

The regulatory B subunit

There are four unrelated families of B subunits that have been identified to date: B *(B55, PR55,* or *PPP2R2),* B′ *(B56, PR61,* or *PPP2R5),* B″ *(PR72* or *PPP2R3),* and B‴ *(PR93/PR110)*. All together 15 genes encode 26 B subunits of PP2A, which can potentially assemble more than 100 distinct PP2A complexes (Janssens and Goris 2001; Sontag 2001; Zhou, Pham et al. 2003; Sablina and Hahn 2008; Eichhorn, Creyghton et al. 2009). These subunits are differentially expressed both spatially and temporally, and little is known about how these differences affect the overall function of PP2A.

- B subunit- B55

The B subunit family (55kDa) is encoded by four genes, which is expressed in a tissue-specific manner. The B55α and B55δ subunits are widely expressed in different tissues whereas B55β (β and β2) and B55γ

are highly expressed in brain and are thought to be developmentally regulated as B55β decreases and B55γ increases after birth [Janssens and Goris 2001; Eichhorn, Creyghton et al. 2009). PP2A-B55 inhibition is an important step in mitosis, where depletion of this PP2A complex accelerates entry into mitosis and blocks exit from mitosis (Hunt 2013). Failure to inhibit PP2A-B55, especially B55δ, causes a cell cycle arrest in the G2 phase (Mochida, Ikeo et al. 2009; Hunt 2013). Greatwall (Gwl, drosophila or Xenopus) or microtubule-associated serine/threonine kinase-like MASTL (human), an essential mitotic kinase, inactivates PP2A-B55 during mitotic entry to maintain the phosphorylation of cyclin B–Cdk1 substrates until mitotic exit (Mochida, Ikeo et al. 2009; Hunt 2013; Wang, Galan et al. 2013). PP2A-B55 is also involved in regulating interphase intermediate filaments such as Vimentin, which is necessary for cell morphological changes during differentiation, migration, and spreading (Turowski, Myles et al. 1999). B55 regulates the association of Vimentin with the cytoskeleton and B55 knockdown leads to hyperphosphorylation and disassembly of Vimentin (Turowski, Myles et al. 1999). PP2A-B55 is also involved in cell signaling. Increased expression of B55α and B55δ was associated with increased levels of phosphorylation of MEK1/2, ERK1 and ERK2; this was through activation of Raf1 by direct binding of these PP2A complexes to Raf1 and removal of its inhibitory phosphorylation at Ser-259 (Adams, Coffee et al. 2005). Therefore, both B55α and B55δ regulatory subunits play an important role

in positively regulating Raf1-MEK1/2-ERK1/2 signaling (Adams, Coffee et al. 2005). PP2A-B55α was also shown to selectively control Akt phosphorylation at Thr-308 but not Ser-473 in NIH3T3 fibroblast and FL5.12 cells, and regulate cell proliferation and survival (Kuo, Huang et al. 2008). The survival of FL5.12 cells, a pro-B-cell lymphoid cell line that is dependent on IL3, was also increased after knockdown of B55α and IL-3 deprivation (Kuo, Huang et al. 2008).

- B' subunit- B56

The B′ subunits (56kDa) are encoded by five genes, which are 80% identical and differ at both the N- and C- termini (Janssens and Goris 2001; Eichhorn, Creyghton et al. 2009). B56α and B56γ (γ1, γ2, and γ3) are widely expressed in different tissues but are more abundant in heart and skeletal muscle (Janssens and Goris 2001). B56β (β1 and β2) and B56δ are expressed in the brain and their expression is increased upon retinoic-acid induced differentiation of neuroblastoma cells, suggesting that their expression is regulated during brain development (Janssens and Goris 2001; Eichhorn, Creyghton et al. 2009). Despite the fact that B56γ3 and B56δ are the only isoforms that have nuclear localization signals, all isoforms can shuttle to the nucleus (Seshacharyulu, Pandey et al. 2013). The B56 subunits are well studied as they regulate cell division, apoptosis, planer cell polarity, and many important developmental and tumorigenesis pathways. PP2A-B56 is thought to be a tumor suppressor and play an

important role in human cell transformation. This was initially discovered through expression of some viral oncoproteins such as SV40 small t (ST) and polyoma virus middle T (PyMT) and small t (PyST) antigen that were required to complete human cell transformation (Ruediger, Roeckel et al. 1992). It was shown that these viral oncoproteins compete with B56 and disrupt its interaction with PP2A, therefore inactivating B56 function (Yang and Phiel 2010). Later, a siRNA screen showed that knockdown of B56α or B56γ was necessary for human cell transformation (Sablina, Hector et al. 2010). Additional studies have shown that B56α regulates MYC and Akt and B56γ regulates Wnt during transformation (Arnold and Sears 2006; Sablina, Hector et al. 2010; Yang and Phiel 2010).

- B'' subunit

The B″ subunits include four families: PR72 and PR130 that are splice variants, PR59, G5PR, and PR70 and its splice variant PR48 (Janssens and Goris 2001; Eichhorn, Creyghton et al. 2009). PR72 is exclusively expressed in heart and skeletal muscle whereas PR130 is expressed in all tissues, but is more abundant in heart and muscle (Janssens and Goris 2001). PR59 is a mouse specific subunit, with no known human ortholog, and is expressed in testis, kidney, liver, brain, heart, lung, but not in skeletal muscle (Janssens and Goris 2001; Eichhorn, Creyghton et al. 2009). PR59 and PR72 are 56% identical and PR59 and PR48 are 68% identical (Janssens and Goris 2001). All

members of the B″ family contain two EF hand calcium-binding domains, which bind Ca^{2+} and regulate their interaction with the core enzyme and subsequent phosphatase activity (Eichhorn, Creyghton et al. 2009). The B″ subunits are involved in multiple cellular processes, including modulation of neuronal signaling, Wnt signaling, regulation of calcium-channel phosphorylation, and tumor suppression (Sablina, Hector et al. 2010; Wlodarchak, Guo et al. 2013). The PR72, PR70, and PR48 subunits have been demonstrated to play a role in cell cycle progression by positively regulating the tumor suppressor retinoblastoma (RB) and cell division control 6 (Cdc6) proteins, which are both crucial for the G1/S transition (Yan, Fedorov et al. 2000; Davis, Yan et al. 2008; Magenta, Fasanaro et al. 2008; Eichhorn, Creyghton et al. 2009; Wlodarchak, Guo et al. 2013). PR72 was shown to act as a negative regulator of the Wnt signaling cascade by interacting with Naked, a Wnt antagonist (Creyghton, Roel et al. 2005). PR72 was also shown to be required during early embryonic development to regulate cell morphogenetic movements during body axis formation (Creyghton, Roel et al. 2005). Interestingly, PR130 was found to positively modulate Wnt signal transduction by opposing the action of PR72 and restricting the activity of Naked (Creyghton, Roel et al. 2006). However, it is unknown how different signaling pathways regulate transcription of these two opposing subunits from different promoters.

- B’’’ subunit

The B‴ subunits (striatin family) are encoded by three genes: *PR93 (SG2NA, S/G2 nuclear autoantigen, STRN3)*, *PR110 (striatin, STRN)*, and *zinedin (STRN4)* (Janssens and Goris 2001; Hwang and Pallas 2014). They are mainly expressed in the nervous system (Benoist, Gaillard et al. 2006), however, their expression has been detected in other tissues (Hwang and Pallas 2014). They are Calmodulin-binding proteins (CaM) and enhance calcium (Ca^{2+})-dependent signaling (Seshacharyulu, Pandey et al. 2013). The B’’’ subunits have roles in many cellular functions such as signaling, cell cycle control, apoptosis, vesicular trafficking, Golgi assembly, cell polarity, cell migration, neural and vascular development, and cardiac function (Hwang and Pallas 2014). They form a complex with germinal center kinase III (GCKIII) kinases together with PP2A and other components called striatin-interacting phosphatase and kinase (STRIPAK) complexes (Hwang and Pallas 2014). It has been demonstrated that STRIPAK negatively regulates Hippo signaling during development in drosophila (Ribeiro, Josue et al. 2010) along with many other functions that are recently being uncovered such regulating ERK and Esterogen receptor signaling (Hwang and Pallas 2014).

Mouse Models

To study the physiological effect of PP2A, many mouse models have been generated.

Mouse model for the A subunit

In the Aα homozygous knockout mouse, development was blocked before E10.5 and in a conditional knockout mouse, Aα expression was continuously required for adult mice to live as they died upon Aα depletion after 6 days (Ruediger, Ruiz et al. 2011).

The discovery of different mutations in Aα and Aβ- which are almost all deficient in binding to either B or B and C subunits - in various human tumors including cancer of the breast, lung, colon, skin, and ovaries suggested that PP2A plays a role in cancer (Wang, Esplin et al. 1998; Calin, di Iasio et al. 2000; Takagi, Futamura et al. 2000; Jones, Wang et al. 2010; Ruediger, Ruiz et al. 2011). Different mouse models were generated based on mutations found in tumors: The Aα mutant E64D mutation was found in human lung carcinoma and the E64G mutation was found in a breast carcinoma. Both of these mutations were defective in B56 binding. Also another knockout mouse was generated that expressed a truncated Aα, which was defective in the B and C subunit binding. They all showed that indeed the mutant Aα increased the incidence of cancer by 50 to 60% in lungs; this was dependent on p53 and did not increase the

incidence of cancer when p53 was suppressed (Ruediger, Ruiz et al. 2011).

Mouse model for the C subunit

The PP2A Cα and Cβ share 97% similarity in their amino acid sequence. However complete loss of Cα in mice resulted in embryonic lethality phenotype and Cβ could not compensate for the Cα loss (Gotz, Probst et al. 1998). Later it was shown that this could be explained by differential localization of Cα and Cβ. During normal early embryonic development Cα was predominantly localized to plasma membrane of inner cell mass whereas Cβ was present in the cytoplasm and nucleus (Gotz, Probst et al. 2000). Surprisingly, embryos developed normally until post-implantation, around embryonic day 5.5/6.0 when gastrulation started, showing that initial development was not impaired in these mice (Gotz, Probst et al. 1998). In Cα depleted cells, both E-cadherin and β-catenin were redistributed from the plasma membrane to the cytoplasm, which was accompanied by accelerated degradation (Gotz, Probst et al. 2000). These results clearly demonstrated that PP2A is involved in Wnt signaling during early embryonic development.

Conditional knockout of Cα in hematopoietic and endothelial cells (Tie2Cr transgenic mice) showed that Cα depletion perturbed fetal liver erythropoiesis and caused sever anemia (Chen, Gu et al. 2011). This was

due to a decrease in the survival of erythroid cells through impaired STAT5–Bcl-xL and not a decrease in the fetal liver hematopoietic stem and progenitor cell pool (Chen, Gu et al. 2011).

Generation of conditional Cβ null mice showed that Cβ is not required for mouse development and reproduction, as these mice were healthy and fertile (Gu, Qi et al. 2012).

Leu309 located in the C-terminus of the C subunit is subject to post-translational modifications including phosphorylation and methylation and the L309A mutation was shown to be defective in binding distinct subunits *in vitro* and in yeast (Schild, Isenmann et al. 2006). L309A mutant expression in the Harderian gland of transgenic mice showed a delayed postnatal development and hypoplasia of the gland, causing enophthalmos (slit-eye phenotype) (Schild, Isenmann et al. 2006). Similar to the results from the Cα knockout mice, E-cadherin and β-catenin were both shifted to the cytosol and their expression was reduced (Schild, Isenmann et al. 2006). Interestingly, the levels of some of the B subunits were changed: B55α was reduced to 46% and B56ε to 38%, whereas, B56γ and PR59 were increased 2.5- and 3-fold (Schild, Isenmann et al. 2006). This suggests that the amount and availability of the C subunit affects the expression of these subunits.

Mouse model for the B subunit

Of the many different regulatory subunits discovered so far, there have been only three mouse models generated: B56γ, B56δ, and G5PR. The transgenic B56γ mouse was produced by the overexpression of the full-length B56γ cDNA cloned into the lung-specific human surfactant protein promoter and a SV40 ST poly-A cassette (Everett, Kamibayashi et al. 2002). The mice died neonatally due to lack of normal lung structure and suppressed β-catenin expression suggesting a role for B56γ in association with Wnt signaling during lung development (Everett, Kamibayashi et al. 2002).

Another mouse model of B subunit reported a role for B56δ in the central nervous system (Louis, Martens et al. 2011). B56δ knockout mice were viable and fertile with no overt growth abnormalities but Tau was hyperphosphorylated in restricted brain areas depending on age (Louis, Martens et al. 2011).

Conditional knockout of G5PR in B-cells identified a role for G5PR in maturation or life span of B-cells in periphery (Xing, Igarashi et al. 2005). Lack of G5PR caused an abnormal activation of Bim during B-cell stimulation through BCR-mediated signal transduction, which led to prolonged mitochondrial membrane destabilization and activation-induced cell death (AICD) (Xing, Igarashi et al. 2005).

Generation of a T-cell specific knockout of G5PR showed thymic atrophy, with significant decrease in thymocyte numbers (particularly CD4 and CD8 double-positive thymocytes) and mature single-positive cells (Xing, Wang et al. 2008). Same to the B-cell mouse model, loss of G5PR did not affect proliferation or differentiation of cells but increased susceptibility to apoptosis caused by prolonged activation of JNK, which was associated with the augmented expression of FasL (Xing, Wang et al. 2008). This study also demonstrated a unique apoptotic signal in thymocyte that was different from that of mature B-cells undergoing AICD reported previously.

The specific role of other B subunits in development and diseases (particularly cancer) has yet remained unexplored. In chapter 3, I characterize a novel mouse model of B56α knockout and its role in cell homeostasis and transformation. I report that B56α plays a role in tissue homeostasis and loss of B56α predisposes cells to aberrant cell proliferation.

Interacting Proteins: Tumor Suppressors and Oncoproteins

In addition to the B subunits that regulate PP2A function, various viral and cellular proteins can also control PP2A activity (Figure 1.4) and each of these proteins has tumor suppressive or oncogenic activity. Proteins encoded by viruses such as PyMT and PyST antigens, and SV40 ST antigen, can act as regulatory subunits and change PP2A activity (Arroyo and Hahn 2005). They can replace the B subunit from the core complex and inhibit PP2A activity, which subsequently results in cell transformation through discrete pathways (Rodriguez-Viciana, Collins et al. 2006; Eichhorn, Creyghton et al. 2009). SV40 ST can activate PI3K/Akt but not the MAP kinase pathway, while PyST activates the MAP kinase pathway. PyMT activates both the MAPK and PI3K pathways (Rodriguez-Viciana, Collins et al. 2006).

Cellular proteins such as SET, CIP2A, ANP32, ARPP-16/19, and TPRL1 regulate PP2A activity. α4, PTPA, PME-1, and LCMT1 are important for both PP2A biogenesis and function and the coordination of these many interacting proteins demonstrates that PP2A activity is under a tight control in cells. The importance of each of these proteins and their implications in different pathogenesis, specifically cancer, are discussed below.

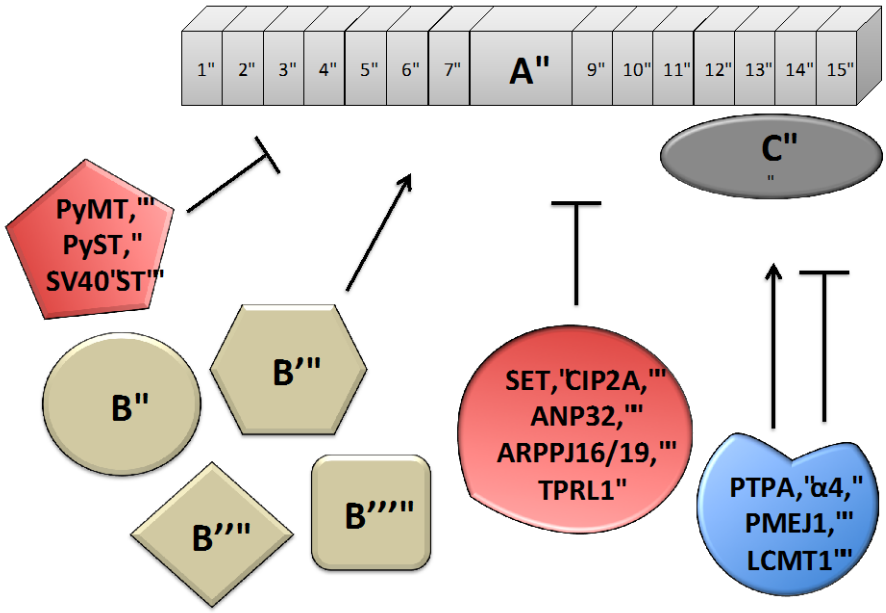

Figure 1.4: PP2A activity is regulated by interacting with various viral and cellular proteins and also post-translational modifications.

❖ SET

The oncoprotein SET (or inhibitor 2 of PP2A, I2PP2A) was initially identified as a fusion protein with NUP214 (SET-NUP214 or SET-CAN) in Acute Undifferentiated Leukemia where 270 amino acids of the total 277 amino acids of SET were present (Adachi, Pavlakis et al. 1994). SET was purified from bovine kidney and shown to localize to the nucleus, outer layer of the nuclear membrane, and cytoplasm (Adachi, Pavlakis et al. 1994; Li, Guo et al. 1995). SET inhibits PP2A activity by binding to at least the C subunit of PP2A (Li, Guo et al. 1995). High levels of SET mRNA

expression have been observed in malignant brain tumors, tumors of the head and neck region, testicular cancers, and in different types of hematological malignancies (Neviani, Santhanam et al. 2005; Westermarck and Hahn 2008; Christensen, Chen et al. 2011; Agarwal, Mackenzie et al. 2014). SET is a key modulator of DNA replication, chromatin remodeling, gene transcription, and cell cycle, and it is unknown whether some of these functions are dependent on its activity toward PP2A (Nagata, Kawase et al. 1995; Okuwaki and Nagata 1998; Seo, McNamara et al. 2001; Canela, Rodriguez-Vilarrupla et al. 2003; Nowak, Pai et al. 2003). SET has a broad range of targets: it induces c-Jun and AP-1 transcriptional activity (Al-Murrani, Woodgett et al. 1999), co-operates with BCR-ABL in CML-BC (Blast Crisis) and positively regulates MYC, STAT5, ERK1/2, Akt, and JAK2, NFκB, nm23-H1 and migration through Rac-1 (Neviani, Santhanam et al. 2005; ten Klooster, Leeuwen et al. 2007; Switzer, Cheng et al. 2011). Because of its overexpression in many types of cancer and its regulation of many key oncoproteins, SET has become an interesting and potential target for therapeutics, which will be discussed later in this chapter and in more details in chapter 2.

- CIP2A

Another important PP2A interacting protein is Cancerous Inhibitor of PP2A (CIP2A). The oncoprotein CIP2A (initially known as P90) was discovered to play an important role in cell transformation, by inhibiting

PP2A-B56α activity resulting in increased MYC stability and activity (Junttila, Puustinen et al. 2007). Although CIP2A expression is not essential for normal mouse growth and development (Ventela, Come et al. 2012), CIP2A overexpression has been demonstrated in various types of tumors including in head and neck squamous cell carcinoma, gastric, colon, breast and prostate tumors and its expression correlates with clinical progression and outcome (Junttila, Puustinen et al. 2007; Li, Ge et al. 2008; Come, Laine et al. 2009; Vaarala, Vaisanen et al. 2010; Teng, Yang et al. 2012). In addition, a CIP2A hypomorph MMTV-neu-driven mouse model has shown an impaired mammary tumor development and progression (Laine, Sihto et al. 2013). Many studies have shown an important role for CIP2A in promoting tumor growth, resistance to apoptosis and senescence-inducing therapies (Junttila, Puustinen et al. 2007; Laine, Sihto et al. 2013; Lee, Jeong et al. 2013). In addition to MYC, CIP2A promotes the phosphorylation and activity of additional oncoproteins, including E2F1, Akt, and JNK2 (Niemela, Kauko et al. 2012; Laine, Sihto et al. 2013; Lee, Jeong et al. 2013), making it a very favorable potential therapeutic target.

- ANP32a

Other PP2A cellular inhibitors are less characterized. ANP32a (or inhibitor 1 of PP2A, I1PP2A), and SET were both purified and characterized as a PP2A inhibitor from bovine kidney (Li, Guo et al. 1995).

ANP32a inhibits PP2A by binding to the C subunits (Li, Makkinje et al. 1996) and is largely localized to the cytosol (Li, Makkinje et al. 1996). Both SET and ANP32a homo- and heterodimerize with other proteins in the cell and regulate many functions including histone acetyl-transferase inhibition, chromatin remodeling, and mRNA stabilization (Santa-Coloma 2003) though it is unclear if these functions are dependent upon their PP2A inhibition activity.

- cAMP-regulated phosphoproteins

cAMP-regulated phosphoproteins ARPP-16, ARPP-19 (derived from the same gene by alternative splicing), and the closely related ENSA (α-endosulfine) were recently identified as potential PP2A inhibitors (Walaas, Hemmings et al. 2011; Hunt 2013). During mitosis in the oocyte, phosphorylated ARPP-19 and ENSA (by Greatwall kinase) bind specifically to B55δ, but not other types of B subunits (Hunt 2013). Failure to inhibit PP2A-B55 causes G2 phase cell cycle arrest. A similar role for ARRP-19 and ENSA in regulation of mitosis has been demonstrated in Hela cells (Gharbi-Ayachi, Labbe et al. 2010). ARRP-19 becomes phosphorylated by mammalian homolog of Greatwall kinase, termed microtubule-associated serine/threonine kinase-like (MASTL)- likely the MAST3 isoform- during the cell cycle and inhibits PP2A (Voets and Wolthuis 2010).

❖ TIPRL1

TIP or TIPRL1 is a ubiquitously expressed inhibitor of the C subunit of PP2A as well as Protein Phosphatases 4 and 6 (PP4 and PP6) (McConnell, Gomez et al. 2007). A study showed that TIPRL1 was involved in DNA damage and repair signaling and by inhibiting PP2A, it increased the phosphorylation state of an unknown protein substrate (neither Chk1 or Chk2) of ataxia-telangiectasia mutated (ATM)/ATM- and Rad3- related (ATR) kinases (McConnell, Gomez et al. 2007). It is proposed that TIPRL1 might be involved in PP2A biogenesis or degradation after holoenzyme disassembly following cellular stress; TIPRL1 can proactively inhibit active PP2A C, which protects cells from unrestricted activity of the released C subunit that can be detrimental (Sents, Ivanova et al. 2013).

❖ α4

α4 (immunoglobulin-α-binding protein 1 (IGBP1)), or phosphatase 2A-associated protein of 42 kDa (Tap42) in yeast), is another PP2A interacting protein in cells that forms a stable complex by binding directly with the C subunit, independent of the A or B subunits (Sents, Ivanova et al. 2013). Based on published reports on the effects of α4 on PP2A activity, α4 is thought to regulate the assembly and maintenance of the C subunit rather than just increasing or inhibiting its activity (Kong, Fox et al. 2004; Kong, Ditsworth et al. 2009; Sents, Ivanova et al. 2013). However,

there has been some conflicting data on the affect of α4 on PP2A. α4 depletion led to a loss not only of PP2A C, but also PP4, PP6, and PP2A A, and subsequent loss of PP2A activity. On the other hand, α4 overexpression increases the C subunit and decreases the ubiquitinated C subunit suggesting that α4 protects the C subunit from degradation (Kong, Ditsworth et al. 2009). α4 knockout in mice was lethal and conditional gene targeting of α4 showed a fundamental role for α4 in proliferation and survival of B and T lymphocytes, embryonic fibroblasts, adipocytes, and hepatocytes (Kong, Fox et al. 2004; Sents, Ivanova et al. 2013). Loss of α4 resulted in profound apoptosis (initiated by p53 and c-Jun-dependent transcriptional mechanisms) and impaired cell spreading and migration (reduced activation of Rac-1) (Kong, Fox et al. 2004; Sents, Ivanova et al. 2013). It is known that α4 binds and inhibits the enzymatic activity of either newly synthesized C or C released from the PP2A complexes following cellular stress, which is required for its biogenesis and reincorporation into active PP2A holoenzymes (Kong, Ditsworth et al. 2009). Furthermore, it has been demonstrated that the C–α4 complex consists of an additional member, the ATP-dependent T-complex polypeptide-1 ring complex (TRiC)/chaperonin-containing T-complex polypeptide complex (CTT) chaperone family (TRiC/CCT), which is potentially helping the proper folding of α4 and or the C subunit (Sents, Ivanova et al. 2013).

In addition to regulating PP2A biogenesis, a small number of specific substrates have been discovered for the PP2A-α4 complex such

as mitogen-activated protein kinase kinase 3 (MEK3) and the RING-domain ubiquitin E3 ligase midline-1 (Mid1) (Liu, Prickett et al. 2001; Prickett and Brautigan 2007).

PP2A Regulation by Post-Translational Modifications

The C subunit of PP2A is one of the most highly conserved proteins, particularly at its C-terminal TPDYFL (304–309) tail, which is subject to various post-translational modifications such as carboxymethylation and phosphorylation (Janssens, Longin et al. 2008; Sents, Ivanova et al. 2013). The regulation of PP2A by these post-translational modifications is discussed below.

Regulation by carboxymethylation

Reversible carboxymethylation occurs on the free carboxyl group of the C-terminal Leucine 309 (Leu309) residue of PP2A C (Favre, Zolnierowicz et al. 1994; Xie and Clarke 1994). This modification is catalyzed by the S-adenosylmethionine-dependent enzyme LCMT1 (leucine carboxyl methyltransferase-1), which is mainly localized to the cytoplasm, Golgi region and late endosomes, and reversed by the methylesterase PME-1, which is predominantly localized to the nucleus (Lee and Stock 1993; Ogris, Du et al. 1999; Longin, Zwaenepoel et al.

2007; Janssens, Longin et al. 2008; Longin, Zwaenepoel et al. 2008). This correlates with localization of methylated or demethylated PP2A C (Turowski, Fernandez et al. 1995). The importance of this methylation was investigated during cell cycle progression, where demethylated Leu309 was found in the cytoplasm of G1 cells and in the nucleus of S and G2 cells (Turowski, Fernandez et al. 1995). Others have investigated its potential role in Alzheimer's disease pathogenesis (Longin, Zwaenepoel et al. 2008). Several studies have shown that LCMT1 activity is essential for cellular functions and loss of LCMT1 induces apoptosis and is linked to neurodegenerative diseases (Stanevich, Jiang et al. 2011). Structural analysis has shown that LCMT1 binds directly to the PP2A active site and prevents uncontrolled phosphatase activity of the PP2A holoenzyme or free C subunit by: 1) regulating the assembly of functional PP2A holoenzymes and preventing formation of dominant negative inactive holoenzymes, and 2) facilitating the efficient transition of activated PP2A to substrate-specific holoenzyme (Stanevich, Jiang et al. 2011). Although it was initially shown that Leu309 methylation is an essential prerequisite for B55 subunit recruitment (Sents, Ivanova et al. 2013), conflicting results show that methylation of Leu309 is not a prerequisite for holoenzyme assembly (Ikehara, Ikehara et al. 2007). It is now accepted that it only increases the binding affinity for the B55 and then B56 subunits (Xu, Chen et al. 2008) meaning that once Leu309 in methylated, it preferably recruits the B55 subunit.

Knockdown of either LCMT1 or PME-1 in mice were shown to be lethal, suggesting that these enzymes have indispensable functions, and strengthening the concept that proper control of PP2A activity is critical in cells (Hunt 2013). Although PME-1 overexpression increases demethylation of Leu309, the effect that this has on cell functions is still not clear. PME-1 is reported to stabilize a specific fraction of (newly synthesized) inactive PP2A independent of its methylesterase activity (Turowski, Fernandez et al. 1995). Although it was originally thought that demethylation by PME-1 inactivates PP2A, this seems to not hold true because there are active demethylated PP2A complexes in cells; therefore PME-1 is more considered to be a stabilizing factor after PP2A inactivation (unknown mechanisms) (Longin, Jordens et al. 2004).

Regulation by cis/tras isomerization

The newly synthesized C subunit is inactive due to possessing a different conformation and interacting with PME-1 (Turowski, Fernandez et al. 1995; Fellner, Lackner et al. 2003; Leulliot, Vicentini et al. 2006). The peptidyl prolyl isomerase PTPA (Phosphotyrosyl phosphatase Two A Phosphatase Activator) (encoded by PPP2R4) acts as a switch to convert the inactive C subunit to the active conformation (Leulliot, Vicentini et al. 2006). Structural studies have shown that a mutant PTPA without isomerase activity fails to reactivate the PME-1-bound inactive form of

PP2A *in vitro* (Hunt 2013). It is important to note that reactivation requires PTPA activity and not methylation, and LCMT1 methyltransferase activity cannot reactivate the inactive enzyme form (Longin, Jordens et al. 2004).

Although the study of PP2A interacting proteins has been done in many different experiment settings, the activation and assembly of PP2A seems to occur in a tightly controlled stepwise manner which involves at least five major PP2A regulators/interacting proteins: α4, the A subunit, PTPA, PME-1, and LCMT1 (Figure 1.5). While α4 binds to the monomeric newly synthesized and inactive C subunit, the A subunit should compete with α4 to be able to act as the scaffold protein for the PP2A holoenzyme (Kong, Ditsworth et al. 2009). PME-1 has a dual regulatory function: 1) it binds to and stabilizes the inactive PP2A C, and 2) prevents premature methylation through its methylesterase activity (Ogris, Du et al. 1999). PTPA then activates the inactive PP2A C subunit (either with α4 or A subunit) potentially by a prolyl cis/trans isomerase activity (Longin, Jordens et al. 2004). Finally, LCMT1 carboxymethylates the activated PP2A C which can facilitate binding to B subunits (Sents, Ivanova et al. 2013).

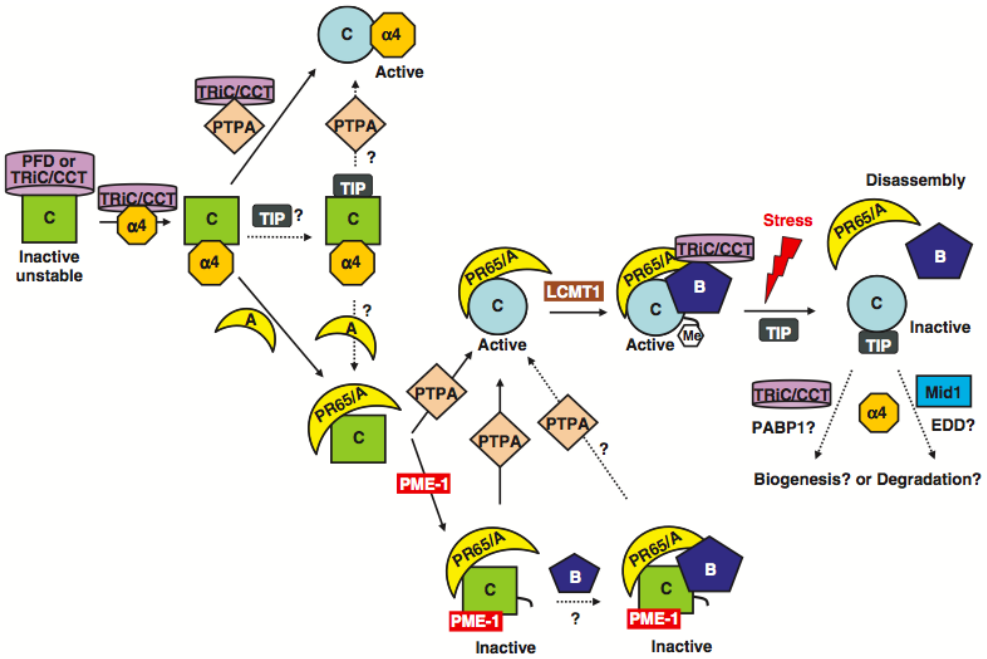

Figure 1.5: The PP2A biogenesis occurs in a tightly controlled stepwise manner (Sents, Ivanova et al. 2013).

Regulation by phosphorylation

Tyrosine (Tyr) and Threonine (Thr) residues can also become phosphorylated in the C subunit, which is associated with PP2A inactivation. A known mechanism for this modification occurs on Tyr307 in response to activation of various receptor and non-receptor kinases such as Lyn, Fyn, Jak2, Src (Lechward, Awotunde et al. 2001). This temporary inactivation of phosphatases is thought to allow the signals to pass from the receptors to cellular effectors at the beginning of the signal transduction pathway. Thr304 phosphorylation occurs through an 'auto-

phosphorylation-activated protein kinase' which is less understood (Guo and Damuni 1993; Guo, Reddy et al. 1993; Janssens, Longin et al. 2008). Mutational studies have shown that Thr304 phosphorylation might specifically inhibit holoenzyme assembly with the B55 subunits, whereas Tyr307 phosphorylation might selectively affect interaction with particular B56 subunits (Sents, Ivanova et al. 2013). In all these cases, dephosphorylation can occur through PP2A auto-dephosphorylation mechanisms (Janssens, Longin et al. 2008). It still remains unclear how exactly these modifications change the dynamic of holoenzyme assembly under physiological conditions.

PP2A's Regulation of Cellular Processes

The role of PP2A in normal cell growth

PP2A regulates many cellular processes involved in stem cell self-renewal and differentiation, cell cycle and proliferation, cell survival, and apoptosis.

There are new evidence suggesting that PP2A plays an important role in regulating human embryonic stem cells (hESC), where both the A and C subunit levels and PP2A activity gradually increase during the course of hESC differentiation (Yoon, Jun et al. 2010). It has been shown that overexpression of PP2A C promotes hESC differentiation whereas inhibition of PP2A with Okadaic Acid (OA) maintains hESC self-renewal in

the absence of basic fibroblast growth factor (bFGF) that is normally needed for hESC maintenance in culture (Yoon, Jun et al. 2010). PP2A regulates many key factors that are important for stem cell self-renewal including PI3K/Akt, Wnt/β-catenin, and MYC. It was demonstrated that PP2A activation resulted in a decrease in phosphorylation of Akt, GSK-3β, and MYC, and decreased expression of the transcription factors Oct-4, Sox2, and MYC (Yoon, Jun et al. 2010)- which were shown to be required for the generation of induced pluripotent stem cells iPSC (Takahashi, Tanabe et al. 2007). OA treatment on the other hand, almost completely blocked the interaction of PP2A with Akt, GSK-3β, and MYC, and the enhanced expression of Oct-4, Sox2, Akt, MYC, and the inactive phosphorylated form of GSK-3β (Yoon, Jun et al. 2010). In another study using Drosophila PP2A, PP2A inhibited self-renewal of neuroblasts primarily by regulating asymmetric localization and activation of Numb; and neuroblasts expressing PP2A mutants had neural stem cell over growth (Wang, Chang et al. 2009). A new study has shown a role for PP2A-B55α in stimulating ESCs to differentiate into FLK1+ cardiovascular progenitors (Honarpour, Rose et al. 2014). Given that PP2A regulates many proteins involved in stem cell self-renewal and maintenance, further research is needed to address the function of specific PP2A complexes in regulating stem cells.

PP2A controls proliferation, growth, and cell survival by regulating cellular pathways. PP2A-B55 inhibition was shown to be an important step

in mitosis where failure to inhibit PP2A-B55, especially B55δ, causes a cell cycle arrest in G2 phase (Mochida, Ikeo et al. 2009; Hunt 2013). Greatwall inactivates PP2A-B55 at mitotic entry to promote the phosphorylation of cyclin B–Cdk1 substrates until mitotic exit (Mochida, Ikeo et al. 2009; Hunt 2013; Wang, Galan et al. 2013). The B56 family members are also present throughout mitosis; through interaction with Shugoshin (Sgo1) proteins, they have been implicated in the maintenance of sister chromatid cohesion. They also ensure proper chromosome congression by antagonizing Aurora B kinase activity and modulating BUBR1 at the kinetochore to establish stable kinetochore–microtubule attachment at the metaphase plate (Funabiki and Wynne 2013). Moreover, *in vitro* studies have shown that B'' overexpression results in cell cycle arrest at the G1 phase by positively regulating the tumor suppressor retinoblastoma (RB) and cell division control 6 (Cdc6) proteins that are both crucial for the G1/S transition (Yan, Fedorov et al. 2000; Davis, Yan et al. 2008; Magenta, Fasanaro et al. 2008; Eichhorn, Creyghton et al. 2009; Wlodarchak, Guo et al. 2013).

Signaling pathways regulated by PP2A

PP2A regulates various cell signaling proteins including MAPK, MYC, PI3K/Akt, Wnt/β-catenin, p53, and Bcl-2, which are important for proliferation, growth and survival/apoptosis (Figure 1.6). The role of PP2A

in regulating these signaling pathways are discussed below and summarized in table 1.1.

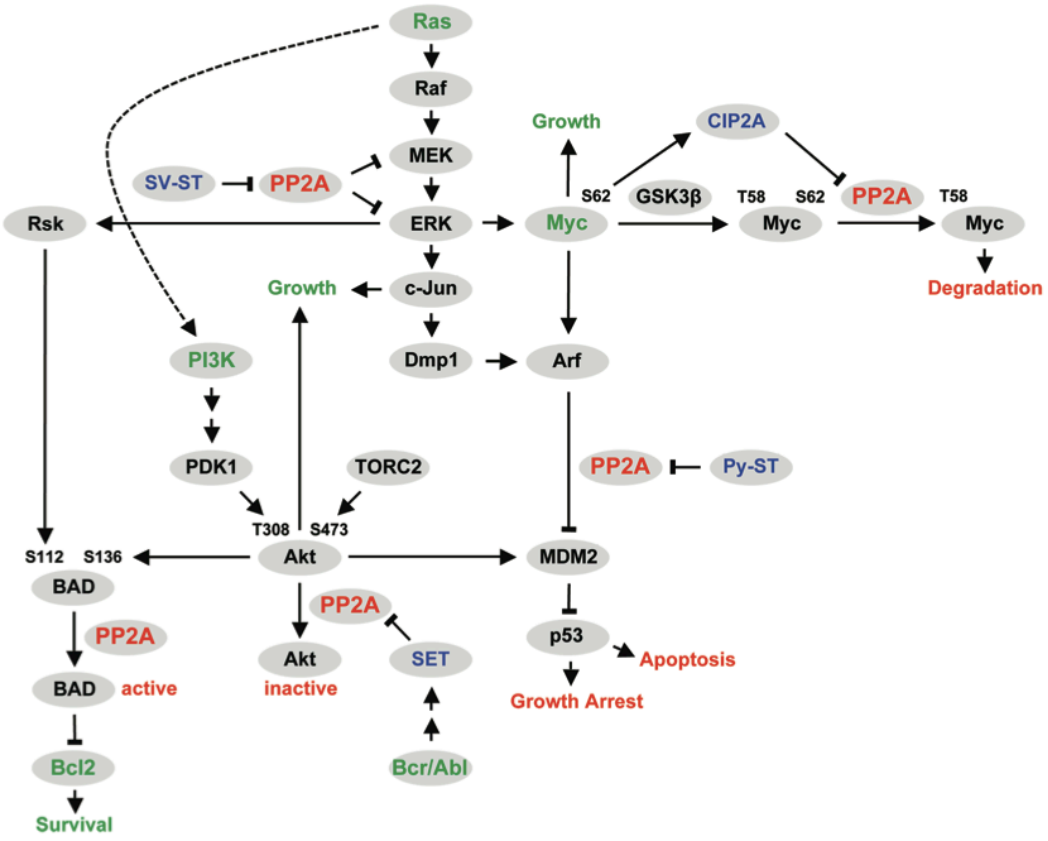

Figure 1.6: PP2A regulates various cell signaling pathways that are important for proliferation, growth, survival and apoptosis (Walter and Ruediger 2012).

❖ MAPK Signaling

Oncogenic Ras plays an important role in cancer development and activates the Raf-MEK-ERK kinase cascade. PP2A can either inhibit or activate the MAPK signaling and this is context dependent (Figure 1.7).

B55α or B55δ were shown to positively regulate MAPK signaling by dephosphorylating the inhibitory phosphorylation site on Raf-1 and Kinase Suppressor of RAS (KSR1), but B55γ functioned as a negative modulator of c-SRC activity and inhibited MAPK signaling (Ory, Zhou et al. 2003; Adams, Coffee et al. 2005; Eichhorn, Creyghton et al. 2007; Eichhorn, Creyghton et al. 2009). PP2A also inhibits the Raf-MEK-ERK kinase pathway by dephosphorylating MEK (B subunit not known) and ERK, and downstream effectors such as MYC (Arnold and Sears 2006; Eichhorn, Creyghton et al. 2009). B56β and B56γ were both shown to directly dephosphorylate ERK and decrease its activity (Letourneux, Rocher et al. 2006; Eichhorn, Creyghton et al. 2009; Kawahara, Maenaka et al. 2013). MYC protein stability is regulated by B56α where PP2-B56α dephosphoryates the phosphorylation of Ser62, which is important for increasing MYC stability and transcriptional activity (Figure 1.2) (Arnold and Sears 2006; Farrell, Pelz et al. 2013).

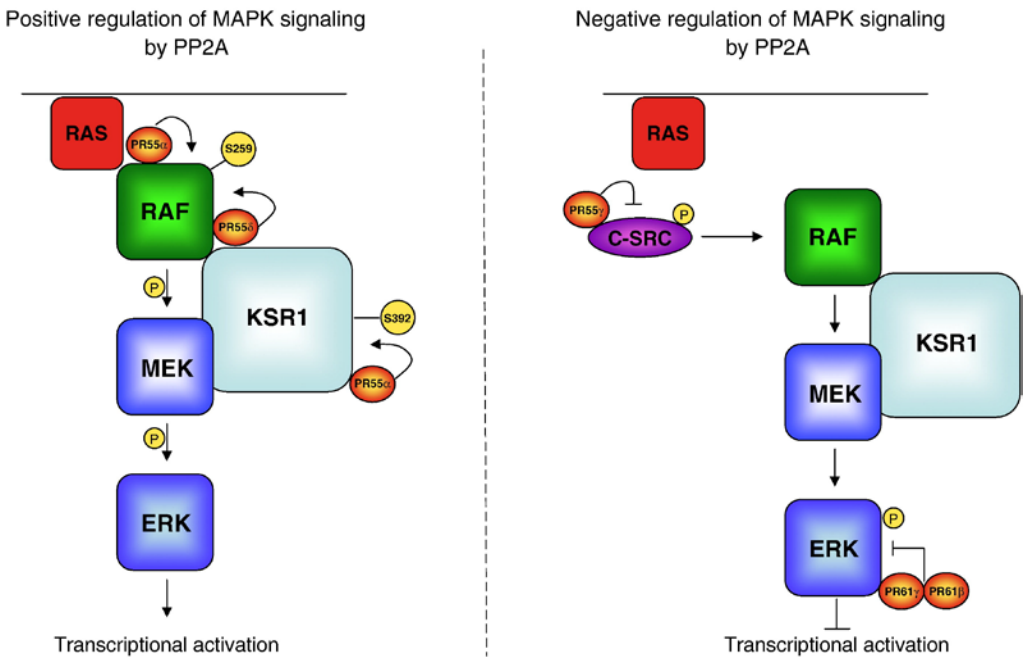

Figure 1.7: PP2A can be both inhibit or activate the MAPK signaling by interacting with different regulatory subunits (Eichhorn, Creyghton et al. 2009).

❖ PI3K/Akt Signaling

PI3K/Akt plays an important role in cell growth, proliferation, and apoptosis and is one of the most frequently activated pathways in cancer. Akt is activated by phosphorylation at Thr308 and Ser473, and PP2A acts as a negative regulator of the Akt pathway by promoting the dephosphorylation of these sites. The PP2A-B55α holoenzyme has been shown to directly interact with Thr308 and dephosphorylate it (Eichhorn, Creyghton et al. 2009; Walter and Ruediger 2012). Knockdown of B56γ resulted in elevated levels of Ser473 phosphorylation, although it remains unclear whether Akt is a direct target of B56γ (Sablina, Hector et al. 2010).

❖ Wnt Signaling

The WNT signaling cascade is required for proper embryogenesis and development, and deregulation of this pathway leads to uncontrolled cellular proliferation and tumorigenesis. PP2A also plays a major role in the Wnt/β-catenin signaling pathways (Figure 1.8). A mouse model of B56γ knockout showed suppressed β-catenin expression providing evidence for genetic association of B56γ with Wnt signaling (Everett, Kamibayashi et al. 2002). It has been shown that PP2A (B56α, B56β, B56γ, PR72) plays a negative role in Wnt signaling by associating with a number of Wnt signaling components such as APC, Axin, and Naked (Everett, Kamibayashi et al. 2002; Creyghton, Roel et al. 2005). B56α, B56β, and B56γ were all shown to interact with Axin and inhibit Wnt signaling during vertebrate development (Seeling, Miller et al. 1999). B56γ inhibited formation of APC–Axin complexes leading to destabilization of β-catenin (Everett, Kamibayashi et al. 2002; Baek and Seeling 2007; Eichhorn, Creyghton et al. 2009). PR72 can negatively regulate Wnt signaling through its interaction with Naked, the Wnt antagonist (Creyghton, Roel et al. 2005; Eichhorn, Creyghton et al. 2009). However, PP2A can also stabilize β-catenin directly by forming a complex with E-cadherin and β-catenin at the plasma membrane, which then results in stabilization of both proteins (Gotz, Probst et al. 2000). B55, B56ε, and PR130 subunits were shown to be positive regulators of Wnt signaling (Yang, Wu et al. 2003; Creyghton, Roel et al. 2006; Eichhorn, Creyghton

et al. 2009). All together, these findings imply that different PP2A complexes may have opposing outcomes on pathways in cell. Whether this is dependent on cell-type, cell cycle, or differentiation stage remains unknown. Interestingly, in studying the role of PP2A on Wnt signaling, one study showed that B56α was moderately expressed before the mid-blastula transition (MBT) in *Xenopus laevis,* reduced during gastrulation and neurulation, and again increased during organogenesis, while B56γ was expressed at low levels until organogenesis (Baek and Seeling 2007). B56α was enriched in the ventral hemisphere pre-MBT, while B56γ was ventrally enriched post-MBT (Baek and Seeling 2007). More studies are needed to better understand the spatiotemporal activity of different PP2A complexes on signaling pathways in different cells and conditions.

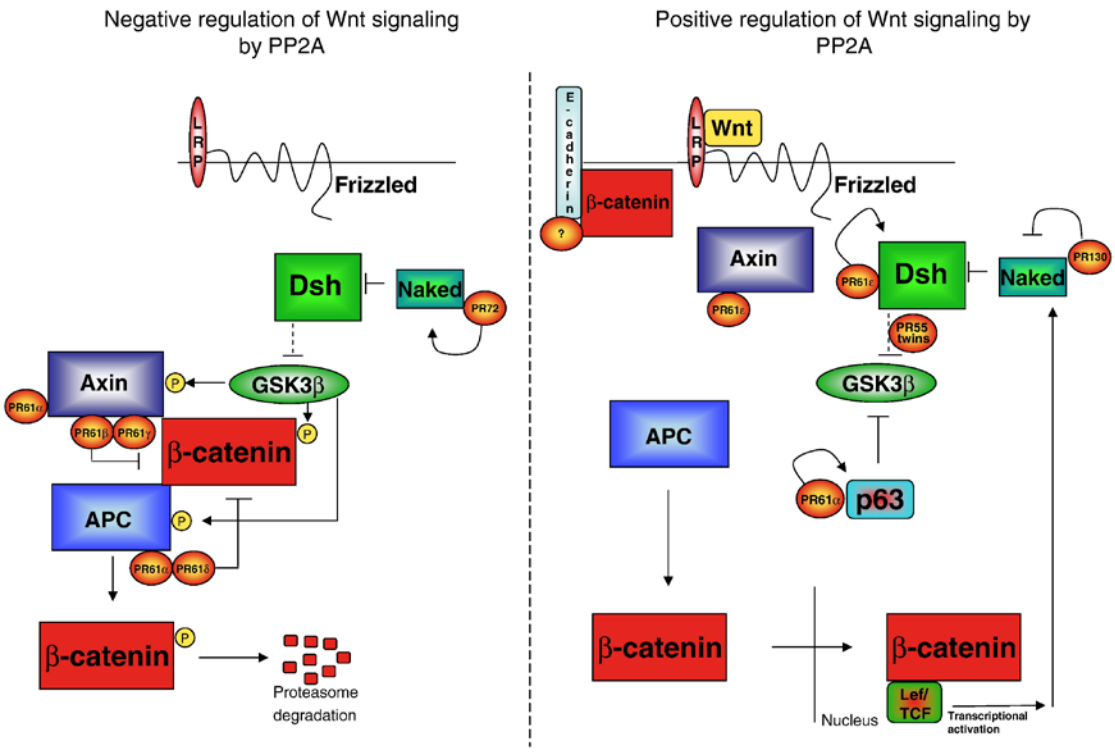

Figure 1.8: PP2A can inhibit or activate the Wnt signaling by interacting with different regulatory subunits (Eichhorn, Creyghton et al. 2009).

❖ p53

The tumor suppressor p53 plays an important role in regulating various types of stress such as DNA damage, by inducing growth arrest or apoptosis. PP2A (B56γ) has been shown to directly dephosphorylate p53 at Thr55 to increase its stabilization, which leads to increased apoptosis following DNA damage (Li, Cai et al. 2007). PP2A also controls the MDMD2-p53 feedback loop following p53 activation and therefore acts as a negative regulator of p53. B56α or B56β have been shown to form a complex with cyclin G, a p53 target, and dephosphorylate MDM2 at

Thr216 and therefore activate MDM2, which leads to ubiquitination and enhanced degradation of p53 (Harris and Levine 2005).

- Bcl-2

The majority of studies suggest that PP2A plays a pro-apoptotic role in cells. Three key components of programmed cell death, the pro-apoptotic BAD and BAX and anti-apoptotic Bcl-2, are also regulated by PP2A. B56α co-localizes with Bcl-2 at the mitochondrial membrane and suppresses its pro-survival activity (Ruvolo, Deng et al. 2001; Arnold and Sears 2008). PP2A can also activate BAD but the specific holoenzyme complex involved has of yet to be determined (Arnold and Sears 2008). Interestingly it was also shown that nicotine induces proliferation and anti-apoptosis effects through activation of Akt, phosphorylates and inactivates the pro-apoptotic activity of BAX, and that this was inhibited by PP2A (C subunit) overexpression; PP2A (no known subunit) dephosphorylates BAX by direct interaction and activates its pro-apoptotic function (Xin and Deng 2006).

Overall these findings indicate that PP2A is involved in fundamental cellular processes such as proliferation, development, and survival; therefore it is important to understand the individual role of different B subunits in different cell types and contexts. This will aid in identification of critical subunits that are aberrantly regulated during different

pathogenesis, and may ultimately lead to the development of novel therapeutic agents for various diseases.

Table 1.1. PP2A regulatory subunits involved in cell signaling

PP2A interaction protein	
	old and Sears 2008)
BAX	

The role of PP2A in cell transformation

PP2A was first suggested to act as a tumor suppressor based on the fact that Okadaic acid inhibited the PP2A C subunit, promoted cell transformation, and gave rise to tumors (Eichhorn, Creyghton et al. 2009). Transformation of rodent cells requires the co-expression of SV40 LT antigen, which inhibits p53 and pRB, and oncogenic H-Ras (Michalovitz, Fischer-Fantuzzi et al. 1987). In contrast, human cell transformation requires more components: LT, H-Ras, hTERT (human telomerase catalytic subunit), and SV 40 ST, which inhibits PP2A activity (Sablina and Hahn 2008). Transformation has been well studied in HEK-TER cells, in which primary fibroblast cells express SV40 LT, H-Ras, and hTERT and require the addition of SV40 ST to complete transformation (Hahn, Dessain et al. 2002; Sablina and Hahn 2008). Mutations in the domains of ST that are required for its inhibition of PP2A, abrogate its transforming activity in HEK-TER cells (Hahn, Dessain et al. 2002; Sablina and Hahn 2008), demonstrating that PP2A inhibition is an important step in transformation. Interestingly, a gene expression analysis has shown that ST regulates both PP2A-dependent and PP2A-independent genes, but the majority of the genes essential for cell transformation were PP2A-dependent; the PP2A-independent genes may enhance, but not be essential, for transformation (Moreno, Ramachandran et al. 2004).

To identify which PP2A complexes are involved in this process, a siRNA screen showed that B56α, B56γ, and PR72/PR130 were the only B

subunits found to be critical for regulating human cell transformation (Sablina, Hector et al. 2010). PP2A complexes containing these B subunits regulate MYC, Wnt, and PI3K/Akt signaling, respectively (Arnold and Sears 2006; Sablina, Hector et al. 2010).

Because of a broad range of PP2A functions in cells it is not surprising that various aspects of cellular function are affected by ST. ST promotes cell transformation by enhancing cell proliferation, upregulating anti-apoptotic pathways, and changing the cytoskeleton (Sablina and Hahn 2008). ST promotes transition from G1 to S phase by activating several pathways and transcription factors such as the MAPK cascade, which eventually upregulates AP-1 transcriptional activity, CREB, Sp-1, E2F and increases MYC's transcriptional activity by stabilizing it (Arnold and Sears 2006; Sablina and Hahn 2008). ST expression also upregulates anti-apoptotic targets of NF-κB, including *ALDH1, SERPINB2*, and *EPR-1* (survivin) as well as the PI3K/Akt pathway (Moreno, Ramachandran et al. 2004; Sablina and Hahn 2008). Moreover, ST induces dramatic F-actin rearrangement resulting in altered cytoskeleton through increasing Rac-1-induced membrane ruffling, Cdc42-induced filopodia formation, and loss of RhoA-dependent stress fibers (Sablina and Hahn 2008). It has also been shown that genes involved in adhesion and motility such as *SPP1* (osteopontin) and *PXN* (paxillin) are upregulated in cells expressing ST, whereas genes involved in cell-cell adhesion and junction such as *ICAM-*

1, VCAM-1, *JUP* (plakoglobin), and *CLDN11* (claudin 11) are down-regulated (Moreno, Ramachandran et al. 2004; Sablina and Hahn 2008).

PP2A's Contribution to Human Pathogenesis

PP2A in malignancies

Given the highly diverse cellular functions of PP2A, and that its inhibition is required for cell transformation, it is not surprising to find that PP2A activity is downregulated in human cancers. Loss of PP2A activity has been reported to occur through loss, mutation, or epigenetic regulation of the subunits and/or overexpression of the cellular inhibitors or interacting proteins. Mutation of the *PPP2R1A* (Aα) or *PPP2R1B* (Aβ) subunits of PP2A has been shown to occur at a low level in breast cancers (Calin, di Iasio et al. 2000; Sablina and Hahn 2008). Alterations in the A subunits that impair integration of the C and/or B subunits have been observed in primary breast cancers and lung carcinomas, and in melanoma cell lines (Calin, di Iasio et al. 2000; Ruteshouser, Ashworth et al. 2001; Esplin, Ramos et al. 2006; Marsh, Healey et al. 2007; Sablina and Hahn 2008). Reduced expression of Aα has also been reported in approximately 40% of both glioblastomas and oligodendrogliomas with little or no mutation in *PPP2R1A* or *PPP2R1B* (Colella, Ohgaki et al. 2001). Somatic mutations in *PPP2R1B* have been reported in 15% of

lung, 8–15% of colon, and 13% of breast cancers (Wang, Esplin et al. 1998; Sablina and Hahn 2008).

Many studies reported mutations in the *PPP2R2A (B55α)* subunit in luminal B breast cancers, somatic deletion in 67.1% of prostate tumors, and decreased expression of B55α in non-small cell carcinoma (Cheng, Liu et al. 2011; Curtis, Shah et al. 2012; Kalev, Simicek et al. 2012). In addition, *PPP2R2B* (B55β) was shown to be epigenetically inactivated by DNA hypermethylation in colorectal cancer (Tan, Lee et al. 2010) and B55γ was downregulated in human glioma cells and brain tumors (Fan, Chen et al. 2013).

Comparing the primary melanomas with their metastasized tumors showed that B56α was downregulated in the metastasized tumors (Mannava, Omilian et al. 2012). Moreover, another study discovered many mutations throughout the *PPP2R5C* (B56γ) gene in several cancers (melanoma, lung, etc.) that were classified into three categories: mutations that had "no effect", "loss of AC core interaction", and "loss of substrate interaction" (Nobumori, Shouse et al. 2013). Identifying these types of mutations in other subunits are important, as many mutations might have no association with any physiological functions.

In addition to alterations in specific PP2A subunits, endogenous inhibitors of PP2A, such as SET and CIP2A, have been shown to be upregulated in a variety of cancers. CIP2A is overexpressed in head and neck squamous cell carcinoma, colon cancer, and 39% of breast tumors

(Westermarck and Hahn 2008; Come, Laine et al. 2009). SET is overexpressed in malignant brain tumors, tumors of the head and neck region, testicular cancers and different types of hematological malignancies (Westermarck and Hahn 2008; Christensen, Chen et al. 2011; Agarwal, Mackenzie et al. 2014). Understanding how PP2A is inactivated in different tumors can provide a better stratification for therapeutic strategies developed to re-activate PP2A. In chapter 2, I discuss such a therapeutic strategy from our investigation into the interplay between MYC, PP2A, and SET or CIP2A to better understand the mechanisms that contribute to MYC overexpression in breast cancer. We report that increased SET or CIP2A expression occurs in breast cancer, associated with decreased PP2A activity and contributing to MYC oncogenic activity; and inhibiting either of them can be a potential therapeutic approach.

PP2A in neurodegenerative disorders

PP2A is not only deregulated in cancers: in the nervous system, the changes in PP2A activity are linked with neurodegenerative disorders, particularly Alzheimer's disease. Deregulated hyperphosphorylation of the microtubule-associated protein, Tau, is a major feature of this disease, which results in the Tau aggregation and neurofibrillar tangles and triggers neuronal death (Virshup and Shenolikar 2009). PP2A-B55α dephosphorylates Tau and regulates the activity of several Tau kinases

(Virshup and Shenolikar 2009). Defective B55α expression or decreased methylated PP2A (L309) has been linked to increased Tau phosphorylation in Alzheimer's disease (Zhou, Gustafsson et al. 2008; Virshup and Shenolikar 2009). New studies suggest that compromised PP2A activation can occur through increased SET and SET translocation to cytoplasm in the neurons of Alzheimer's disease brains (Chohan, Khatoon et al. 2006). This suggests a role for other PP2A interacting proteins in other disorders and provides the opportunity for application of PP2A activating or SET antagonist compounds for therapy.

PP2A in other disorders

Recently, Parkinson's disease has also been associated with abnormal hyperphosphorylation because of increased activity of leucine rich repeat kinase 2 (LRRK2) and increased phosphorylation of α-Synuclein (α-syn). Specifically, PP2A-B55α has been shown to reduce α-syn phosphorylation, which results in its reduced aggregation and neuronal preservation, and therefore behavioral improvements in an α-syn transgenic mouse model (Braithwaite, Voronkov et al. 2012).

Recent studies also suggest that PP2A becomes hyperactivated in liver, muscle, retina, and the pancreatic islet as a result of glucolipotoxicity and diabetes, raising the potential therapeutic benefits of PP2A inhibiting

compound in diabetes and/or other disorders of metabolism (Kowluru and Matti 2012). More studies are required to uncover these functions.

Pharmacological Modulation of PP2A

Recently, multiple pharmacological modulators of PP2A have been shown to have anti-tumor activity both *in vitro* and *in vivo.* Some of these compounds have PP2A activating functions while others are inhibitors of PP2A (Figure 1.9). Ceramide, FTY720, OP449, and Forksalin are among the PP2A activating compounds that have been tested in many models of cancer. On the other hand, various natural compounds have been identified that inhibit PP2A function such as Okadaic acid, norcantharidin, and Fostriecin.

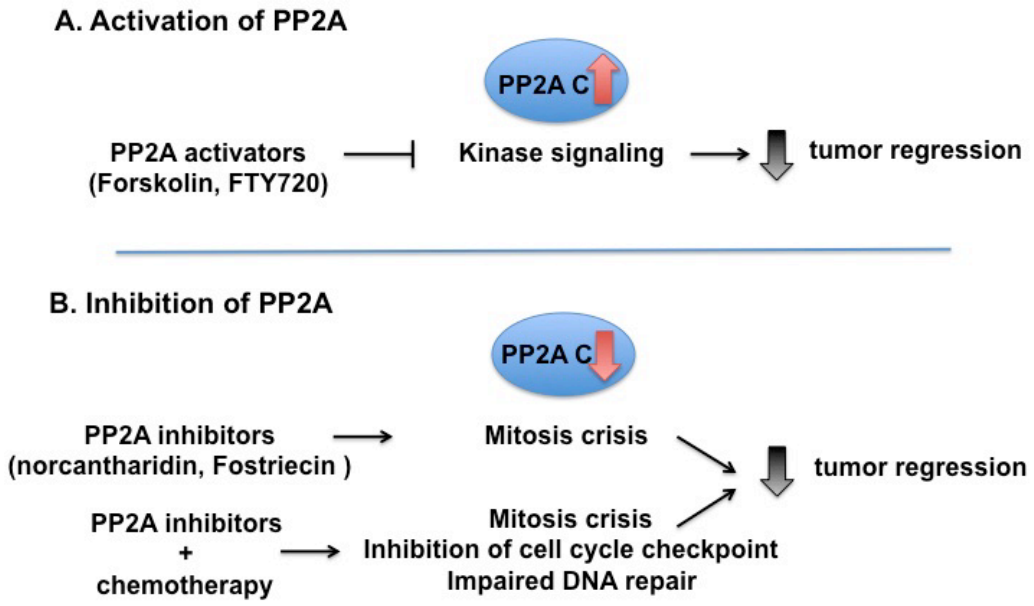

Figure 1.9. Protein Phosphatase 2A as a potential target for anticancer therapy.

❖ Ceramide

Ceramide is a product of sphingolipid metabolism that is found ubiquitously in eukaryotic cell membrane and is activated in response to inflammation, cell stress, and apoptosis (Lin, Chen et al. 2006). Many studies have shown a pro-apoptotic role for Ceramide in pathology of apoptosis-based diseases where many kinases (p38 MAPK and JNK) and phosphatases such as PP2A become active (Lin, Chen et al. 2006). It is also defined as a tumor suppressor lipid and many studies have shown great interests for its therapeutical implications (Lin, Chen et al. 2006). Ceramide was shown to interact with SET, release PP2A from SET inhibition, and therefore affect MYC phosphorylation and degradation (Mukhopadhyay, Saddoughi et al. 2009). Similar to Ceramide, other

sphingoids, such as sphingosine and its derivatives also act as allosteric PP2A activators *in vitro* (Chalfant, Szulc et al. 2004).

- FTY720

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3- diol; Fingolimod, Novartis), is a synthetic sphingosine analogue and known immunosuppressant that has been FDA approved for treatment of patients with refractory multiple sclerosis. FTY720 was shown to exert anti-tumor function in CML cancer, GIST, and lung (Neviani, Santhanam et al. 2007; Saddoughi, Gencer et al. 2013). FTY720 directly binds SET, reactivates PP2A, and therefore suppresses lung tumor cell growth and induces cell death by necroptosis (Saddoughi, Gencer et al. 2013).

- OP449

OP449 (COG449), an ApoE mimetic peptide, was designed to interfere with SET, and release PP2A from inhibition by SET (Christensen, Chen et al. 2011). OP449 was shown to have anti-proliferative effects in models of glioma, CLL, AML, CML, and pancreatic cancers and decrease MYC, ERK, NFκB, and Rac-1 (Christensen, Chen et al. 2011; Switzer, Cheng et al. 2011; Agarwal, Mackenzie et al. 2014; Farrell, Allen-Petersen et al. 2014).

- Forskolin

Forskolin, is a chemical activator of PP2A, was first used to induce adenylate cyclase activation, and therefore increase cAMP levels and upregulate protein kinase A (PKA) (Kalev and Sablina 2011). Later, 1,9-dideoxyforskolin, a Forskolin derivative that does not affect cAMP levels, showed that this PP2A activation (independent of cAMP) could inhibit growth and induce apoptosis in myeloid and lymphoid leukemic cells (Neviani, Santhanam et al. 2005). The mechanism of PP2A activation by Forskolin is not understood well.

PP2A inhibition has been suggested as an alternative strategy for treating cancer. The rationales for using PP2A inhibitors are: 1) PP2A inhibition in cancer cells induces catastrophe and cell death as a result of improper cell cycle progression and accelerates checkpoint activation (Kalev and Sablina 2011); 2) because of PP2A's role in DNA damage and repair pathway, combination of PP2A inhibition with a DNA damaging agent or DNA repair inhibitor can sensitize cancer cells to chemotherapy; and 3) in actively dividing cells, PP2A inhibition associates with increased toxicity which can be a strategy for cancer therapy.

There are several natural compounds that inhibit the catalytic subunits of different serine/threonine phosphatases such as Okadaic acid (OA), norcantharidin, and Fostriecin. Due to the specific "hydrophobic cage" within the binding pocket of PP2A C, OA shows a higher affinity for PP2A compared to other phosphatases (Kalev and Sablina 2011).

- Cantharidin

Another natural PP2A (and PP1) inhibitor Cantharidin, has been used for a long time as an anti-cancer agent by Chinese for the treatment of hepatoma and oesophageal carcinoma. However, due to high toxicity, Cantharidin analogues have been produced such as Norcantharidin and LB1.2. Specifically LB1.2. was designed to inhibit PP2A vs. PP1 and has less toxicity (Lu, Kovach et al. 2009; Kalev and Sablina 2011). In one study, inhibition of PP2A by LB.1.2 in combination with temozolomide, blocked cell cycle arrest and increased cell cycle progression, which causes marked glioblastoma xenograft regression (Lu, Kovach et al. 2009).

- Fostriecin

Fostriecin, another PP2A inhibitor, was shown to have less toxicity compared to some other PP2A inhibitors and anti-tumor function. Fostriecin suppresses tumor growth by inducing premature cell cycle entry and therefore results in cell death (Kalev and Sablina 2011). However, because of short half-life in plasma measured during a phase I clinical trial in 20 patients, it showed no effects on tumor growth (Kalev and Sablina 2011).

Statement of Thesis

Given that MYC overexpression can happen through increased protein stability and impaired protein degradation, and that despite the important role of PP2A in cell transformation, most of PP2A inhibition assays for cell transformation is done *in vitro*, the overall objective of this thesis is to test the hypothesis that PP2A (-B56α) activity is important for normal cell homeostasis through controlling the activity of downstream targets such as MYC and suppression of PP2A, either through loss of the tumor suppressor B subunits or overexpression of cellular inhibitors such as SET and CIP2A, results in cell transformation.

To test this hypothesis I have investigated the mechanisms of PP2A inhibition in cancer (chapter 2) and also generated a mouse model of PP2A-B56α knockout (chapter 3).

In chapter 2, I test how two cellular inhibitors of PP2A, SET and CIP2A, are overexpressed in human breast cancer and how inhibiting either of these proteins decreases the growth of breast cancer cells *in vitro* and *in vivo.* I also test the therapeutic potential of SET antagonist, OP449, on MYC activity in breast cancer cell lines and the outcome of treatment with this compound.

In chapter 3, I characterize a novel mouse model of PP2A-B56α knockout and identify the physiological effect of B56α loss.

Together this thesis identifies the role of PP2A in cancer pathogenesis and a broad application of PP2A as a novel therapeutic target.

Chapter Two

Targeting c-MYC by Antagonizing PP2A Inhibitors in Breast Cancer

(Janghorban et al. 2014, PNAS)

Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer

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Abbreviations: PP2A, protein phosphatase 2A; CIP2A, Cancerous Inhibitor of PP2A

Running title: Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer

A detailed list of contributors is described in the “Appendices” section.

Abstract

The c-MYC oncoprotein is stabilized and activated by phosphorylation at Serine 62 (S62) in breast cancer. Protein phosphatase 2A (PP2A) is a critical negative regulator of c-MYC through its ability to dephosphorylate S62. By inactivating c-MYC and other key signaling pathways, PP2A plays an important tumor suppressor function. Two endogenous inhibitors of PP2A, the SET oncoprotein (I2PP2A, Inhibitor-2 of PP2A) and CIP2A (Cancerous Inhibitor of PP2A), inactivate PP2A and are overexpressed in several tumor types. Here I show that SET is overexpressed in about 50-60% and CIP2A in about 90% of breast cancers. Knockdown of SET or CIP2A reduces the tumorigenic potential of breast cancer cell lines both *in vitro* and *in vivo*. Treatment of breast cancer cells *in vitro* or *in vivo* with OP449, a novel SET antagonist, also decreases the tumorigenic potential of breast cancer cells and induces apoptosis. I show that this is, at least in part, due to decreased S62 phosphorylation of c-MYC and reduced c-MYC activity and target gene expression. Because of the ubiquitous expression and tumor suppressor activity of PP2A in cells, as well as the critical role of c-MYC in human cancer, we propose that activation of PP2A (here accomplished through antagonizing endogenous inhibitors) could be a novel anti-tumor strategy to post-translationally target c-MYC in breast cancer.

Significance Statement

Increased kinase activity and suppressed phosphatase activity are hallmarks of oncogenic signaling. The c-MYC transcription factor, a master driver of human cancer, is stabilized and activated by persistent Serine 62 phosphorylation. The tumor suppressor Protein Phosphatase 2A (PP2A) targets this site and negatively regulates c-MYC. Here, we show that two cellular inhibitors of PP2A, SET and CIP2A, are overexpressed in breast cancer, and depletion or inhibition of SET or CIP2A reduces c-MYC expression and activity, and decreases the tumorigenic potential of breast cancer cells. These findings strongly suggest that inhibiting SET or CIP2A in order to reactivate PP2A may be an effective therapeutic strategy for targeting c-MYC in breast cancer.

Introduction

The c-MYC (MYC) oncoprotein is overexpressed in human breast cancer and this is associated with poor clinical outcome (Chrzan, Skokowski et al. 2001; Wolfer, Wittner et al. 2010). Expression of MYC is regulated at multiple levels, including protein stability, which is increased in several cancer types (Chrzan, Skokowski et al. 2001; Malempati, Tibbitts et al. 2006; Zhang, Farrell et al. 2012). MYC stability is regulated in part by sequential and interdependent phosphorylation at two conserved residues, Threonine 58 (T58) and Serine 62 (S62) (Sears, Nuckolls et al. 2000). MYC is phosphorylated at S62 (pS62) through the Ras/Raf/MEK/ERK pathway or CDK activation in response to growth signals and this modification increases its stability and oncogenic activity (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004; Hann 2006; Wang, Cunningham et al. 2011). When growth signals cease, GSK3, in a manner dependent upon prior phosphorylation at S62, phosphorylates T58 (pT58) (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). T58 phosphorylation facilitates PP2A-mediated dephosphorylation of pS62 and recruitment of the E3 ubiquitin ligase SCF^{Fbw7} to initiate proteasomal destruction of MYC (Arnold and Sears 2006; Arnold and Sears 2008). This process is facilitated by AXIN1, which helps nucleate a destruction complex for MYC at target gene promoters (Arnold, Zhang et al. 2009; Farrell, Pelz et al. 2013). Our previous work has shown that MYC stability

is increased in breast cancers and that this correlates with high pS62- and low pT58-MYC (Zhang, Farrell et al. 2012).

PP2A is a ubiquitously expressed, heterotrimeric Serine-Threonine (S/T) phosphatase that mediates 30-50% of cellular S/T phosphatase activity (Westermarck and Hahn 2008). Target specificity of PP2A is directed by a variable regulatory (B) subunit, and we have shown that B56α is the isoform that directs PP2A to MYC (Arnold and Sears 2006; Westermarck and Hahn 2008). Human cell transformation requires inhibition of PP2A activity and, in an siRNA screen, B56α, B56γ, and PR72/PR130 were the only B subunits shown to be critical for regulating human cell transformation (Sablina, Hector et al. 2010). PP2A complexes containing these B subunits regulate MYC, Wnt, and PI3K/Akt signaling, respectively (Arnold and Sears 2006; Sablina, Hector et al. 2010).

While loss of PP2A activity is critical for tumor growth, mutations in PP2A subunits are very rare in breast cancers (Calin, di Iasio et al. 2000; Sablina and Hahn 2008). Alterations in the A subunit that impair integration of the C and/or B subunits have only been observed in breast cancers at a low frequency (Calin, di Iasio et al. 2000; Esplin, Ramos et al. 2006; Marsh, Healey et al. 2007; Sablina and Hahn 2008), suggesting that other mechanisms can affect PP2A activity and, subsequently, MYC protein levels. Indeed, endogenous inhibitors of PP2A such as SET and CIP2A have been shown to be upregulated in a variety of cancers (Westermarck and Hahn 2008; Come, Laine et al. 2009). CIP2A is

overexpressed in head and neck squamous cell carcinoma, colon cancer, and 39% of breast tumors (Westermarck and Hahn 2008; Come, Laine et al. 2009). CIP2A interacts directly with the N-terminus of pS62-MYC and impairs its degradation by inhibiting PP2A-B56α activity (Junttila, Puustinen et al. 2007). SET binds to at least the C subunit of PP2A and inhibits its activity (Li, Guo et al. 1995). SET is overexpressed in malignant brain tumors, tumors of the head and neck region, testicular cancers, and different types of hematological malignancies (Westermarck and Hahn 2008; Christensen, Chen et al. 2011; Agarwal, Mackenzie et al. 2014), but whether its expression is altered in breast cancer has not been reported.

Here I show that SET and CIP2A are upregulated in breast tumors at both the mRNA and protein levels, and that knockdown of either SET or CIP2A decreases the tumorigenic potential of breast cancer cells *in vitro* and *in vivo*. SET inhibition with OP449, a SET antagonist (Christensen, Chen et al. 2011), also reduces growth and tumorigenic potential of these cells *in vitro* and *in vivo*, and induces apoptosis. SET inhibition, either by RNA interference or OP449, decreases pS62-MYC levels and MYC transcriptional activity. Overall, our study suggests that activating PP2A by inhibiting SET and/or CIP2A may be an important therapeutic strategy that post-translationally targets MYC in breast cancer.

Results

SET and CIP2A are Frequently Overexpressed in Human Breast Cancer

Previously, we have shown that pS62-MYC, a more stable and active form of MYC, is highly expressed in breast cancers (Wang, Cunningham et al. 2011; Zhang, Farrell et al. 2012). In addition, we found that expression of AXIN1, which nucleates the MYC degradation complex, is decreased in some breast tumors (Zhang, Farrell et al. 2012). To investigate additional mechanisms that lead to MYC overexpression, I focused on SET and CIP2A, two oncogenic cellular inhibitors of PP2A, the phosphatase that removes S62 phosphorylation to destabilize MYC. First, I interrogated the expression of SET, CIP2A, and MYC in multiple breast cancer cell lines by qRT-PCR. I found that SET and CIP2A mRNA levels were high in breast cancer cells compared to the immortalized but non-transformed MCF10A cell line (Fig. 2.1A). To confirm this in primary tumor samples, we performed qPCR for SET and CIP2A levels in a cDNA Array of 44 breast tumors and 4 normal samples. We found that SET was overexpressed in about 60% and CIP2A in about 90%, of these tumors (Fig. 2.1B). Similar results were observed in RNA-seq data from breast cancer cell lines where SET and CIP2A expression was elevated in about 50% of cell lines (Fig. S2.1A). In both of these experiments, when tumors were grouped by tumor subtype, we found that high SET expression occurred across all tumor types while high CIP2A expression was

enriched in triple negative tumors on the cDNA array (Fig. 2.1B) and in basal and Claudin-low subtypes in the cell lines, molecularly classified using PAM50 (Fig. S2.1A) (Heiser, Sadanandam et al. 2012). Finally, I measured SET mRNA expression by qPCR in a limited set of primary human breast tumor samples with patient matched adjacent normal tissue and found that 60% of samples showed higher expression of SET in tumor as compared to the matched normal (Fig. S2.1B). Together these data demonstrate that SET is overexpressed in about 50-60% and CIP2A in about 90% of breast cancers and CIP2A overexpression associates with triple negative, basal, and Claudin-low tumor subtypes.

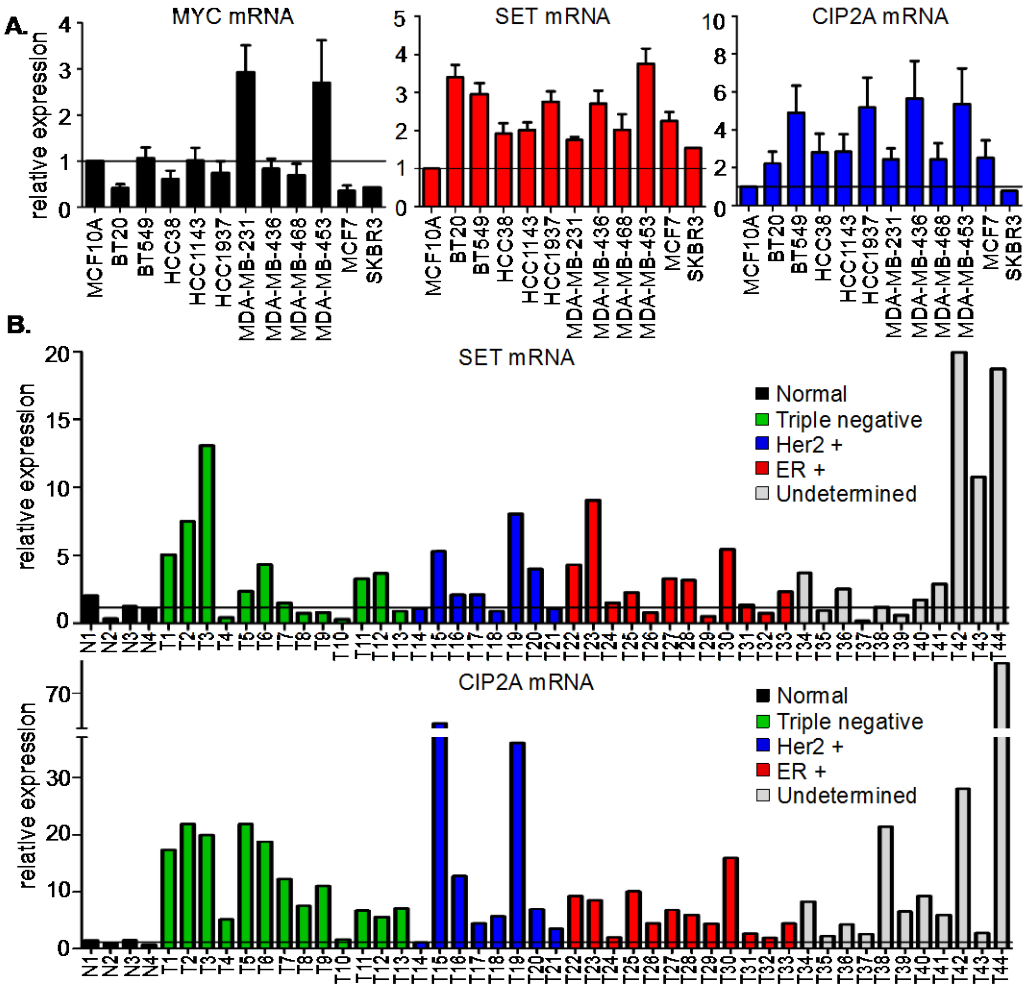

Figure 2.1. SET and CIP2A are frequently overexpressed in human breast cancer. (A) qRT-PCR analysis of MYC, SET, and CIP2A mRNA expression in 12 breast cell lines grown in 0.1% serum. Relative expression is calculated by ΔCT normalized to MCF10A. (B) qPCR analysis of SET and CIP2A expression in 44 breast tumors and 4 normal samples grouped by histologic subtypes, obtained from TissueScan Breast Cancer and Normal Tissue cDNA Array (Array 4).

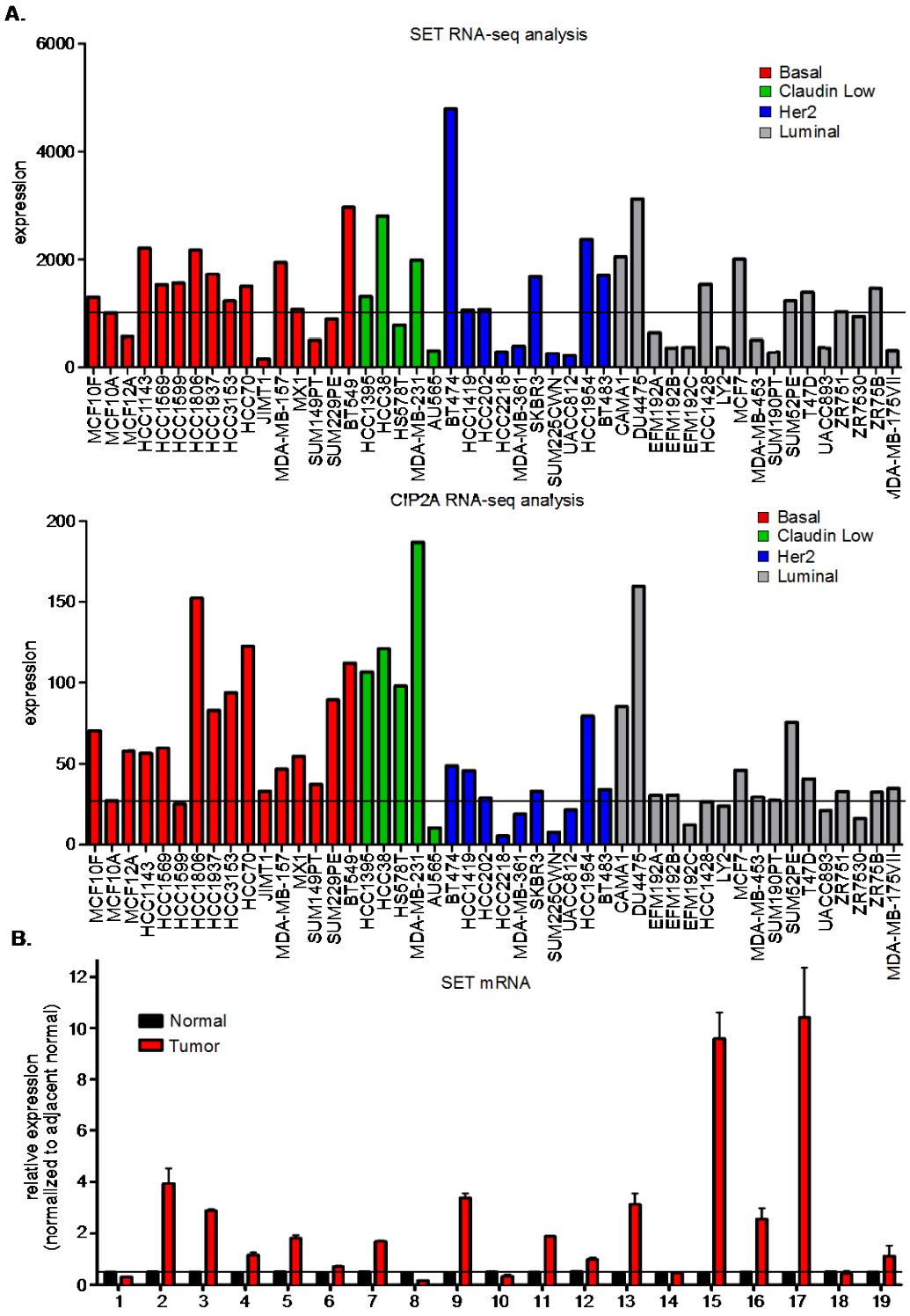

Figure S2.1. SET and CIP2A are frequently overexpressed in human breast cancers. (A) SET and CIP2A expression from RNA-seq

(GSE48216) in breast cancer cell lines grouped by their subtypes using PAM50 (Heiser, Sadanandam et al. 2012). Cells were grown in complete serum media. (B) qPCR analysis of SET expression, normalized to ACTIN expression, in 19 breast tumor samples relative to their patient matched normal samples. The error bars represent SD from the triplicate qRT-PCR reaction. Samples have been previously described (Zhang, Farrell et al. 2012).

Increased SET, CIP2A, and pS62-MYC Protein Levels Occur in Human Breast Cancer

I next measured SET, CIP2A, MYC, and pS62-MYC protein levels in a panel of breast cancer cell lines. As shown in Figure 2.2A, compared to immortalized non-transformed MCF10A cells, many of the breast cancer cell lines showed higher expression of these proteins (quantified in Fig. 2.2A). In addition, increased pS62-MYC positively correlated with increased SET and CIP2A expression (Fig. S2.2A). To extend this observation to human breast tumors, I performed immunofluorescence to assess SET, CIP2A, pS62-MYC, and pT58-MYC levels in breast tumors and compared to patient matched adjacent normal tissue. I found that SET, CIP2A, and pS62-MYC levels were higher in tumors whereas pT58-MYC, which would be associated with increased PP2A activity, was lower (Fig. 2.2B). Together, these data show that SET, CIP2A, and pS62-MYC proteins are commonly overexpressed in human breast cancers.

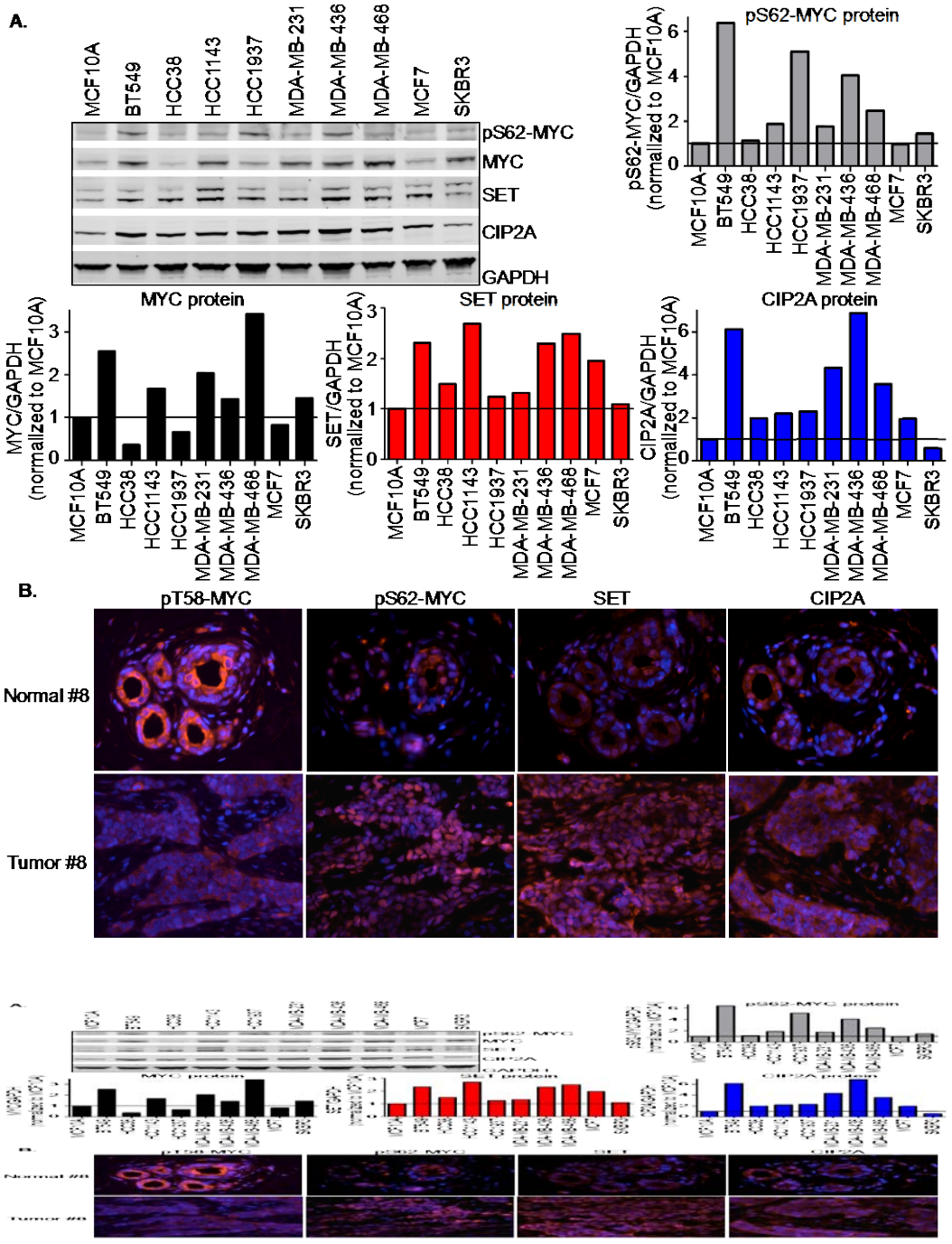

Figure 2.2. Increased SET, CIP2A, and pS62-MYC protein levels occur in human breast cancer. (A) Representative Western blots of SET, CIP2A, pS62-MYC, and MYC protein expression in 10 breast cell lines grown in 0.1% serum. Quantification of SET, CIP2A, pS62-MYC, and

MYC protein expression over GAPDH was done using a LICOR scanner and software. Quantification is graphed relative to expression in MCF10A. (B) Immunofluorescence of serial FFPE sections of breast tumors stained for pT58-MYC, pS62-MYC, SET, and CIP2A compared to their adjacent normal breast tissue. The graphs represent quantification of the mean staining intensity per epithelial cell for each protein over DAPI across multiple regions of interest, and then normalized to the adjacent normal. Error bars represent SD. *P < 0.05, **P < 0.01, and ***P < 0.001.

A.

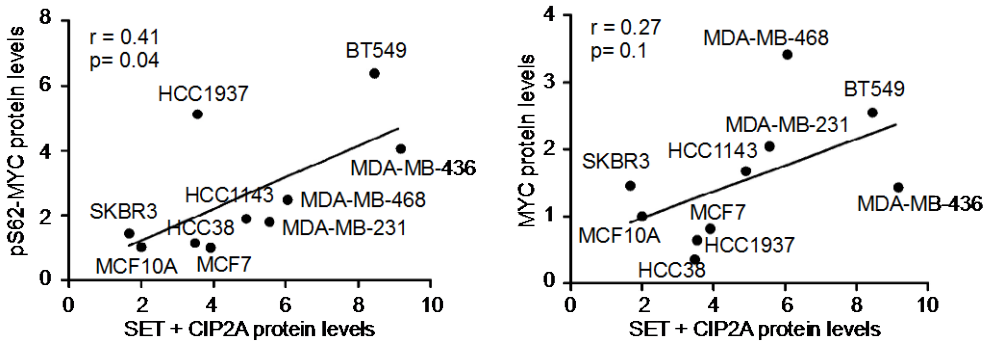

Figure S2.2. High SET and CIP2A levels trend with high pS62-MYC and total MYC expression in breast cancer cell lines. (A) Correlation analysis of pS62-MYC and MYC protein with the sum of SET and CIP2A protein levels from Figure 2A. Quantification and normalization as in Figure 2A.

SET or CIP2A Knockdown Decreases the Tumorigenic Potential of Breast Cancer Cells

In order to better understand the impact of SET and CIP2A overexpression in breast cancer, we performed transient knockdown experiments in multiple breast cancer cell lines. I transfected MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells with SET, CIP2A, or non-targeting (NT) siRNAs and measured population expansion capacity over three days (Fig 2.3A). I observed a modest, but significant decrease in population expansion upon SET or CIP2A knockdown when cells were grown in 2-dimensional (2D) culture as compared to NT siRNA transfected cells (Fig. 2.3A). To determine whether decreased expression of SET or CIP2A affected anchorage-independent growth of breast cancer cells, siRNA transfected cells were plated for soft agar colony forming assays. While only a modest decrease in growth in 2D was observed (Fig. 2.3A), there was a substantial decrease in colony number in soft agar following knockdown of either protein (Fig. 2.3B). Because I were able to observe long-term knockdown (12 days) in 2D (Fig. S2.3A) and reduced soft agar growth (Fig. 2.3B), I examined the effects of SET or CIP2A loss on tumorigenic potential *in vivo* by xenografting cells into the mammary gland of NSG mice after transfection with siRNA. I found a significant decrease in tumor growth with knockdown of either SET or CIP2A in all three cell lines (Fig. 2.3C). Interestingly, MDA-MB-436 cells, which maintained

longer-term knockdown in comparison to the other cells (Fig. S2.3A), displayed the most dramatic decrease in tumor growth.

To extend this analysis, I developed stable clones with shRNA-mediated knockdown of SET in MDA-MB-231 cells. I utilized clones with significant knockdown (Fig. S2.3B) to test cell population expansion as well as ability to form colonies in soft agar. Similar to the experiments with transient SET knockdown, stable knockdown of SET decreased the rate of cell expansion in 2D (Fig. S2.3C), but more significantly inhibited the ability of these cells to form colonies in soft agar (Fig. S2.3D). Together, these results show that SET or CIP2A knockdown in breast cancer cells reduces their oncogenic potential *in vitro* and *in vivo*.

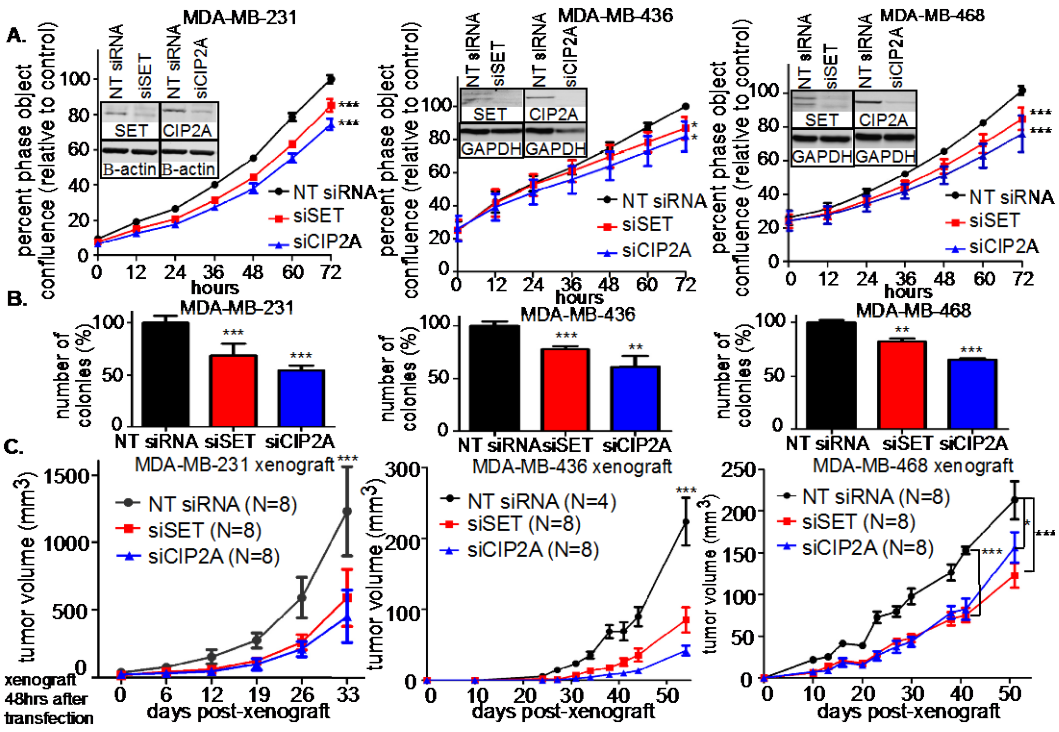

Figure 2.3. SET and CIP2A knockdown decreases tumorigenic potential of breast cancer cell lines. (A) Population expansion analysis of the indicated cell lines over 72 hours after transfection with SET or CIP2A siRNA compared to the control NT siRNA from 3 independent experiments using live cell imaging and IncuCyte analysis software. Representative Western blots show knockdown. (B and C) Soft agar colony assay and xenograft of these cells into the 4th mammary glands of NSG mice. Experimental details and statistics are described in the methods.

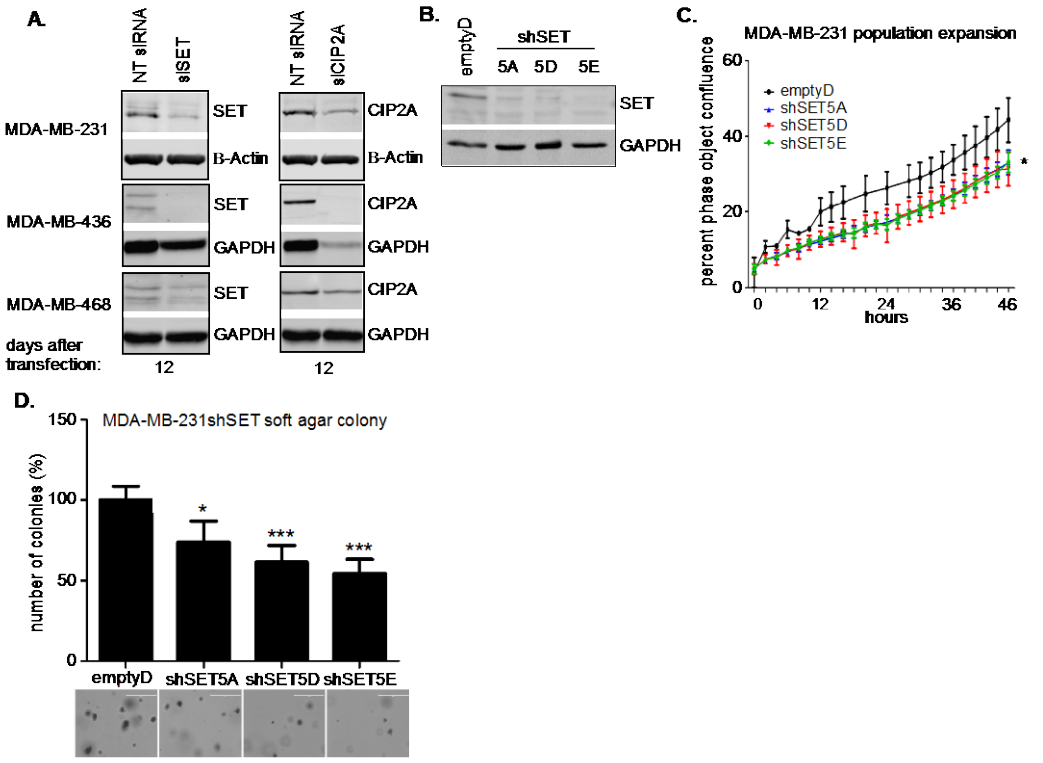

Figure S2.3. SET knockdown decreases growth of breast cancer cell lines. (A) Western blot analysis of SET and CIP2A in MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells after siRNA-mediated SET or CIP2A knockdown for 12 days. (B) Western blot analysis of SET in MDA-MB-231 stable clones with control (emptyD) or SET knockdown using shRNAs. (C) Population expansion was analyzed by live cell imaging using IncuCyte technology. (D) Soft agar colony forming assay for MDA-MB-231-shSET clones. The error bars in C and D represent the SD from three independent experiments.

The SET Antagonist OP449 Decreases the Growth of Breast Cancer Cells and Induces Apoptosis

Given that depletion of two endogenous inhibitors of PP2A, SET and CIP2A, could reduce the oncogenic potential of breast cancer cells, development of a targeted therapeutic that inhibits either of these proteins could be clinically important. Since inhibitors of CIP2A are not currently available, we have collaborated with Oncotide Pharmaceuticals to test their novel SET antagonist, OP449. OP449 (previously referred to as COG449) is a dimer of a chimeric peptide composed of an ApoE mimetic domain that binds to SET, which is fused to antennapedia, a protein transduction domain (Switzer, Cheng et al. 2011). OP449 interacts with SET in cells, causing the release of SET from PP2A and an increase in PP2A activity as demonstrated in both leukemic cells as well as in some solid tumor cell lines, including MDA-MB-231 (Christensen, Chen et al. 2011; Switzer, Cheng et al. 2011).

To begin to address the therapeutic potential of SET inhibition using OP449 as a pharmacological antagonist, I treated several breast cancer cell lines as well as immortalized MCF10A cells with OP449 for 24 hours and analyzed cell viability by trypan blue exclusion. OP449 treatment was cytotoxic in a dose-dependent manner (Fig. 2.4A). Flow cytometry for Annexin V in MDA-MB-231 cells treated with OP449 revealed that OP449 induced apoptosis in these cells as early as 6 hours (Fig. 2.4B). We next isolated cells from two fresh invasive breast

carcinoma samples and examined the effect of OP449 on these primary cells. Cells were treated with OP449 for 4 days, and cell colony expansion was measured over time. While vehicle-treated samples for both patients showed significant proliferation, colony expansion was completely inhibited with OP449 treatment at concentrations above 1.25μM (Fig. 2.4C). To determine whether OP449 reduced anchorage independent growth of breast cancer cells, MDA-MB-231, HCC38, SKBR3, MDA-MB-436, and MDA-MB-468 cells were grown in soft agar and treated with OP449. OP449 significantly reduced the anchorage-independent growth of these cells (Fig. 2.4D). Because OP449 directly inhibits SET, and SET has targets in addition to PP2A, I examined the specificity of OP449 function for PP2A activity. I used siRNA to knock down the PP2A catalytic C subunit in MDA-MB-468 cells and treated with OP449. I found that with 30% knockdown of PP2A C levels, these cells became partially insensitive to OP449 (Fig. 2.4E).

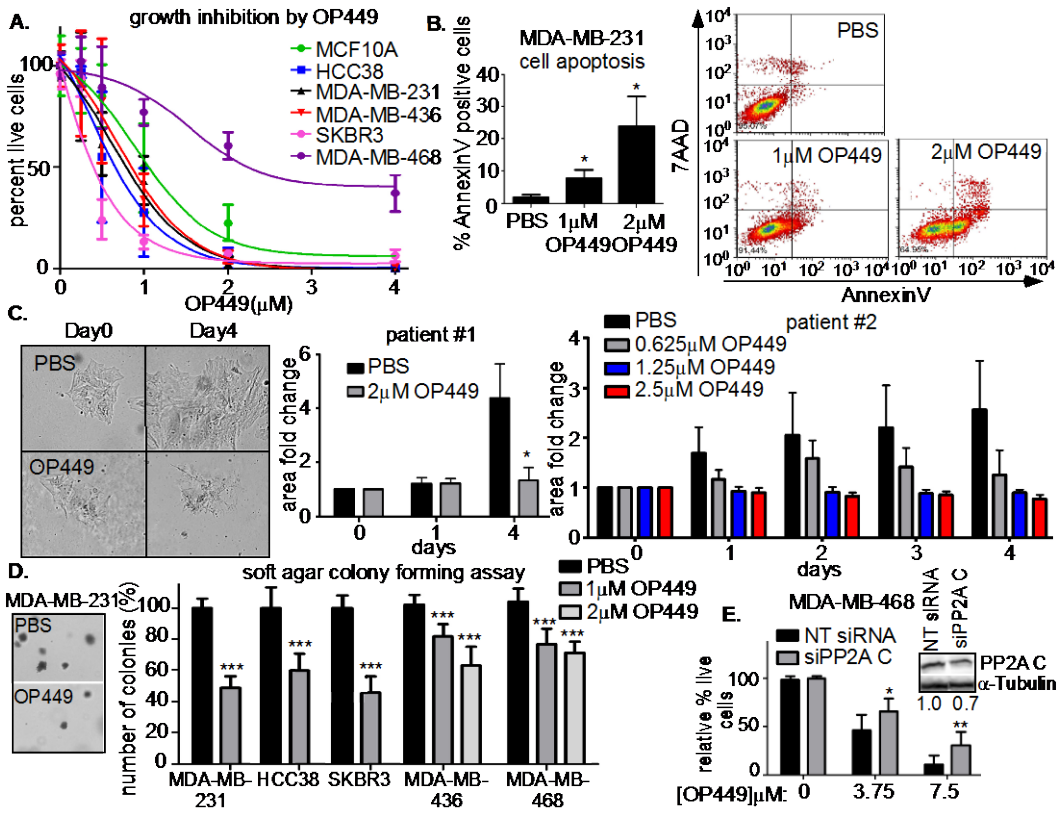

Figure 2.4. The SET antagonist OP449 decreases the growth of breast cancer cells and induces apoptosis. (A) Cytotoxicity of multiple breast cancer cell lines after 24 hours of OP449 treatment. Cell viability was assessed by trypan blue exclusion. (B) Apoptosis assay for MDA-MB-231 cells using AnnexinV-7AAD after 6 hours treatment with OP449 or PBS control. (C) Cell colony expansion of two primary invasive breast carcinoma samples treated with OP449 or PBS. Fold colony area +/- SD is shown. The images are representative cell colonies (patient #1) taken at day 0 and 4 days with and without OP449 treatment. (D) Soft agar colony assay of breast cancer cell lines after treatment with OP449 or PBS. Representative images of colonies are shown. (E) MTS assay for MDA-

MB-468 cells transfected with control NT siRNA or siRNA against PP2A C subunit for 48 hours. Cells were then treated with OP449 for another 48 hours. A representative Western blot of knockdown is shown. Error bars represent SD from three independent experiments except C, as indicated, and E, which is from two experiments performed in triplicate. *P < 0.05, **P < 0.01, and ***P < 0.001.

OP449 Decreases S62-phosphorylated MYC and MYC Transcriptional Activity Contributing to Cytotoxicity in Breast Cancer Cells

OP449 has been shown to downregulate PP2A-regulated pathways including NFκB, Rac1, nm23-H1, STAT5, and Akt (Switzer, Cheng et al. 2011; Agarwal, Mackenzie et al. 2014). Because MYC is negatively regulated by PP2A and previous reports showed reduced expression of pS62-MYC and MYC upon CIP2A inhibition (Junttila, Puustinen et al. 2007; Westermarck and Hahn 2008; Come, Laine et al. 2009; Khanna, Bockelman et al. 2009), we wanted to know whether SET inhibition could also decrease pS62-MYC levels. I therefore measured pS62-MYC and total MYC levels after knocking down SET or CIP2A in MDA-MB-231 and MDA-MB-436 cells. I observed a decrease in pS62-MYC levels in both cell lines and total MYC levels in MDA-MB-436 cells (Fig. 2.5A). Total MYC levels did not change in some of the cell lines likely due to these cells having lost Fbw7-regulated MYC degradation (Mao, Kim et al. 2008). I also observed decreased pS62-MYC levels in the stable SET knockdown clones from MDA-MB-231 cells (Fig. 2.5B). To test whether OP449 also affects pS62-MYC levels in breast cancer cells, MDA-MB-231, HCC38, MDA-MB-436, and MDA-MB-468 cells were exposed to OP449 for 4 hours and lysates were immunoblotted. I observed decreased pS62-MYC after OP449 treatment in these cells (Fig. 2.5C). Because OP449 functions at least in part through affecting PP2A activity (Fig. 2.4E and (Christensen, Chen et al. 2011; Switzer, Cheng et al. 2011)), and PP2A has targets in

addition to MYC, I examined the specificity of PP2A activation after OP449 treatment on MYC S62 phosphorylation using okadaic acid (OA), which specifically inhibits PP2A at low doses. SKBR3 cells were starved for 24 hours and then treated for 2 hours with OP449 in the presence or absence of OA. I then examined the effects on pS62-MYC following stimulation with EGF for 10 minutes. I found that, as expected, OP449 treatment inhibited the induction of pS62-MYC by EGF (Fig. 2.5D, top panel). However, in the presence OA, pS62-MYC levels were elevated in control cells and did not change with OP449 treatment (Fig. 2.5D, +OA, bottom panel).

To examine the effect of OP449 treatment on MYC transcriptional activity, a chromatin immunoprecipitation (ChIP) assay was performed in MDA-MB-231 and MDA-MB-468 cells after OP449 treatment. Consistent with decreased expression of the transcriptionally active pS62-MYC (Hydbring, Bahram et al. 2010; Farrell, Pelz et al. 2013) (Fig 2.5C), quantitative ChIP (qChIP) analysis showed that OP449 treatment decreased MYC binding to the promoters of its target genes, Nucleolin, E2F2, and 5s rRNA (Fig. 2.5E). To gain a more comprehensive understanding of the effect of OP449 treatment, or SET or CIP2A knockdown on MYC transcriptional activity, we performed RNA-seq to compare gene expression in MDA-MB-231 cells in control vs. treated or knockdown cells. I then used Gene Set Enrichment Analysis (GSEA) (Subramanian, Tamayo et al. 2005) to test whether a curated gene set of validated targets of c-MYC transcriptional activation (Schaefer, Anthony et

al. 2009) was affected by treatment or knockdown. I found that this MYC gene signature was enriched in control cells versus OP449 treated cells and in control siRNA versus SET or CIP2A knockdown cells (Fig. 2.5F and Fig. S2.4), indicating suppression of MYC target gene expression with OP449 treatment, or SET or CIP2A knockdown. Together, these data show that treatment with OP449, or SET or CIP2A knockdown, causes a decrease in pS62-MYC protein and this leads to a global decrease in MYC's transcriptional activity.

PP2A has many targets that likely contribute to tumor growth. To examine how much of OP449's activity is through its effect on pS62-MYC, I took advantage of our MCF10A-TR-MYC inducible cell lines, in which either wild-type (WT) or a mutant form of MYC (T58A) can be induced with doxycycline (Dox). MYC^{T58A} cannot be phosphorylated at T58 and is resistant to PP2A-mediated S62 dephosphorylation, maintaining constitutive pS62 (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004; Arnold and Sears 2006). Ectopic MYC was induced for 4 hours prior to treatment with OP449 for 48 hours. Although expression of MYC^{WT} on its own was mildly toxic in MCF10A cells, OP449 still induced cell death, while expression of the PP2A resistant MYC^{T58A} mostly rescued this effect (Fig. 2.5G). Together, these results demonstrate that MYC activity is suppressed by OP449 treatment through its effects on PP2A and this in part underlies its cytotoxic activity.

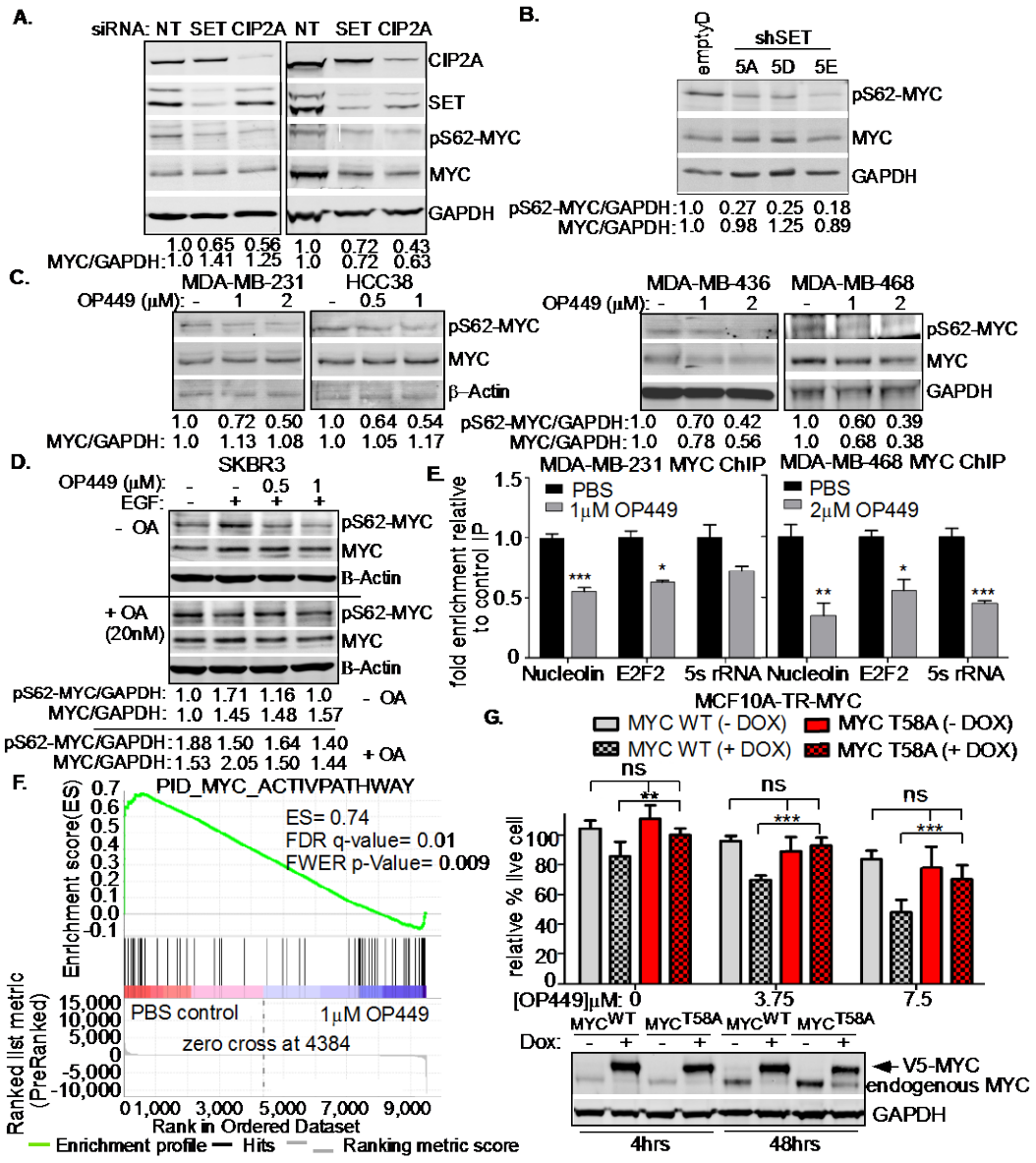

Figure 2.5. OP449 decreases S62-phosphorylated MYC and MYC transcriptional activity contributing to cytotoxicity in breast cancer cells. (A) Western blot analysis of pS62-MYC and MYC proteins in MDA-MB-231 and MDA-MB-436 cells after siRNA knockdown of SET or CIP2A. The levels of pS62-MYC and total MYC were quantified on a LICOR scanner and calculated over GAPDH. (B) Western blot analysis of pS62-

MYC and MYC protein in MDA-MB-231 stable clones with control or SET knockdown (shown in Fig. S2.3B). (C) Western blot analysis of pS62-MYC and MYC after treatment of the indicated cells with OP449 for 4 hours. (D) Western blot analysis of pS62-MYC and MYC from SKBR3 cells starved for 24 hours, and then treated with OP449 without or with 20nM Okadaic acid (OA) for 2 hours, followed by 10 minutes EGF (100ng/ml) treatment. (E) qChIP for MYC at the Nucleolin, E2F2, and 5s rRNA promoters after 24 hours of treatment with OP449. The fold enrichment of bound DNA was graphed as the fold enrichment in MYC IP relative to the fold enrichment in IgG control IP. (F) Gene Set Enrichment Analysis of a MYC gene signature (Schaefer, Anthony et al. 2009) in RNA-seq data from MDA-MB-231 cells treated with 1µM OP449 or PBS for 12 hours. The positive enrichment score (ES) and statistical values are listed. (G) MTS assay for MCF10A-TR-MYC cells treated with 1µg/ml doxycycline (Dox) for 4 hours to induce ectopic expression of wild-type (MYC^{WT}) or mutant MYC (MYC^{T58A}). Cells were then treated with different concentrations of OP449 as indicated for 48 hours. Representative Western blot of ectopic MYC in these cells with 4 or 48 hours of Dox treatment. Error bars represent SD. *P < 0.05, **P < 0.01, and ***P < 0.001.

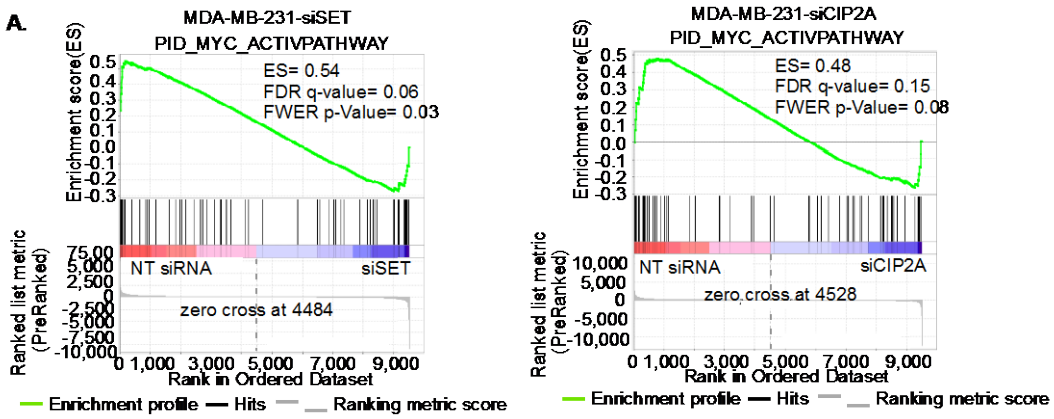

Figure S2.4. Global MYC transcriptional activity is decreased after SET or CIP2A knockdown. MYC gene set enrichment analysis using the MYC gene list published in (Schaefer, Anthony et al. 2009) comparing SET or CIP2A knockdown with control siRNA in MDA-MB-231 cells. The positive enrichment scores (ES), FDR-adjusted q-values, and FWER p-values are listed showing the MYC gene signature enrichment in control versus knockdown cells.

OP449 Treatment Decreases Tumor Growth *in vivo* and Increases PP2A Activity in Tumors

Since we observed that OP449 treatment could decrease the growth of breast cancer cell lines and induce apoptosis *in vitro*, we next wanted to test whether OP449 has anti-tumorigenic properties *in vivo.* MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells were xenografted into the mammary glands of NSG mice. Once tumors were palpable, mice were randomized into two groups and treated intraperitoneally with 5mg/kg of OP449 or PBS control, three times per week. Tumor size was determined by repeated caliper measurement. I found that tumor growth was slowed in mice treated with OP449 as compared to controls (Fig. 2.6A). For MDA-MB-231 tumors, all mice were sacrificed when control tumors reached 2cm in diameter, and tumors were harvested and fixed in formalin for histological analysis. Consistent with our data in cell lines, this analysis revealed that the OP449 tumors had an increased number of apoptotic cells as measured by TUNEL assay and a decreased number of proliferating cells as measured by Ki67 staining (Fig. 2.6B and 2.6C). Following on these results, we performed a second study with MDA-MB-231 tumors, treating them three times per week for forty days and again observed decreased tumor growth with OP449 treatment (Fig. S2.5A). On the last day, we injected mice with OP449 or the vehicle control 2 hours prior to sacrifice. Tumors were dissected, flash frozen, and used for Western blot analysis of OP449 in the tumors and for PP2A activity

assays. We observed OP449 specifically in the tumors of treated mice (Fig. 2.6D) and a significant increase in PP2A activity in the OP449 treated tumors compared to PBS treated tumors (Fig. 2.6E).

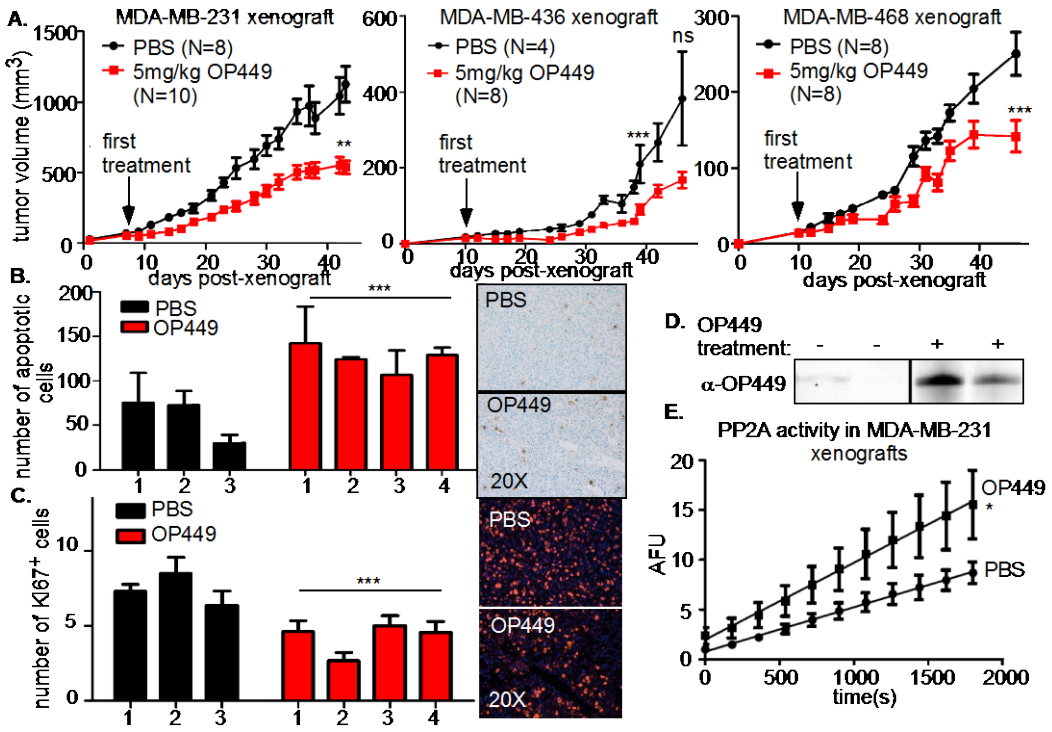

Figure 2.6. OP449 suppresses breast tumor growth *in vivo* associated with increased PP2A activity. (A) Tumor growth curve for MDA-MB-231, MDA-MB-436, and MDA-MB-468 xenografts in the 4th mammary gland of NSG mice following treatment with OP449 or PBS. (B) TUNEL assay and (C) IF for Ki67 for MDA-MB-231 harvested xenografts from A. The mean and SD of total apoptotic cells in 75 random fields or Ki67 positive cells in 25 fields for three control mice (6 tumors) and four OP449 treated mice (8 tumors) is graphed. (D) Western blot analysis of OP449 with peptide specific antibody in lysates from MDA-MB-231 xenografts (Fig. S2.5A). (E) Tumor lysates from D were used to measure PP2A activity as described previously (Christensen, Chen et al. 2011).

To further address the pharmacokinetic, distribution, and plasma stability of OP449, studies were conducted to detect OP449 protein in plasma isolated from OP449-infused rats after one-hour infusion of 2 mg/kg. Western blotting indicated that OP449 is immediately detectable and then rapidly cleared from the blood, without the appearance of a degradation product (Fig. S2.5B). We extended this analysis by incubating OP449 in rat plasma *in vitro* for 12, 24 and 48 hours at 37°C followed by Western blotting and observed that OP449 was stable in isolated plasma with a half-life of >24 hours (Fig. S2.5C). The rapid clearance of OP449 *in vivo* from the blood stream was associated with its detection in tissues, which persists for more than 6 hours. Together these results suggest that OP449 can decrease the growth and the tumorigenic potential of breast cancer cells *in vivo*, and this is associated with increased apoptosis and decreased proliferation. In addition, OP449 has *in vivo* bioavailability sufficient for treating tumors and eliciting a pharmacologically induced increase in PP2A activity in the treated tumors.

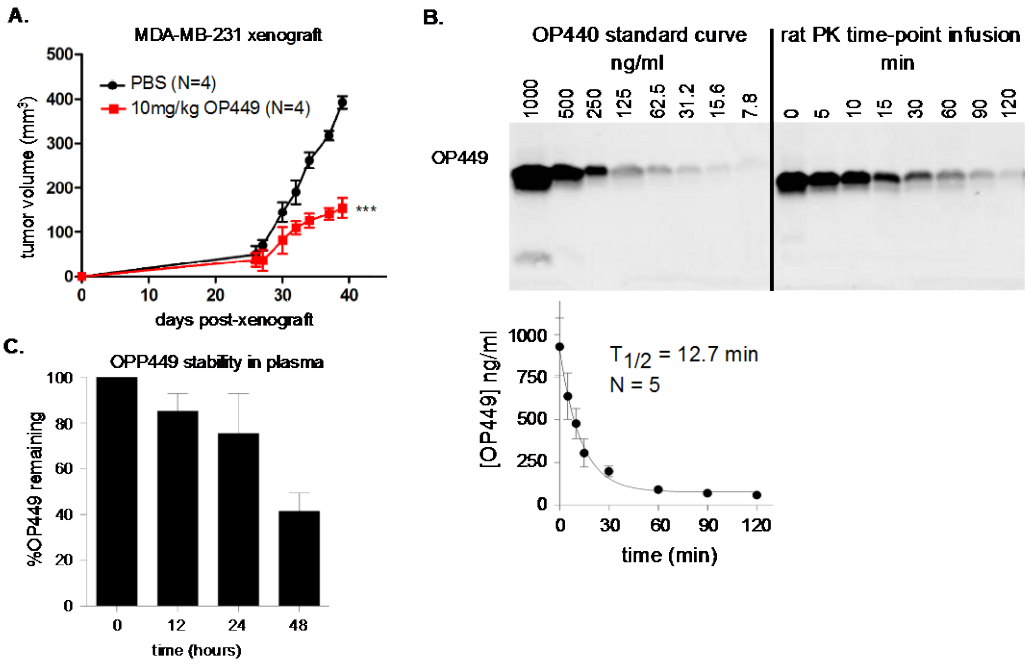

Figure S2.5. Xenograft tumor growth and pharmacokinetics of OP449. (A) Tumor growth curve for MDA-MB-231 xenografts in the 4th mammary gland of NSG mice following treatment with OP449 or PBS. Mice were injected with 10mg/kg of OP449 or PBS intraperitoneally (IP) three times per week. Tumors were harvested and frozen in liquid Nitrogen 2 hours after the final injection and used for Western blot analysis in Fig. 6D and PP2A activity assay in Fig. 6E**.** (B) Pharmacokinetic profile of OP449 following infusion into the femoral vein of non-sedated rats. 2mg/kg OP449 was infused into the femoral vein of a cannulated rat over a period of 1 hour. At the end of the infusion, blood was drawn at the indicated times from a jugular vein cannula, plasma was prepared, run on PAGE and Western blotted with an anti-OP449 antibody (right blot). A standard curve of OP449 in rat plasma is shown from the

same gel (left blot) as the experimental samples for quantification of OP449 in the rat plasma from infused animals. The graph represents the average plasma OP449 levels over time from 5 rats. (C) OP449 stability in plasma. Rat plasma with OP449 was incubated at 37° C for the indicated times and OP449 levels were quantified by Western blot.

Discussion

In analyses of oncogenic mechanisms in breast cancer, accumulation of the MYC protein has been observed in 46% of primary breast tumors (Deming, Nass et al. 2000; Chrzan, Skokowski et al. 2001). Many mechanisms have been proposed to explain the elevated levels of MYC protein in tumors. Previous work from our lab has shown that increased levels of MYC can result from disruption of the normal MYC protein degradation pathway rather than increased expression at the mRNA level (Zhang, Farrell et al. 2012). Our research also revealed that PP2A-B56α is a critical negative regulator of MYC protein stability through its dephosphorylation of Serine 62 (Arnold and Sears 2008). This is highly relevant because Hahn et al. demonstrated that inhibition of PP2A activity is required for cell transformation (Sablina and Hahn 2008; Sablina, Hector et al. 2010). Furthermore, we have shown that this requirement for PP2A inhibition can be partially replaced by expression of the more stable MYC^{T58A} mutant (Yeh, Cunningham et al. 2004). In this report, I studied the role of two cellular inhibitors of PP2A, SET and CIP2A, in breast cancer to understand if their expression 1) is common in tumors, 2) regulates tumorigenesis, 3) affects the level and/or activity of pS62-MYC, and 4) could be exploited as potential targets for breast cancer therapy.

I found that SET and CIP2A are frequently overexpressed in breast cancer cell lines at both the mRNA and protein levels. In addition, I found that CIP2A, SET and pS62-MYC, but not pT58-MYC are frequently co-

overexpressed in human primary tumor samples relative to matched normal tissue. For CIP2A, these findings confirmed a previous report that CIP2A directly regulates MYC S62 phosphorylation and stability (Junttila, Puustinen et al. 2007) and that CIP2A is overexpressed in human breast tumors (Come, Laine et al. 2009). In contrast, our finding that SET is frequently overexpressed in breast cancers is novel and suggests that overexpression of PP2A inhibitors may play an important role in the development of human breast cancer. Interestingly, I found that CIP2A overexpression correlated with the triple negative breast cancer subtype, a result that was further supported with RNA-seq data from human breast cancer cell lines. This result is important, as the Claudin-low breast cancer subtype associates highly with upregulation of the MYC/MAX network (Heiser, Sadanandam et al. 2012). This correlation suggests that CIP2A could potentially be used as a diagnostic biomarker for more malignant, MYC-driven tumor types. While SET was overexpressed in greater than 50% of all breast cancer samples tested, there was no significant correlation between increased SET levels and any breast cancer subtype, suggesting that SET plays a broader role in sustaining oncogenic signaling in breast cancers.

It has been shown that CIP2A deletion results in a reduction of oncogenic potential for multiple tumors (Junttila, Puustinen et al. 2007; Khanna, Bockelman et al. 2009; Wang, Li et al. 2011). Furthermore, SET knockdown was shown to negatively affect tumorigenesis in blast crisis

CML (Neviani, Santhanam et al. 2005). Here I show that in breast cancer SET and CIP2A knockdown reduces cell growth *in vitro* and significantly attenuates tumor growth *in vivo*. Furthermore, loss of these PP2A inhibitors decreased the level of pS62-MYC in these cells and suppressed global MYC-driven gene expression. These results suggest that inhibiting either SET or CIP2A could be a viable strategy to post-translationally target MYC and inhibit tumor growth in breast cancer.

While no known inhibitors of CIP2A have been described, previous work has demonstrated that the SET antagonist OP449 (originally named COG449) activates PP2A, is cytotoxic to primary CLL cells, and decreases lymphoma xenograft tumor growth (Christensen, Chen et al. 2011). To explore the therapeutic potential for PP2A activation through SET inhibition as an approach for breast cancer therapy we treated breast cancer cells with OP449 and measured growth and oncogenic potential *in vitro* and *in vivo*. OP449 treatment resulted in increased apoptosis and decreased proliferation *in vitro* and *in vivo*. Interestingly, we found that although MYC^{WT} overexpression was additive with OP449 treatment in inhibiting the growth of MCF10A cells, the MYC^{T58A} mutant, which is resistant to PP2A and has constitutively high S62 phosphorylation was able to rescue this effect. Importantly, OP449 inhibited tumor growth at doses that were not toxic to mice, and other studies using OP449 showed selective inhibition of cancer growth while normal fibroblasts remained unaffected (Switzer, Cheng et al. 2011). Furthermore, we showed here

that OP449 has *in vivo* bioavailability, and can be detected in the tumors of treated mice and treated tumors have a resultant increase in PP2A activity.

In summary, inhibiting SET (and potentially CIP2A) may provide an important therapeutic strategy for the treatment of breast cancer by targeting MYC in a way that has not yet been achieved by other means. Furthermore, SET antagonism with OP449 will facilitate downregulation of other PP2A regulated pathways including NFκB, nm23-H1, Akt, and Rac-1 (Switzer, Cheng et al. 2011), all of which likely contribute to the potent growth inhibitory effects we observed here in breast cancer. This makes OP449 a favorable drug because in addition to targeting MYC, which may have opposing effects on metastasis (Liu, Radisky et al. 2012), OP449 can facilitate downregulation of these other oncogenes. These results further validate the use of SET antagonists and PP2A activators as a novel strategy for breast cancer therapy.

Materials and Methods

Cell Culture, knockdown, and cell population expansion assay

All cell lines were purchased from ATCC except SKBR3, which was a gift from Dr. Joe Gray (OHSU, OR). Cell culture and knockdown methods are described in SI methods. The cytotoxicity assay was performed in reduced serum (0.1%) due to observed precipitation of OP449 at higher serum concentrations. Acquisition and culturing of primary tissue is described in SI Methods (IRB approval No: 3330). The cell population expansion assay was performed on an IncuCyte Zoom (Essen Bioscience), and detailed information on this assay is described in SI methods.

Western blot, chromatin immunoprecipitation (ChIP), and immunofluorescence (IF)

Western blot, ChIP, and IF analyses were performed as described previously (Arnold, Zhang et al. 2009; Zhang, Farrell et al. 2012; Farrell, Pelz et al. 2013) and as detailed in SI Methods. Patient samples used for IF analysis were obtained from the OHSU Cancer Pathology Shared Resource (IRB approval No: 6478).

Quantitative RT-PCR (qRT-PCR) and RNA sequencing (RNA-seq)

The TissueScan Breast Cancer cDNA Array (Array 4), TaqMan primers, and RNA-seq are described in SI methods. cDNA from patient

samples (Fig. S2.1B) was provided by Dr. Dexi Chen and described in (Zhang, Farrell et al. 2012).

Orthotopic xenografts of breast cancer cell lines and OP449 pharmacokinetics

Orthotopic xenograft of breast cancer cell lines is described in SI methods. Briefly, cells were xenografted into the 4th mammary gland of NOD/SCID/γ-chain null (NSG) mice. For the treatment study, once tumors were palpable, mice were divided into two groups and treated with OP449 or vehicle control (PBS) three days/week. The xenografted tumors were harvested to perform TUNEL, Ki67, PP2A activity, and OP449 detection assays (described in SI methods). OP449 pharmacokinetic studies are described in SI methods.

Statistics

Standard deviation (SD) for all graphs was calculated from three independent experiments (unless otherwise stated in the figure legend) using GraphPad Prism 5. P values were analyzed by Student t test, with a two-tailed method (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Acknowledgments

We would like to thank Dr. Joe Gray for providing the SKBR3 cell line and the RNA-seq data from a panel of breast cancer cell lines, Dr. Dexi Chen for providing cDNA from breast cancer samples, Dr. Nicholas Wang and Darcie Babcock for help with dose response analysis, Dr. Paul Spellman for helpful suggestions, Dr. Xiaoyan Wang for technical help and all members of the Sears lab for editing the manuscript and other helpful suggestions. Sequencing for the RNA-seq data was performed in the OHSU Integrated Genomics Laboratory and Gene Profiling Shared Resource.

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Supporting Information

SI Materials and Methods

Cell Culture and transfection

Breast cancer cell lines were grown in the following media containing 10%FBS and 1X pen/strep: RPMI-1640 for HCC38, HCC1143, HCC1937, and BT549; DMEM for MDA-MB-436, MDA-MB-468, and SKBR3; and EMEM for BT20. MCF10A, MDA-MB-231, and MCF7 were grown as described in (Zhang, Farrell et al. 2012) and generation of stable MCF10A-TR-MYC cells were described in (Farrell, Pelz et al. 2013).

Transient knockdowns were performed using siRNAs to SET (M-019586-01), CIP2A (M-014135-00), and PP2A C (M-003598-010005) and DharmaFECT1 transfection reagents according to the protocol provided (Dharmacon). Non-targeting siRNA (NTsiRNA) (D-001206-14) was used as a control. For the xenograft assay, cells were transfected with siRNAs 2 times over 2 days prior to transplant. MDA-MB-231-shSET stable clones were made using a shRNA-encoding plasmid obtained from Sigma (TRCN0000063717). Empty vector (SHC001) was used as a control. Briefly, cells were transfected using Lipofectamine2000 (Invitrogen) and stable clones were generated after selection with puromycin.

Quantitative-PCR (qPCR)

RNA was isolated from breast cancer cell lines using TRIzol reagent (Invitrogen) and cDNA was made as described previously (Zhang, Farrell et al. 2012). qPCR was then performed using TaqMan primers for c-MYC (Hs00905030_m1), SET (Hs00853870_g1), CIP2A (Hs00405413_m1), ACTIN (Hs99999903_m1), and 18s (Hs03003631_g1).

TissueScan Breast Cancer and Normal Tissue cDNA Array (Array 4) was obtained from Origene. The array contained dried cDNAs from 48 samples including 4 normal. SET and CIP2A were measured by using TaqMan probes. 18s was used as an internal control. The relative fold change was measured by the $\Delta\Delta(C_T)$ method between each tumor sample and the average of the normal samples in expression: $\Delta(C_T)$= C_T(SET or CIP2A)- C_T(18S); $\Delta\Delta(C_T)$= $\Delta(C_T)$ – average ($\Delta(C_T)$ of normal samples); Fold change= $2^{\Delta\Delta(C_T)}$.

RNA sequencing (RNA-seq) and Gene Set Enrichment Analysis

RNA-seq data for Fig. S2.1A can be accessed at the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/, accession no. GSE48216) (Daemen, Griffith et al. 2013).

For RNA-seq data used in Fig.2.5F and Fig.S2.4A, total RNA was isolated with TRIzol (Invitrogen) from MDA-MB-231 cells following treatment with vehicle (PBS) or 1μM OP449 for 12 hours, or following

transfection with control NT, SET, or CIP2A siRNA for 48hours. RNA-seq was performed as described previously (Farrell, Pelz et al. 2013). Briefly, data were aligned using Bowtie version 0.12.7 to Human genome version hg19, and custom R scripts and software were used to count tags that aligned to the exons of UCSC RefSeq gene models. The chi-square statistic was calculated for each gene model and the Benjamini & Hochberg (1995) false discovery rate adjustment was applied. Each sample was normalized based on the total reads that aligned to exons to calculate fold change and expression difference between classes. The c-MYC gene set published in (Schaefer, Anthony et al. 2009) was used to analyze MYC enrichment in the ranked (by expression difference between classes) gene list using GSEA version 2.0.14. The RNA seq data can be accessed at the GEO (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE58008).

Antibodies for Western blot, Chromatin immunoprecipitation (ChIP), and immunofluorescence (IF)

Western blot analysis was performed as described previously (Arnold, Zhang et al. 2009). Immunoblots were visualized using the Odyssey IR imager (LI-COR) that can detect both Fluor 680 and IRDye 800 secondary antibodies (1:10000). Quantification of Western blots was done using the Odyssey IR software, version 1.2 (LI-COR).

Antibodies used include: MYC Y69 (ab32072, 1:1000; Abcam), MYC N262 (2µg for ChIP; Santa Cruz), monoclonal pS62-MYC (1:500; BioAcademia), pT58-MYC (A00242; 1:50 for IF; GenScript), SET (A302-262A; 1:2000 for Western blot and 1:1000 for IF; Bethyl), CIP2A (1:50000 for Western blot and 1:2000 for IF; a gift from Dr. Jukka Westermarck; University of Turku and Abo Akademi University, Finland), GAPDH (AM4300; 1:10000; Ambion). For immunofluorescence we used our pS62-MYC antibody (1:100) we developed as previously described (Zhang, Farrell et al. 2012) and quantification of IF was done using the ImageJ software.

Soft agar colony forming assay

20,000-25,000 cells were plated in 0.35% Nobel agar on top of 0.7% Nobel agar mixed with 2X complete media. For treatments, 2X drug was added to cells at the time of plating and cells were retreated three times per week. Cells were fixed in 0.005% crystal violet overnight at 4^{o} C after 3-4 weeks and colonies were counted from 20 random fields using the EVOS FL cell imaging system (Advanced Microscopy Group).

AnnexinV-7AAD apoptosis assay

Apoptosis assay was performed according to the BD Pharmingen protocol. MDA-MB-231 cells were treated for 6 hours with OP449 or PBS. Following trypsinization, cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer at a concentration of $1x10^{6}$ cells/ml. 5µl

of FITC-AnnexinV and 5µl of 7AAD was added to 100µl of the suspended cells. Cells were incubated at room temperature for 15 minutes in dark and then 400µl of the 1X Binding Buffer was added to each tube. Cells were analyzed by the FACS Calibur flow cytometer and FCS Express software (De Novo).

Cell population expansion and cell viability assays

The cell population expansion assay performed on the IncuCyte (Essen Bioscience) uses captured images to measure live cell content change over time. Cells were transfected with siRNA for 48 hours and plated for live cell imaging and Western blotting. 5000 MDA-MB-231, 8000 MDA-MB-436, and 7000 MDA-MB-468 cells were plated in 96-well plates grown in complete media. Cells were incubated in the IncuCyte for 3 days. Live cell images were taken every 2 hours and percent phase object confluence was calculated using IncuCyte Zoom software.

The cell viability assays were done using the MTS assay according to manufacturer's protocol (Promega).

Growth inhibition of primary breast tumor cell samples

Primary invasive breast carcinoma tissue from two patients was obtained from a needle core biopsy through the OHSU Biolibrary with informed patient consent (institutional review board approval No: 3330). Tissue was dispersed through collagenase/hyaluronidase digestion and plated on collagen-I coated 6-well culture dishes in DMEM/F12

supplemented with 10% FBS, 0.5 mg/ml hydrocortisone, 20ng/ml EGF, and 10µg/ml insulin. Cultures were allowed to grow for 24-48 hours and then imaged (day 0). Cells were treated with varying doses of OP449 or PBS for four days. Cell colonies were visualized over time on an EVOS Fl inverted digital microscope and colony area was quantified using ImageJ software.

Xenograft of human breast cancer cell lines

Mice were handled in accordance with the Oregon Health and Science University Institutional Animal Care and Use Committee. 1×10^6 (for SET/CIP2A knockdown) or 2×10^6 (for OP449 treatment) of MDA-MB-231, MDA-MB436, and MDA-MB-468 cells in 25%matrigel+75%complete media were xenografted into the fourth mammary gland of NOD/SCID/γ chain null (NSG) mice. For the treatment study, once tumors were palpable, mice were randomly divided into two groups and treated via intraperitoneal (IP) injection with 5mg/kg (for Fig. 2.6A) or 10mg/kg (for Fig. S2.5A) of OP440 three days/week or vehicle control (PBS). Tumors were measured by a digital caliper and tumor volume was calculated by $(W \times L^2)/2$.

TUNEL and Ki67 analyses

Six xenografted MDA-MB-231 tumors from three control mice and eight tumors from four OP449 treated mice were fixed in formalin and Paraffin embedded. Sections from control and OP449 treated tumors

were assessed by TUNEL staining using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the protocol. The mean and SD of total apoptotic cells in 75 random fields for each tumor set is graphed. For Ki67 staining, sections were analyzed by immunofluorescence using Ki67 antibody (1:1000, Novocastra, NCL-Ki67-MM1).

PP2A activation in orthotopic mammary tumors

MDA-MB-231 cells were orthotopically xenografted into the fourth mammary gland of NSG mice (Fig. S2.5A). Mice were treated with OP449 (10mg/kg I.P.) or PBS three times per week for forty days. Mice were euthanized and tumor harvested 2 hours following the final treatment. Harvested tumors were rapidly dissected, flash frozen in liquid nitrogen, and ground to a fine powder on liquid nitrogen. PP2A assays were performed as described previously (Christensen, Chen et al. 2011).

OP449 detection in orthotopic breast cancer tumors

Tumor lysates from the PP2A assays above were diluted to 5mg/mL total protein and prepared for PAGE by diluting 75µL with Laemmli protein electrophoresis buffer (4X, 25 µL). These protein solutions were heated to 90º C for 5 minutes, separated by SDS PAGE, and transferred onto nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were blocked using 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 3 hours, and then

washed with TBST. The membrane was incubated overnight at 4°C in a rabbit anti-OP449 reactive antibody (DU219) diluted in SuperBlock. The membranes were washed with TBST for 1 hour and incubated with donkey anti-rabbit IRDye 680 secondary antibodies to detect OP449 in the tumor cell lysates. The membranes were washed thoroughly and protein bands visualized and quantitated [Odyssey Infrared scanner (LiCor)].

OP449 pharmacokinetics

Rats were purchased from Charles River Laboratories with a femoral vein and a jugular vein catheter surgically inserted. Rats were slowly infused with OP449 in lactated Ringer's solution for 1 hour at a dose of 2mg/kg through the femoral vein catheter. At the end of the 60 minute infusion period, 200μL of blood was drawn through the jugular vein catheter for each time point and 20μL sodium citrate added to prevent coagulation. Anti-coagulated whole blood was centrifuged at 5,000x g for 5 minutes and plasma was removed. Blood was collected from five rats at 0, 5, 10, 15, 30, 60, 90 and 120 minutes following the completion of the 60 min infusion. A standard curve for quantitation of OP449 was prepared by spiking OP449 into normal rat plasma to a final concentration of 1.0μg/mL and performing 2-fold dilutions. OP449 was quantitated by adding 10μL of the plasma to 65μL of phosphate buffered saline and 25μL of 4x Laemmli protein electrophoresis buffer. Western blot was performed as described above and OP449 was detected by the anti-OP449 reactive antibody (DU219). Stability of OP449 in rat plasma was assessed using plasma

from the T = 0 time points from each rat. Plasma was divided into aliquots and incubated at 37° C for 12, 24, or 48 hours before being quantified by Western blotting. Residual OP449 for each time point was determined by normalizing each time point to the amount in the starting plasma sample.

Chapter Three

Investigating the Activity of the Tumor Suppressor PP2A-B56α Using a Novel Mouse Model

Investigating the Activity of the Tumor Suppressor PP2A-B56α Using a Novel Mouse Model

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Abbreviations: PP2A, protein phosphatase 2A; SCC, squamous cell carcinoma

A detailed list of contributors is described in the “Appendices” section.

Abstract

Protein phosphatase 2A (PP2A), a ubiquitously expressed Serine-Threonine phosphatase, mediates 30-50% of cellular Serine/Threonine protein phosphatase activity. PP2A regulates the activity of many key signaling pathways, including c-Myc (Myc), and plays an important tumor suppressor role in cells. Knockdown of the B56α regulatory subunit of PP2A, which directs PP2A activity toward Myc, promotes cell transformation, indicating that this subunit helps to specify some of PP2A's tumor suppressor activity. Here I report the generation of a novel B56α knockout mouse in order to study the physiologic activity of the PP2A-B56α phosphatase. While this is a whole body knockout, the primary phenotypic effect I observed with B56α loss was spontaneous skin lesion formation. The skin lesions showed hyperproliferation of the epidermis, hair follicles, sebaceous gland, and dermis. Increased levels of Myc-Serine62 phosphorylation and Myc activity were one of downstream effects of B56α loss in the skin lesions. B56α loss also induced papilloma initiation and increased the number of skin and bone marrow stem cells. Further analysis of other tissues showed an increase of immune cells in the spleen, which is suggestive of elevated extramedullary hematopoiesis in the B56α mice. Our model suggests that B56α is important for cells to maintain homeostasis and that B56α loss can lead to increased activity of important oncogenes, such as Myc, leading to aberrant cell growth and defects in stem cell maintenance.

Introduction

Protein Phosphatase 2A (PP2A) is a heterotrimeric Serine-Threonine protein phosphatase that is ubiquitously expressed in eukaryotic cells (Westermarck and Hahn 2008) and mediates 30-50% of cellular Serine/Threonine protein phosphatase activity (Arnold and Sears 2008). PP2A is involved in the regulation of numerous signaling pathways, including stem cell self-renewal, proliferation, differentiation, migration, cell survival, and apoptosis. The PP2A heterotrimeric holoenzyme consists of three major subunits: a catalytic (C) subunit (36 kDa), a structural (A) subunit (65kDa), and a variable regulatory (B) subunit, which directs the PP2A holoenzyme to a specific target and location (Sablina and Hahn 2008; Westermarck and Hahn 2008). In mammals, the A and C subunits are found in two isoforms, α and β. Aα and Aβ are 87% identical, whereas Cα and Cβ share 97% identity (Sablina and Hahn 2008). To date, four unrelated families of B subunits have been identified: B, B′, B″, and B‴ (Zhou, Pham et al. 2003; Sablina and Hahn 2008). All together, 15 genes encode 26 B subunits of PP2A, which can potentially assemble more than 100 distinct PP2A complexes (Janssens and Goris 2001; Sontag 2001; Zhou, Pham et al. 2003; Sablina and Hahn 2008; Eichhorn, Creyghton et al. 2009). All of these subunits have distinct spatial and temporal expression patterns, and details about the different complexes and their activities remain undetermined.

PP2A plays an important tumor suppressor role in cell transformation. To identify which PP2A complex was implicated in this process, a siRNA screen was performed and revealed that B56α, B56γ, and PR72/PR130 were the only B subunits shown to be critical for regulating human cell transformation (Sablina, Hector et al. 2010). PP2A complexes containing these B subunits regulate key oncogenic pathways, including c-Myc (Myc), Wnt, and PI3K/Akt signaling (Arnold and Sears 2006; Sablina, Hector et al. 2010).

Myc is a transcription factor that regulates many genes involved in critical cellular functions such as proliferation, growth, and apoptosis (Meyer and Penn 2008). A high level of Myc expression has been reported in about 70% of human cancers where only about 20% of these tumors have *MYC* gene amplification or translocation (Farrell and Sears 2014). Therefore, increased protein stability through deregulated Myc degradation pathways has been suggested as another mechanism of Myc overexpression in tumors (Malempati, Tibbitts et al. 2006; Zhang, Farrell et al. 2012). Myc stability is regulated in part by sequential and reversible phosphorylation at two conserved residues, Threonine 58 (T58) and Serine 62 (S62), which have opposing effects on Myc stability (Sears, Nuckolls et al. 2000). Following activation of growth signals, Myc becomes phosphorylated at S62 (pS62) through the Ras/Raf/MEK/ERK pathway or CDK activation during the cell cycle. pS62 increases Myc stability and oncogenic activity (Pulverer, Fisher et al. 1994; Chang, Claassen et al.

2000; Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004; Hann 2006; Wang, Cunningham et al. 2011). When growth signals cease, GSK3 becomes active and phosphorylates T58 (pT58), which is dependent upon prior phosphorylation on S62 (Sears, Nuckolls et al. 2000; Yeh, Cunningham et al. 2004). T58 phosphorylation facilitates PP2A-B56α-mediated dephosphorylation of pS62 and recruitment of the proteasomal degradation complex consisting of the E3 ubiquitin ligase SCF^{Fbw7} (Welcker, Orian et al. 2004; Welcker, Orian et al. 2004). This process is facilitated by the scaffold protein Axin1, which helps nucleate a destruction complex for Myc at target gene promoters (Arnold, Zhang et al. 2009; Farrell, Pelz et al. 2013).

In addition to Myc, PP2A-B56α also negatively regulates β-catenin and Bcl-2. It has been shown that B56α is part of the Axin1-mediated degradation complex for β-catenin and B56α overexpression reduces β-catenin expression in mammalian cells and Xenopus embryo explants (Seeling, Miller et al. 1999). B56α was also shown to co-localize with Bcl-2 at the mitochondrial membrane and suppress it pro-survival activity (Ruvolo, Deng et al. 2001; Arnold and Sears 2008). Despites B56α's important role in regulating key oncogenes and cell transformation, no studies have ever reported the function of B56α *in vivo*. Here I report a novel B56α knockout mouse and characterize B56α's physiological function. Although the B56α gene knockout was ubiquitous, the primary phenotypic effects I observed with B56α loss were spontaneous skin

lesion formation with increased immune cell infiltrations in the skin lesions, spleen, and liver. The skin lesions showed hyperproliferation of the epidermis, hair follicles, sebaceous glands, and dermis. The skin lesions also had increased pS62-Myc and expression of a Myc target, Cdk4. Papilloma initiation was induced and the number of skin and bone marrow stem cells was increased in the B56α knockout mice. Because of B56α's role in negatively regulating several key oncogenes such as Myc and β–catenin, our model suggests that B56α is important for homeostasis of these cells, and B56α loss can lead to increased activity of these oncogenes contributing to aberrant cell proliferation.

Results

The homozygous knockout mice have hypomorphic expression of B56α

Our lab has previously shown that PP2A-B56α interacts with the N-terminus of Myc, that overexpression of B56α results in a reduction of Myc levels, and that inhibition of B56α leads to Myc overexpression and an increase in transcriptional activity (Arnold and Sears 2006). More recently, it was shown that B56α activity is required for normal cell proliferation and that loss of B56α induced transformation in human cells (Sablina, Hector et al. 2010). Despite this important role in cell transformation, to our knowledge, the physiologic role of PP2A-B56α *in vivo* has not yet been studied. Thus, we wanted to generate a B56α knockout mouse model. The *PPP2R5A (B56α)* gene was knocked out using the gene trap vector developed by Texas A&M Institute for Genomic Medicine (TIGM) and B56α knockout mice were generated (Fig. 3.1A) (Hansen, Markesich et al. 2008). The gene trap vector, which contains a splice acceptor (SA) site followed by the *lacZ* gene for β-galactosidase (β-gal) expression, integrated into the first intron of B56α. To our surprise, the B56α$^{-/-}$ mice were born healthy in normal ratios, were fertile, and had no obvious developmental defects. To measure the B56α mRNA expression, I performed qRT-PCR using multiple tissues from a group of 8-week old littermates (Fig. 3.1B). I noticed that there was a persistence of low expression of B56α in the homozygous knockout mice. qRT-PCR analysis

of B56α in mouse embryonic fibroblasts (MEFs) showed the same results, and were compared to a no reverse transcriptase (noRT) control to ensure no genomic contamination (Fig. S3.1A). B56α mRNA expression was also measured in the skin of a separate group of mice (Fig. 3.1C). Because there was some expression of B56α in the homozygous knockout mice, I speculated that there could be bypass of the splice acceptor site in the inserted *LacZ* gene trap. To test this I designed primers that amplify only exon 1 or from exon 1 to 3. In the case of complete splice acceptor usage, we would expect to see amplification of exons1-3 only in the wild-type mice (see schematic of exons and primers in Fig. 3.1D). PCR analysis of cDNA from different tissues and MEFs showed that exons1-3 was amplified in the wild-type B56α as expected (Fig. 3.1D). Surprisingly, exons1-3 was also amplified in the B56α$^{-/-}$ cells, to a lesser degree, implying that there was leaky expression of B56α mRNA in the B56α$^{-/-}$ mice. These mice will be henceforth referred to as B56α$^{HM/HM}$.

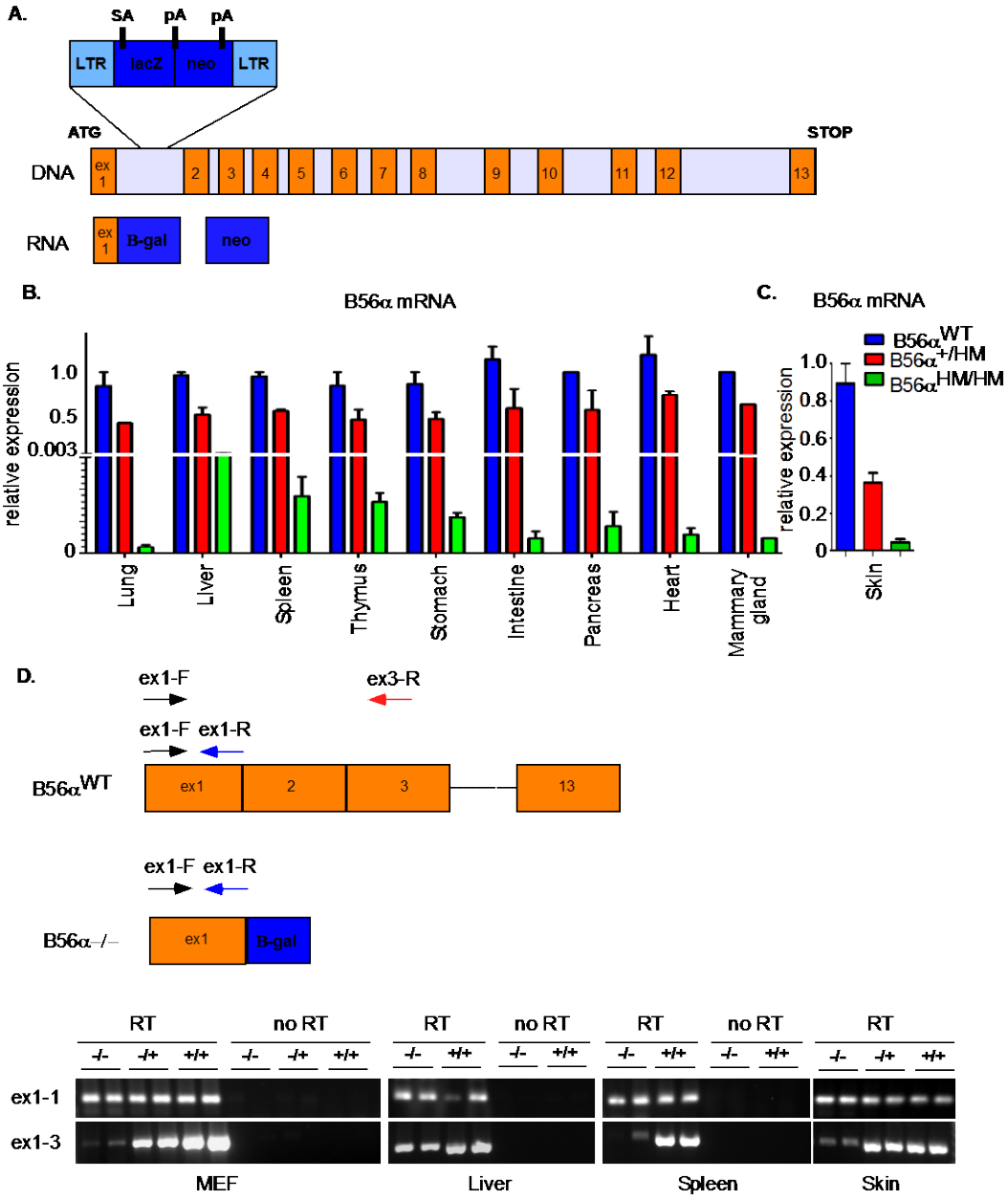

Figure 3.1. Generation of transgenic mice with hypomorphic expression of B56α. A) *PPP2R5A (B56α)* gene is knocked out by a gene-trapping vector. The vector contains a splice acceptor (SA) site immediately upstream of a promoterless *lacZ* gene. Its integration into

intron one leads to a fusion transcript being generated from the upstream exon one of *B56α* and *lacZ* upon transcriptional activation of gene. B) qRT-PCR analysis of B56α mRNA expression in different tissues from 2 mice per genotype. Relative expression is calculated by ΔCT normalized to wilt-type B56α. The lower part of Y-axis in in semi-log. C) qRT-PCR analysis of B56α mRNA expression in normal skin (mice with lesions in Fig. 2). D) PCR analysis of exon1-exon1 and exon1-exon3 transcripts in different tissues and MEFs from three genotypes.

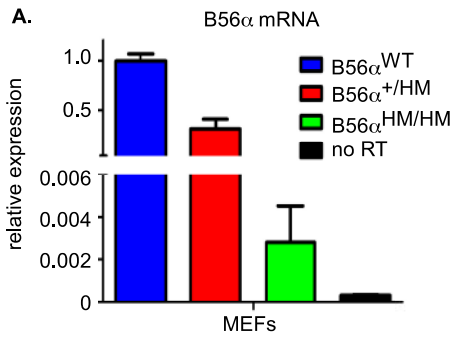

Figure S3.1. B56α expression in the mouse embryonic fibroblast cells. A) qRT-PCR analysis of B56α mRNA expression in MEFs. Relative expression is calculated by ΔCT normalized to wild-type B56α.

B56α knockout mice develop spontaneous skin lesions

To understand the physiological function of B56α, a cohort of the B56α$^{HM/HM}$ mice were generated and aged. Although the hypomorphic expression of B56α was in the whole body, the primary phenotypic effect I observed was spontaneous skin lesion formation with hair loss (Fig. 3.2A). About 30% of the B56α$^{HM/HM}$ mice developed skin lesions between 11 to 21 months of age and had to be taken down because of poor body conditions (Fig. 3.2B). One mouse had no skin lesions, but had a 2cm tumor mass under the skin in the dermis (bottom right H&E in Fig. 3.2C). At the time of sacrifice because of the poor body condition, some of the B56α$^{HM/HM}$ mice had an enlarged liver (mild portal lymphocytic inflammation) or liver lesions that looked undifferentiated (Fig. S3.2A and B). Some mice also had enlarged spleens that will be discussed later (Fig. 3.5). The rest of the B56α$^{HM/HM}$ mice showed no obvious phenotype and looked healthy and normal to the study's endpoint. Only one mouse in the B56α$^{+/HM}$ group had to be sacrificed because of a Harderian gland tumor in the eye (gland within the eye's orbit of mice which does not exist in human)**.** I also found a few B56α$^{HM/HM}$ mice developing slit-eye phenotype (squinting) that I did not further characterize. The H&E staining of the skin lesions showed hyperproliferation of the epidermis, hair follicles, sebaceous glands, and dermis (Fig. 3.2C). Ki67 staining of normal skin showed that there was no difference in the number of proliferative cells between the two genotypes (on the left, Fig. 3.2D). However, the number

of proliferative cells was increased in the skin lesions when compared to normal skin (on the right, Fig. 3.2D and Immunofluorescence pictures).

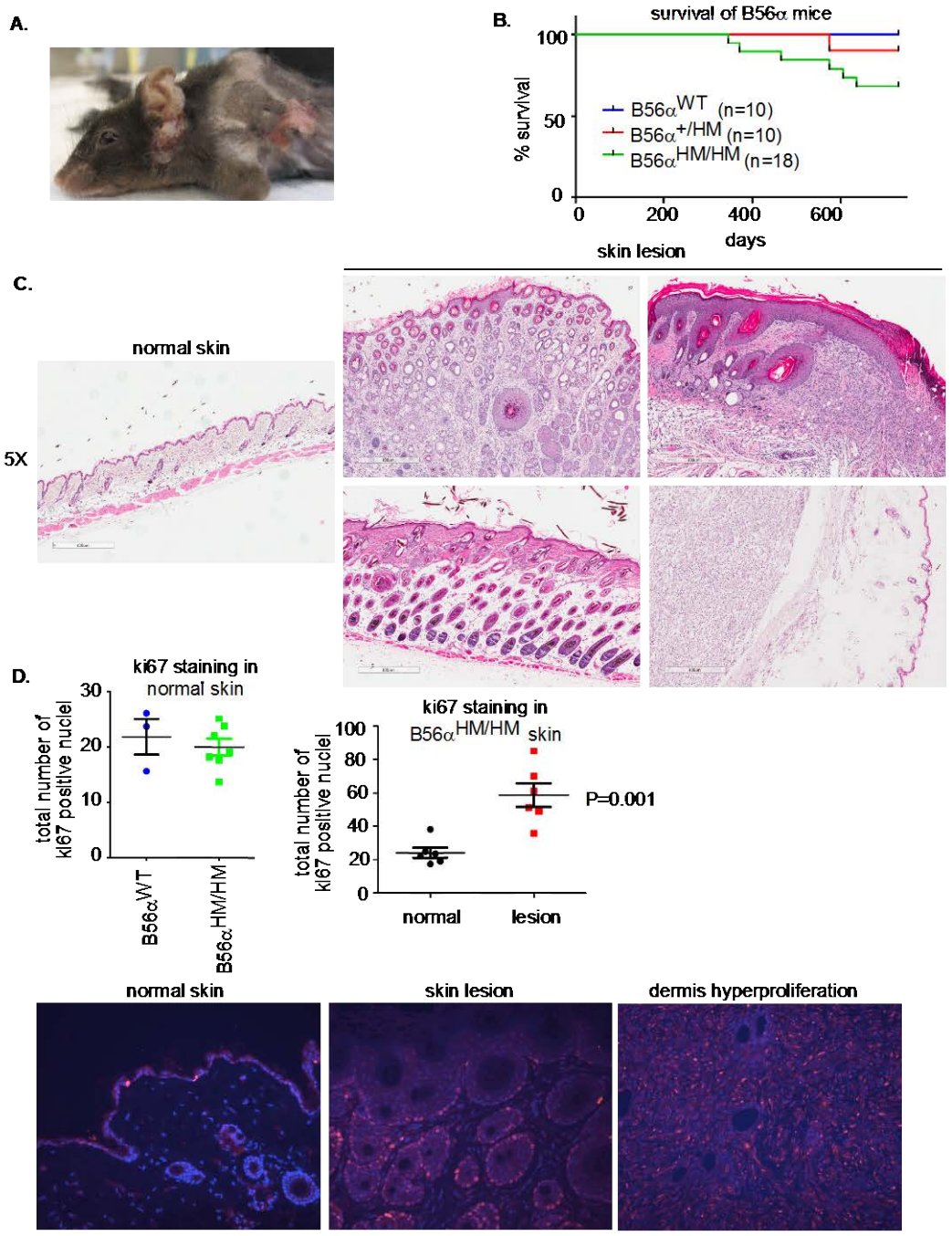

Figure 3.2. B56α knockout mice develop spontaneous lesions. A) A representative picture of spontaneous skin lesions. B) Survival of a cohort of B56α mice. C) H&E staining of skin lesions. D) Ki67 staining in normal

skin of three genotypes, and normal and skin lesions. A representative picture is shown. p-value is from a two-tailed student t test.

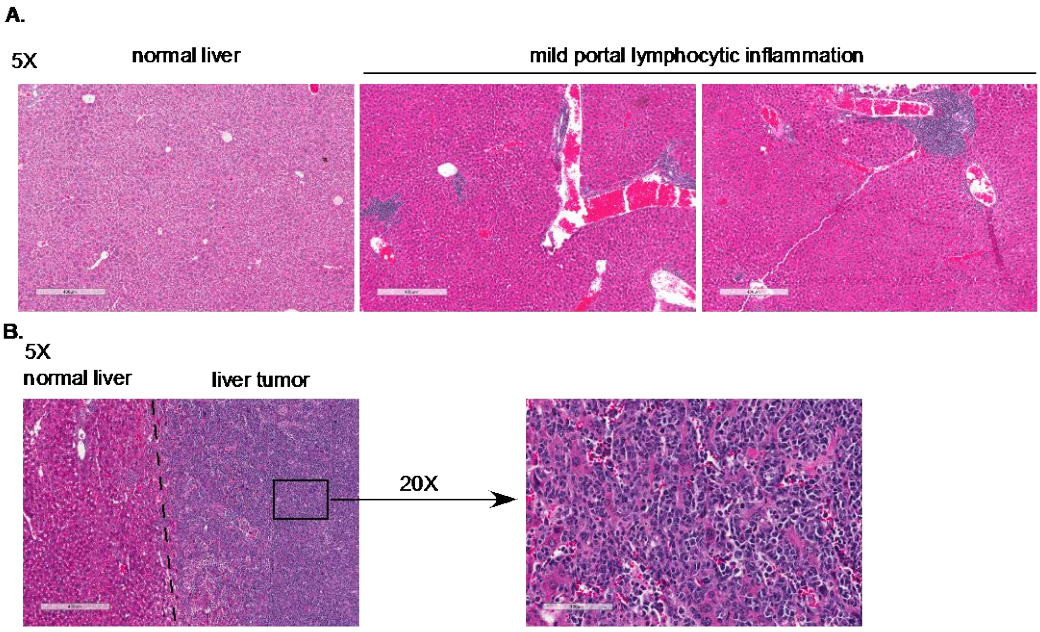

Figure S3.2. B56α knockout mice develop mild portal lymphocytic inflammation. A and B) H&E staining of the liver from mice with skin lesions. B) H&E staining of a liver tumor from one mouse with skin lesions.

Myc is increased in B56α$^{HM/HM}$ skin lesions

Because PP2A-B56α interacts with Myc and dephosphorylates S62, we analyzed pS62-Myc expression in the normal skin and skin lesions. Immunofluorescent analysis showed that pS62-Myc was increased in the skin lesions when compared to the normal skin of B56α$^{HM/HM}$ and wild-type B56α mice (Fig 3.3A). Next we measured Cdk4 expression, a Myc target, in the normal skin samples and found that the levels of Cdk4 expression were similar among the three genotypes (Fig 3.3B). However, Cdk4 was upregulated in the skin lesions compared to the normal skin of B56α$^{HM/HM}$ mice (Fig 3.3C).

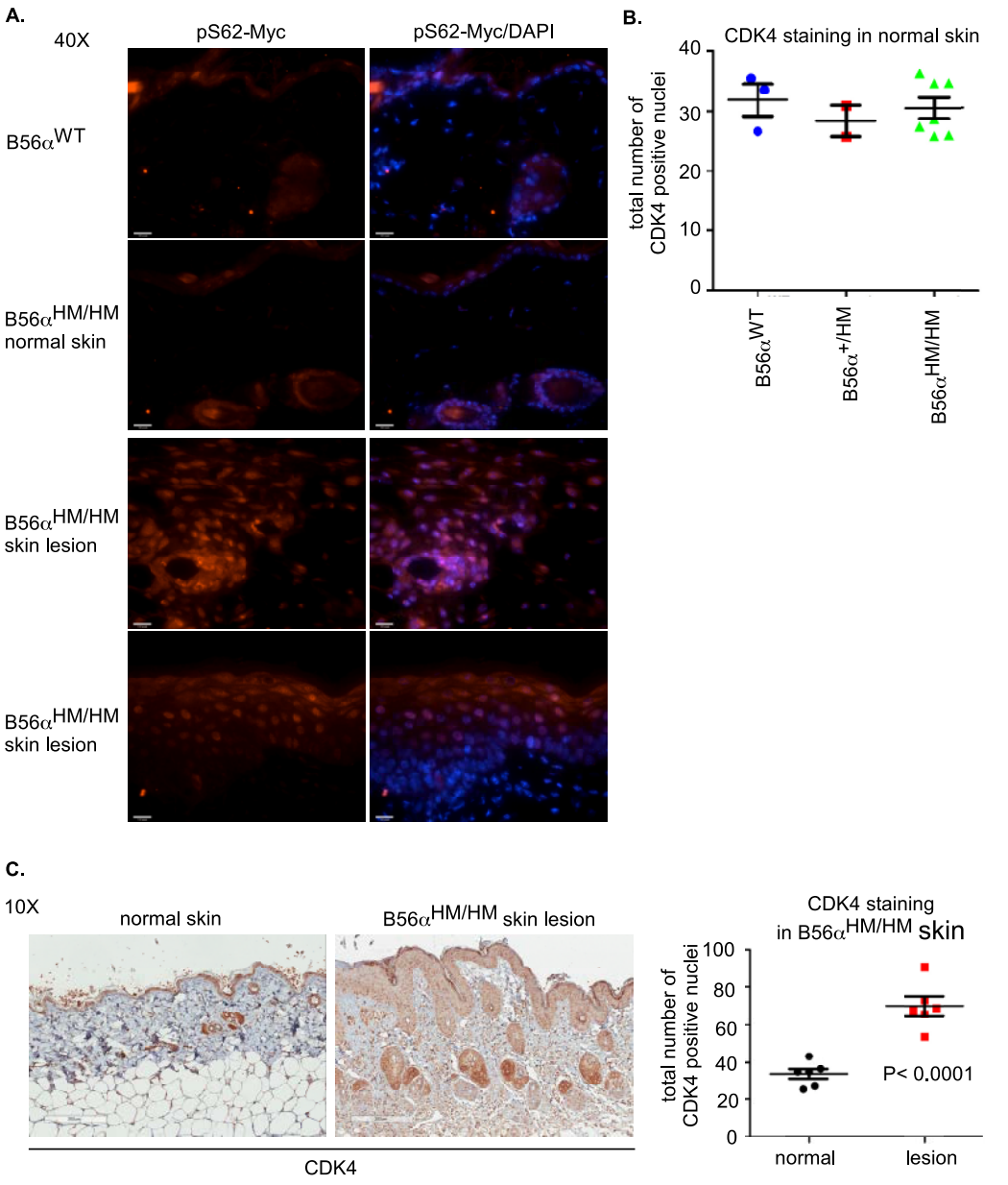

Figure 3.3. Myc is increased in the skin lesions. A) pS62-Myc staining of normal and skin lesions. B) Cdk4 staining in normal skin of three genotypes. C) IHC of Cdk4 and its quantification in skin lesions compared to normal skin. Quantification of nuclear Cdk4 staining was done by using the Aperio ImageScope 11.2.0.780 (Aperio Technologies).

B56α depletion accelerates the formation of skin papillomas in mice and its expression is decreased in human skin lesions

Because the primary effect of B56α depletion was the formation of spontaneous skin lesions, I asked whether B56α played a role in cell transformation of the skin epidermis. To address this, I decided to perform the DMBA/TPA chemical carcinogenesis assay DMBA initiates tumor formation by inducing mutations in critical genes such as Ha-Ras or in stem cells in the bulge region of hair follicles or the basal compartment of interfollicular epidermis; TPA promotes tumor growth by inducing inflammation and changing gene expression and expansion of an initiated stem cell population (Abel, Angel et al. 2009). First, I backcrossed the B56α mice to an isogenic FVB background as they should show a more uniform response and they are reported to be more sensitive to tumor promotion by TPA. FVB mice are also reported to be more susceptible to squamous cell carcinoma (SCC) development (Abel, Angel et al. 2009). The DMBA/TPA treatment of a cohort of mice showed that depletion of B56α induced earlier formation of skin papillomas in the $B56\alpha^{HM/HM}$ mice (Fig. 3.4A). Papillomas formed 43 days after initiating TPA treatment in the $B56\alpha^{+/HM}$ and $B56\alpha^{HM/HM}$ mice, whereas they appeared 71 days after TPA treatment in the wild-type mice. After 20 weeks of TPA treatment, the total number of papillomas was counted, and no significant difference in the total number of papillomas was found between the three genotypes (Fig. 3.4B). In addition, analysis of the total

number of papillomas and SCC conversion also showed no difference between genotypes (Fig. 3.4C).

To understand the relevance of B56α in human skin lesions, qRT-PCR analysis of B56α mRNA expression was performed. B56α mRNA expression was decreased in many types of human skin lesions (Fig. 3.4D) and in about 10 out of 13 human squamous cell carcinoma (SCC) samples (Fig. 3.4E). Western blot analysis of B56α protein levels in the same human SCC samples showed that B56α protein expression was also reduced in these samples (Fig. 3.4F).

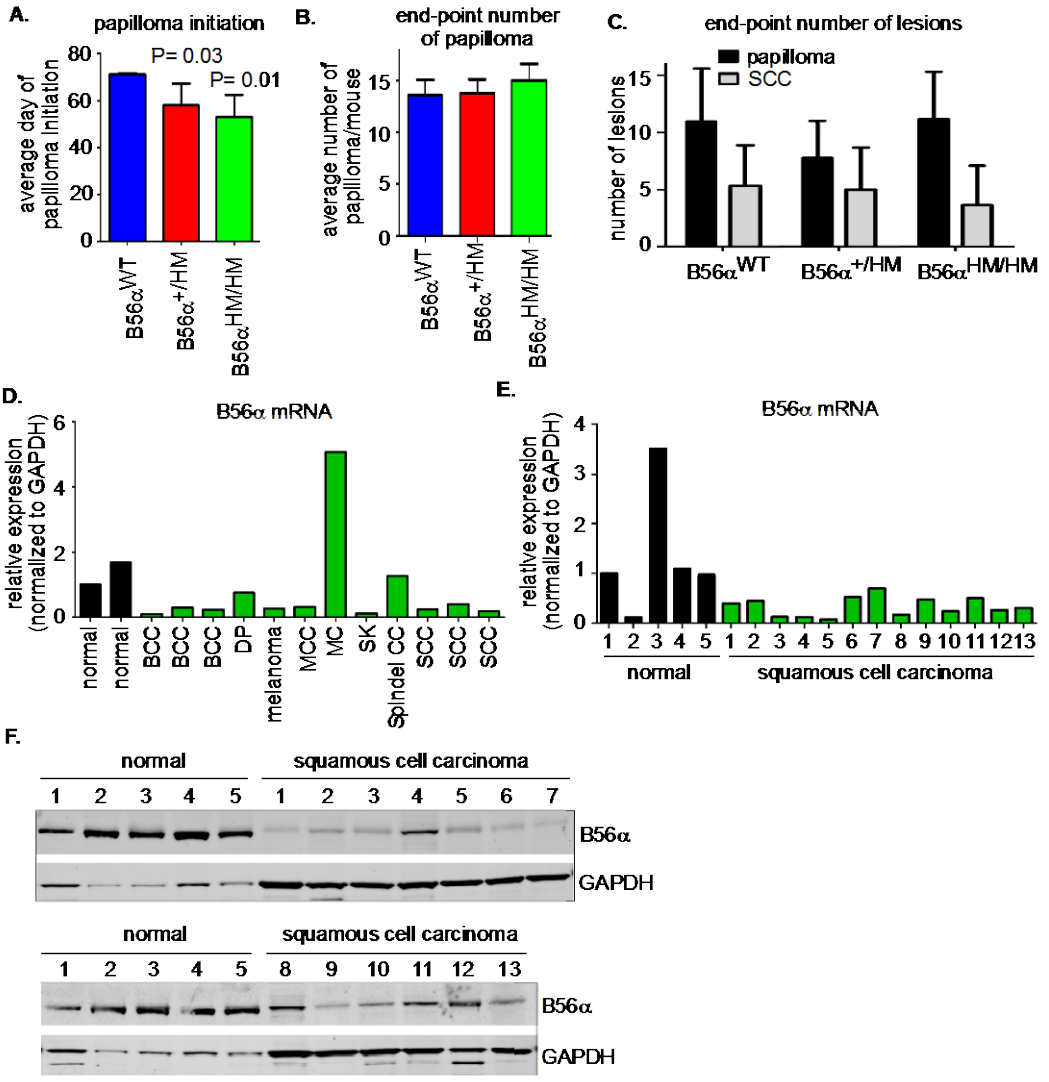

Figure 3.4. B56α depletion induces the formation of skin papilloma in mice and its expression is decreased in human skin lesions. A) Average of days for papilloma initiation after performing the two-stage DMBA/TPA chemical carcinogenesis. B) Average number of papillomas on the end point after performing the DMBA/TPA treatment. C) Total number of papillomas and SCC conversion over the entire cohort of mice. D and E) qRT-PCR analysis of B56α mRNA expression in different skin

lesions (in D) and 13 squamous cell carcinoma (SCC) samples (in E). BCC: Basal Cell Carcinoma, DP: Dermatofibrosarcoma Protuberans, MCC: Merkel Cell Carcinoma, MC: Mucinous Carcinoma, SK: Seborrheic Keratosis, Spindel CC: Spindel Cell Carcinoma. F) Western blot of B56α protein expression in SCC samples used in E.

B56α depletion increases the number of stem cells

New studies suggest that PP2A plays an important role in regulating human embryonic stem cell (hESC), where the levels of both PP2A C and PP2A A, as well as the overall PP2A activity, gradually increase during the course of hESC differentiation (Yoon, Jun et al. 2010). PP2A regulates many key factors that are important for stem cell self-renewal including PI3K/Akt, Wnt/β-catenin, and Myc, but it is still unclear which B subunits are important for this process. Because B56α negatively regulates both β-catenin and Myc, I decided to look at skin stem cell populations. I performed a Bromodeoxyyuridine (BrdU) long-term label-retaining assay in the skin of mice. BrdU incorporates into DNA of highly proliferative cells during neonatal time period and after a chase period, only the slow-cycling stem cells remain labeled (Cotsarelis, Kaur et al. 1999). I injected 10 day olds pups with BrdU and after 75 days harvested dorsal skin tissues. I found that B56α loss increased the number of BrdU long-term label-retaining cells (Fig. 3.5A).

Furthermore, upon necropsy analysis of mice with skin lesions I found that some of the mice had enlarged spleens or livers. H&E staining of spleen tissues indicated that B56α depletion increased immune cell infiltrations in mice with skin lesions, which is suggestive of extra-medullary hematopoiesis (Fig. 3.5B). Extra-medullary hematopoiesis can be caused by changes in the bone marrow, including increased hematopoiesis or fibrosis of bone marrow, which results in hematopoiesis

outside of the medulla of bone. It presents as hematopoietic masses in various typical and atypical body locations including the spleen, liver, lymph nodes, thymus, heart, breasts, prostate, broad ligaments, kidneys, skin, peripheral and cranial nerves, and the spinal canal (Sohawon, Lau et al. 2012). Extra-medullary hematopoiesis is a compensatory mechanism due to ineffective erythropoiesis, fibrosis in bone marrow, or is found in under-transfused patients suffering from myeloproliferative disorders (myelofibrosis, leukemia, lymphoma) (Sohawon, Lau et al. 2012).

To confirm increased hematopoiesis, we performed a Colony-Forming Unit (CFU) assay using bone marrow derived cells harvested from wild-type or B56a$^{HM/HM}$ mice. Cells were infected with a control virus or BCR-ABL (to drive colony formation) and then plated for the CFU assay with or without cytokines (described in (Agarwal, Bumm et al. 2008)). In the no cytokine conditions, B56α$^{HM/HM}$ cells formed more colonies compared to the wild-type B56α with or without BCR-ABL (on the left, Fig. 3.5C), suggesting that B56α reduction increases the number of hematopoietic stem cells. When plated with cytokines, however, the B56α$^{HM/HM}$ cells only formed more colonies compared to the wild-type mice when they were infected with BCR-ABL virus (on the right, Fig. 3.5B). This indicates BCR-ABL cooperates with loss of B56α to transform cells.

The H&E staining of liver from mice with skin lesions showed that these mice also had mild portal lymphocytic inflammation (Fig. S3.2A).

Given that both spleen and liver were enlarged in mice with skin lesions, these results imply that B56α also plays an important role in the immune system, which will require further analyses to elucidate.

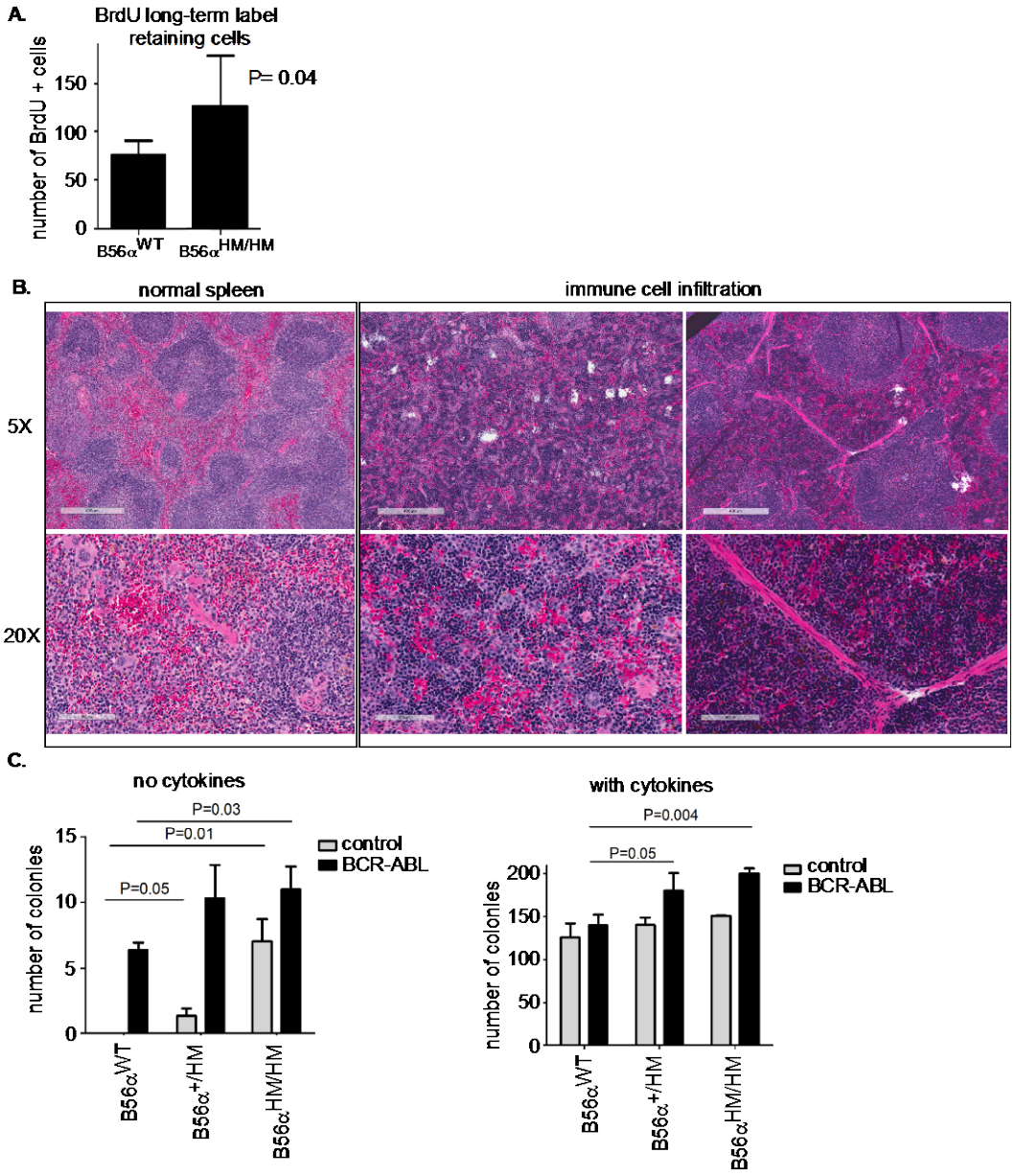

Figure 3.5. B56α depletion increases the number of stem cells. A) Total number of BrdU long-term retaining cells in a cohort of mice (n=8). B) H&E staining of the spleen from mice with skin lesions. C) CFU assay of bone marrow harvested from mice. Cells were infected with viruses 2 times and plated in MethoCult with and without cytokines.

Discussion

B56α plays an important role in regulating PP2A's function toward key oncoproteins such as Myc, β-catenin, and Bcl-2. In this study I report that B56α depletion in a mouse model contributes to the formation of skin lesions, which show hyperproliferation in the epidermis, hair follicles, sebaceous glands, and dermis. Some of the phenotypes we observed in terms of the skin lesions and hair loss resembled the phenotypes of previously published K14.MYC2 mice (Waikel, Kawachi et al. 2001). These mice were shown to gradually lose hair and develop spontaneous ulcerated lesions, which resulted from severe impairment in wound healing. Interestingly, similar to what we found in B56a$^{HM/HM}$ skin lesions, K14.MYC2 mice had hyperproliferative and enlarged sebaceous glands (Waikel, Kawachi et al. 2001). When the K14.MYC2 mice were exposed to chemical (DMBA/TPA) carcinogens, they developed sebaceous adenomas (Honeycutt, Waikel et al. 2010). In contrast, with our DMBA/TPA treatment, we observed earlier development of papillomas, but no difference in total number or progression of those lesions. It is possible that we might have seen similar results to the K14.MYC2 model if we had performed these experiments for a longer time (20 weeks vs. 27 weeks). Another study showed that ectopic expression of MYC (c-Myc-ER-TM) in the suprabasal epithelial layers of the epidermis and hair follicle resulted in desynchronization of the hair growth cycle associated with a marked increase in cell proliferation along the length of the hair outer root

sheath (Bull, Pelengaris et al. 2005). In addition to MYC, aberrant β-catenin activation, which is another target of B56α, has been implicated in de novo hair follicle formation and hair tumors (Gat, DasGupta et al. 1998). It is thought that the follicular bulge retains stem cells that can be a source for both normal hair cycle and sebaceous glands renewal. Both hair follicles and sebaceous glands were increased in our skin lesions, suggesting a role for B56α in maintaining the stem cells within the follicular bulge. When I analyzed the skin lesions I observed that pS62-Myc and its target gene Cdk4 were elevated in the lesions. Normal skin did not have obvious increased pS62-Myc or Cdk4, suggesting that some unknown trigger, potentially mechanical stress, resulted in the accumulation or activation of pathways that led to an increase in pS62-MYC.

Although the skin lesions that developed in the B56a$^{HM/HM}$ mice were benign, the mice had to be sacrificed because of poor body conditions. The majority of the mice with skin lesions had increased immune cell infiltrations suggestive of extra-medullary hematopoiesis in their spleen and some also had lymphocytic inflammation in their livers, suggesting that B56α might regulate homeostasis of the immune system as well. Further flow cytometry analysis is necessary to better dissect out different cell populations in bone marrow, blood, spleen, and thymus.

In previous work, Myc has been shown to control the balance between hematopoietic stem cell (HSC) self-renewal and differentiation in the bone marrow (Wilson, Murphy et al. 2004). Normally, HSCs exist in

proximity to osteoclasts within the bone marrow, and expression of Myc is required to push cells out of their bone marrow niche to become transiently amplifying cells. Myc is then downregulated prior to full differentiation (Wilson, Murphy et al. 2004). Myc depletion has been shown to result in an increased number of stem cells in the bone marrow. In our mouse model, we observed increased immune cell infiltrations, suggesting that B56α loss, which could cause elevated Myc, may then push cells out from bone marrow.

One of the B56α$^{+/HM}$ mice developed a Harderian gland tumor and some of the B56α$^{HM/HM}$ mice had a slit-eye phenotype. I did not characterize this phenotype at the time of manifestation, but I later found that B56α expression could be important in the Harderian gland. It has been shown that in a transgenic mouse model of mutant PP2A C subunit (L309A), Harderian glands have hypoplasia and delayed postnatal development, which results in the slit-eye phenotype (Schild, Isenmann et al. 2006).

Given that the primary phenotype of B56α loss was the formation of skin lesions, I performed two-stage DMBA/TPA carcinogenesis experiments to understand whether B56α depletion could affect papilloma formation. I found that papilloma formation was induced earlier with reduced expression of B56α, but the total number of the papillomas at end stage was not affected, suggesting that B56α might be important for suppressing the initiation of these papillomas, but likely does not impact

the progression step. Whether a complete knockout of B56α would enhance tumor formation still remains to be tested. B56α expression was shown to be suppressed in melanoma tumors and negatively correlated with metastatic tumors (Mannava, Omilian et al. 2012), suggesting that B56α loss might be important for melanoma progression. In addition, B56α controlled oncogene-induced senescence and B56α knockdown suppressed $BRAF^{V600E}$- and $NRAS^{Q61R}$-induced senescence (Mannava, Omilian et al. 2012). I speculate that in our two-stage DMBA/TPA experiment, B56α depletion enhanced tumor formation but at the same time, because of its leaky expression, it enhanced oncogene-induced senescence. Another possibility is that because B56α negatively regulates p53 (Harris and Levine 2005), p53 becomes active in papillomas formed in the $B56\alpha^{HM/HM}$ mice, and therefore suppresses further transformation. Similar to melanoma tumors, I found that B56α expression was reduced in human SCC samples.

Because of new evidence suggesting that PP2A activity gradually increases during the course of hESC differentiation (Yoon, Jun et al. 2010) and that PP2A-B56α regulates many key factors that are important for stem cell self-renewal including Wnt/β-catenin and Myc, I asked whether the number of stem cells in skin and bone marrow were affected by B56α reduction. I found that the number of stem cells was increased in skin and bone marrow of $B56\alpha^{HM/HM}$ mice. This conflicts with the results from the K14.MYC2 mice because these mice had a 75% reduction in the number

of skin stem cells (Waikel, Kawachi et al. 2001). There are two potential contributing factors to these different results, 1) high level Myc expression such as would be driven by the K14 promoter can induce apoptosis, and 2) B56α also negatively regulates β-catenin, which is important for stem cell self-renewal. More experiments have to be done to elucidate the role of B56α in stem cell self-renewal and maintenance.

In summary, I report a novel mouse model of B56α hypomorph and show that, although B56α may not be required for normal development, it is important for normal homeostasis of cells. Potentially due to mechanical stress, the primary phenotype observed in these mice is spontaneous skin lesions. They also have increased immune cell infiltrations suggestive of extramedullary hematopoiesis, and lymphocytic inflammation in the spleen and liver. Whether increased inflammation contributes to the skin lesions or is caused by skin lesions remains to be understood. Increased pS62-Myc and Myc activity is one of the consequences of B56α loss. Our model suggests that B56α is important for cells to maintain their normal homeostasis and B56α loss can lead to increased activity of important oncogenes such as Myc, contributing to aberrant cell proliferation and loss of stem cell homeostasis.

Materials and Methods

The B56α Knockout Mice

Mice were handled in accordance with the Oregon Health and Science University Institutional Animal Care and Use Committee. The B56α knockout mice (pure C57BL/6N background) were generated from embryonic stem (ES) cell clones carrying a gene trapping vector in the *PPP2R5A (B56α)* gene by the Texas A&M Institute for Genomic Medicine (TIGM) (Hansen, Markesich et al. 2008).

Quantitative-PCR (qPCR)

RNA was isolated from homogenized mouse or human tissue using TRIzol reagent (Invitrogen) and cDNA was made as described previously (Zhang, Farrell et al. 2012). qPCR was then performed using TaqMan primers for human B56α (Hs00196542-m1), mouse B56α (Mm00523125-m1), human GAPDH (Hs02786624-g1). The relative fold change was measured by the $\Delta\Delta(C_T)$ method described previously (Farrell, Pelz et al. 2013).

Primers used for PCR analysis of exon1-1 and exon1-3: Forward-ex1 (5'-CGCACCAGUUGCCUGCGC-3'), Reverse-ex1 (5'-GGCAGGGGGTGCAGCTCC-3'), and Reverse-ex3 (5'-GCCAAGAGGCCTCAAGTGTGG-3').

Antibodies for Western Blot, Immunofluorescence (IF), and Immuno-histochemistry (IHC)

Western blot analysis was performed as described previously (Arnold, Zhang et al. 2009). Immunoblots were visualized using the Odyssey IR imager (LI-COR) that can detect both Fluor 680 and IRDye 800 secondary antibodies (1:10000). Antibodies used for western blot include: B56α (sc-6116; 1:200; Santa Cruz Biotechnology) and GAPDH (AM4300; 1:10000; Ambion).

Mouse tissues were collected and fixed in 10% formalin-neutral buffer. Parrafin embedding and Hematoxylin and Eosin (H&E) staining were performed at OHSU Histopathology Core. Antibodies used for IF or IHC: pS62-MYC antibody (1:100) we developed as previously described (Zhang, Farrell et al. 2012), Ki67 antibody (1:1000, Novocastra, NCL-Ki67-MM1), anti-BrdU (1:200, MCA2060, AbD serotec), Cdk4 (sc-260; 1:50; Santa Cruz Biotechnology). IHC images were scanned and quantified by Aperio ImageScope 11.2.0.780 (Aperio Technologies).

Two-stage DMBA/TPA Chemical Carcinogenesis

The back skin of seven-week old mice was shaved 2 days prior dimethylbenz[a]anthracene (DMBA) treatment. After 2 days skin were painted with DMBA (100μg per 100 μl acetone) (D3254, Sigma-Aldrich). After seven days mice were again shaved and treated with 2.5μg 12-O-tetradecanoylphorbol-13-acetate (TPA) (P-1680, LC Laboratories) in 100

µl of acetone per mouse twice a week for twenty weeks. Mice were evaluated twice a week for papilloma development. At the end of TPA treatment all skin papilloma was collected and fixed in 10% formalin-neutral buffer.

BrdU Long-Term Label Retaining Cell Analysis

Newborn pups (10 days old) were injected with 50µl of BrdU (Invitrogen) every twelve hours for a total of four injections. Skin samples were collected 75 days after the injection and fixed in 10% formalin-neutral buffer. IF analysis was performed using anti-BrdU and total number of BrdU-labeled cells were counted from two tissue sections from each mouse.

Colony-Forming Unit (CFU) Assay

The CFU assay has been performed as described in (Agarwal, Bumm et al. 2008). Briefly bone marrow cells were harvested from three genotypes of mice. Cells were infected with $p210^{BCR\text{-}ABL}$ and control retroviruses (described in (Agarwal, Bumm et al. 2008) two times over 48 hours and then plated in triplicate in Methocult H4534 (Stem Cell Technologies, Vancouver, BC, Canada) with or without cytokines (Agarwal, Bumm et al. 2008). Colonies were counted after one week of incubation at 37°C in 5% CO2.

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Chapter Four

Summary and Discussion

Summary

MYC protein overexpression occurs in more than 70% of all human cancers. In contrast, only about 20% of tumors show *MYC* gene amplification. We have shown that increased protein stability is one of the important mechanisms of MYC overexpression in cancer. Our research demonstrated that the MYC protein degradation pathway could become impaired through several mechanisms, including aberrant Axin1 expression, which is the scaffold protein that nucleates the degradation complex for MYC (Zhang, Farrell et al. 2012). Here I have asked whether other members of this degradation complex are deregulated. PP2A-B56α dephosphorylates the stabilizing phosphorylation of MYC at the S62 residue (pS62), and enhances MYC degradation. PP2A is an important tumor suppressor that is inhibited in many cancers (Neviani, Santhanam et al. 2005; Sablina, Hector et al. 2010). PP2A can be inhibited by interacting oncoproteins SET and CIP2A. My research presented in chapter 2, revealed that 50% and 90% of breast tumor samples have high SET and CIP2A mRNA levels, respectively. I confirmed similar mRNA results in breast cancer cell lines (by qRT-PCR and RNA-seq), and found that SET and CIP2A protein levels are also overexpressed. Increased SET and CIP2A levels trended with increased pS62-MYC and total MYC protein. I found that although SET was not associated with any tumor subtypes, CIP2A overexpression was associated with the triple-negative, Caludin-low breast cancer subtype. This suggested that CIP2A could be a

potential biomarker for loss of PP2A tumor suppressor activity in these tumors.

Because SET and CIP2A were overexpressed in breast cancer, I knocked down these proteins in three breast cancer cell lines and showed that knocking down SET or CIP2A decreased the growth and tumorigenic potential of these cells both *in vitro* and *in vivo*. These results suggested that inhibiting SET or CIP2A could be a target for designing therapies for breast cancer.

Using a novel inhibitory peptide mimetic against SET, OP449, I investigated whether OP449 had therapeutic function in breast cancer cells. First I examined the effect of OP449 on the viability of breast cancer cell lines and showed that OP449 decreased cell growth and induced apoptosis both *in vitro* and *in vivo*. OP449 also decreased the growth of cells isolated from primary human breast tumor samples.

To investigate the mechanism of SET and CIP2A activity, I knocked down SET or CIP2A in several breast cancer cell lines and showed that pS62-MYC, and in some cases total MYC, was decreased. Similar to SET/CIP2A knockdown, I showed that OP449 treatment of cells decreased pS62-MYC and total MYC. To investigate if OP449 decreased MYC transcriptional activity, I performed ChIP assays and showed that MYC binding to its target gene promoters was reduced after OP449 treatment. To understand a more global effect of SET/CIP2A inhibition or

OP449 treatment on breast cancer cells, I performed RNA-seq analysis. Using Gene SET Enrichment Analysis (GSEA), I showed that MYC gene targets were un-enriched in the SET or CIP2A knockdown or OP449 treated cells. Finally, to show whether activity of OP449 was through MYC, I used MCF10A-TR-MYC cells that express either wild-type MYC or the oncogenic mutant MYC (MYC^{T58A}) with the addition of Doxycycline. MYC^{T58A} is no longer a target for PP2A and therefore MYC^{T58A} expression should be able to rescue OP449 treated cells. I showed that indeed, MYC^{T58A} decreased the effect of OP449, confirming that MYC was a relevant target for OP449.

To show that OP449 treatment activates PP2A, which then leads to decreased pS62-MYC levels, I treated cells with a PP2A inhibitor compound, Okadaic Acid, and showed that OA could inhibit OP449's effect on decreasing pS62-MYC. Knockdown of the PP2A C catalytic subunit also de-sensitized cells to OP449 treatment showing that OP449 inhibition of SET activates PP2A.

Taken together these results showed that PP2A activity was deregulated in breast cancer by SET or CIP2A overexpression and reactivation of PP2A, either through inhibiting SET or potentially CIP2A, could be a novel anti-tumor strategy to post-translationally target MYC in breast cancer.

My second project described in chapter 3 was to characterize a mouse model of PP2A-B56α knockout. The B56α gene was knocked down using a gene-trapping vector and the resulting mouse was intended to be a whole body knockout. However, I found that the gene-trapping vector was not 100% effective, resulting in some leaky expression of B56α (hypomorphic expression of B56α: $B56\alpha^{HM/HM}$). The primary phenotypic effects of reduced B56α expression was spontaneous skin lesion formation and increased immune cell infiltration. About 30% of the $B56\alpha^{HM/HM}$ mice developed skin lesions between 11 to 21 months, and most of them also showed increased immune cell infiltration suggestive of extramedullary hematopoiesis and some had lymphocytic inflammation in the liver. The skin lesions showed hyperproliferation of the epidermis, hair follicles, sebaceous glands, and dermis. I found that pS62-MYC and its target gene CDK4 were elevated in these lesions. To investigate whether B56α reduction was important for tumor formation in the skin, I performed the two-stage DMBA/TPA chemical carcinogenesis assay and showed that B56α reduction accelerated papilloma initiation. I measured B56α mRNA and protein expression in human squamous cell carcinoma samples and found that B56α expression was reduced in these samples. New data suggests that PP2A activity increases as cells differentiate, so I asked whether B56α loss affected the number of stem cells. I showed that in the B56α hypomorph mice, the number of skin or bone marrow stem cells was elevated. We conclude that, because of B56α's role in negatively

regulating many oncogenes such as c-MYC and β–catenin, B56α is important for normal homeostasis of cells and B56α loss can lead to increased activity of these oncogenes and subsequently aberrant cell proliferation and stem cell maintenance.

Discussion

Many important cellular signaling pathways are regulated by reversible protein phosphorylation. Many kinases and phosphatases have been shown to be impaired in cancer, with kinases being hyperactivated and phosphatases being suppressed. In chapter 1, I discussed the consequences of increased MYC activity through phosphorylation at Serine-62 in breast cancers. The interest in targeting the undruggable MYC prompted me to focus on PP2A, which negatively regulates MYC and enhances MYC degradation. The importance of PP2A in inhibiting human cell transformation *in vitro* has long been established. However, particularly in the case of breast cancer, the mutation analysis of different PP2A subunits has shown little evidence for the requirement of PP2A suppression for transformation. Using the cBioPortal analysis tool (The Cancer Genome Atlas-TCGA), mutations or loss of *PPP2C* (the C subunit), *PPP2R1* (A), and *PPP2R5A* (B56α) does not seem to be the primary reason for PP2A suppression in breast cancers (Figure 4.1) (Cerami, Gao et al. 2012; Gao, Aksoy et al. 2013). In addition to genetic changes, another mechanisms for PP2A suppression can be through epigenetic modification of different PP2A subunits. *PPP2R2B,* encoding B55β, has been shown to be hypermethylated in colorectal cancers, contributing to PDK1-mediated MYC phosphorylation and activation (Tan, Lee et al. 2010). As mutations seem to be low in PP2A subunits, further

studies need to be done to investigate the epigenetic modification of these genes.

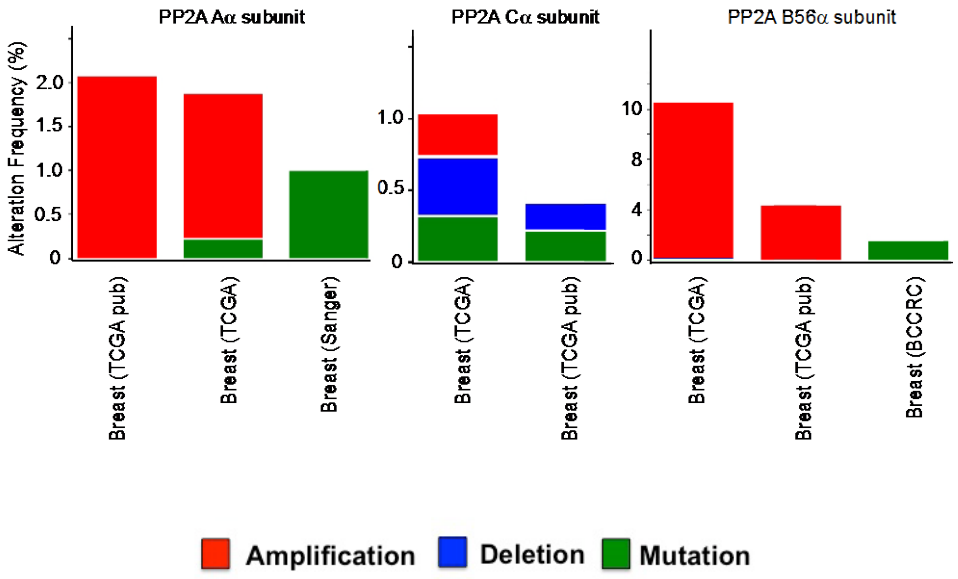

Figure 4.1.Mutation analysis of different PP2A subunits (Cerami, Gao et al. 2012; Gao, Aksoy et al. 2013).

At the protein level, many interacting proteins discussed in chapter 1 and tested in chapter 2, can control PP2A activity. SET and CIP2A in particular have been shown to be overexpressed in many cancers and I investigated their role in controlling PP2A and MYC in breast cancers (presented in chapter 2). The summarized findings from chapter 2 of this thesis are shown in Figure 4.2: PP2A can be suppressed by increased SET or CIP2A levels, contributing in part to MYC stabilization and

oncogenic activity, and inhibiting SET (and potentially CIP2A) will decrease tumor growth and result in tumor regression.

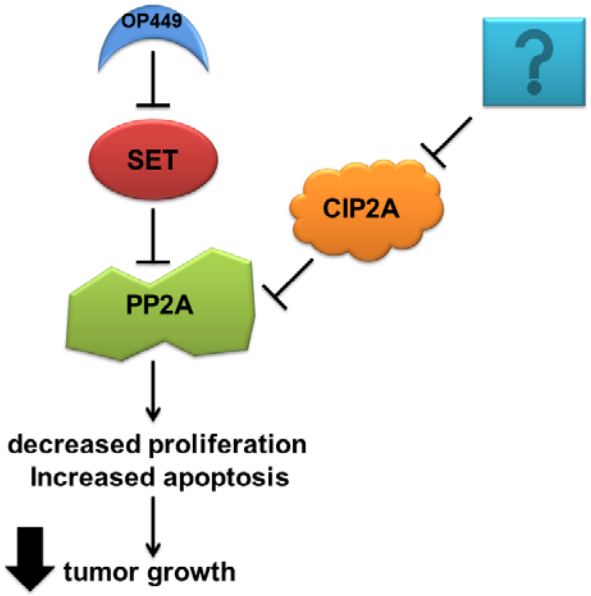

Figure 4.2. PP2A re-activation through inhibiting SET or CIP2A for breast cancer therapy.

SET antagonism with OP449 has been shown to facilitate downregulation of other targets, besides PP2A, including NFκB, nm23-H1, Akt, and Rac-1 (Switzer, Cheng et al. 2011). Rac-1 is an interesting target because Rac-1 has been shown to positively regulate migration and SET membrane recruitment was required for this process (ten Klooster, Leeuwen et al. 2007). It will be important to test whether OP449 can decrease cell migration or metastasis of breast cancer cells. This is

particularly interesting because some literatures suggest that MYC suppresses breast cancer metastasis by direct transcriptional silencing of α_v and β_3-integrin subunits, which are important in metastasis (Liu, Radisky et al. 2012). It is not known how OP449 affects tumor cell migration or whether this involves Rac-1 and/or and MYC.

The effect of OP449 in activating PP2A for breast cancer therapy validates the idea of using PP2A activators. The fact that CIP2A directly regulates MYC and its overexpression correlates with the Claudin-low breast cancer subtype, a subtype that is highly associated with upregulation of the MYC/MAX network (Heiser, Sadanandam et al. 2012), highlights the importance of CIP2A inhibition for this tumor subtype. CIP2A can potentially be used as a diagnostic biomarker for more malignant, MYC-driven tumor types and also can be a potential targeted therapy for these types of tumors. Another study has suggested that in p53-deficient breast cancer, CIP2A expression is enhanced directly by E2F1, which itself is induced by p53 or p21 inactivation (Laine, Sihto et al. 2013). CIP2A activation enhances E2F1 in an E2F1-CIP2A feedback loop and causes resistance to senescence-inducing therapies (Laine, Sihto et al. 2013). Therefore CIP2A can also serve as a prognostic marker for response to senescence-inducing therapies in p53-deficient tumors and potentially inhibiting CIP2A may enhance the response to these therapies.

It is now clear that only a small number of cancer patients will benefit from single therapy due to acquired resistance and that OP449 or

other PP2A activators may need to be used in combination with other therapeutic agents. In the future, it will essential to test whether inhibiting SET by OP449 will affect CIP2A expression or other PP2A cellular inhibitors as a compensatory mechanism for escaping the response to OP449. Another potential compensatory mechanism is downregulation of PP2A subunits, which needs to be tested. In addition, a new study has shown that OP449 in combination with ABL1 tyrosine kinase inhibitors inhibited growth of CML cells from patients who had blast phase disease and patients harboring highly drug-resistant BCR-ABL1 mutations (Agarwal, Mackenzie et al. 2014). It will be important to test whether PP2A activating compounds increase the efficacy of single therapies in breast cancer.

Another important question will be to identify different PP2A complexes that are affected by OP449 or other PP2A activating compounds discussed in chapter 1 including Ceramide, FTY720, and Forksalin. Similar to OP449, Ceramide has been shown to release PP2A from SET inhibition (Mukhopadhyay, Saddoughi et al. 2009). FTY720 directly binds to SET and reactivates PP2A (Saddoughi, Gencer et al. 2013). The mechanism of PP2A activation by Forskolin is not understood well. B56α, B56γ, and PR70 were shown to be important for maintaining normal cell proliferation and loss of these subunits induced cell transformation (Sablina, Hector et al. 2010). It will be interesting to determine how these compounds regulate different PP2A complexes,

specifically if they affect any of these three subunits and whether combining these compounds will have a better therapeutic efficacy.

Using our B56α hypomorph mouse model we will be able to address some of the questions regarding the specificity of PP2A activators and activation of different pathways in response to these drugs. Since my data in chapter 2 showed that MYC was affected by OP449, using this mouse model we will be able to address other targets of OP449. One caveat is that because the mouse is not a full B56α knockout, the drugs might again affect MYC and we will not be able to differentiate between the B56α-dependent and the other compensatory mechanisms. This will still be helpful because if reduced expression of B56α is more relevant that its loss or mutation (according to TCGA data on different tumor types or melanoma/SCC samples), using the PP2A activator is not unreasonable.

Through our B56α mouse model, I found that loss of B56α expression results in the formation of spontaneous skin lesions and increased immune cell infiltration into the skin, liver, spleen and lymph nodes. Several important questions stem from this work. First, is skin a primary tissue that is affected by B56α loss? Reduced expression of B56α expression in human SCC samples, both at the mRNA and protein levels, suggested that B56α could play an important role in normal skin homeostasis. Second, are PP2A activator compounds good therapeutic agents for skin cancers? Another study showed that B56α expression was decreased in human melanoma cancers (Mannava, Omilian et al. 2012)

suggesting that PP2A activators might be useful for treatment of these types of tumors.

Injury to the skin initiates a complex process of events involving inflammation and formation and remodeling of new tissue. P63, one of B56α's targets, has been shown to regulate wound healing in the mouse epidermis and play an important role in maintaining skin stem cells (Suzuki and Senoo 2012; Suzuki and Senoo 2013). Further experiments are required to investigate whether P63 is deregulated in skin lesions and whether impaired wound healing pathways are responsible for formation of spontaneous lesions in the B56α hypomorph mouse model.

Finally, B56α depletion resulted in an increased number of hair follicle stem cells and potentially bone marrow stem cells, shown by their increased colonogenic capacity (shown in chapter 3). B56α regulates many oncogenes including MYC, β-catenin, Bcl-2, p63, and p53 (reviewed in (Eichhorn, Creyghton et al. 2009)). Both MYC, β-catenin, and p63 have been shown to affect maintenance and proliferation of hair follicles in the skin (Gat, DasGupta et al. 1998; Bull, Pelengaris et al. 2005). It will be important to understand how loss of B56α affects the pathways in regulating stem cells or skin lesions. *The overall findings of this research dissertation suggest that PP2A (-B56α) activity is important for normal cell homeostasis and suppression of PP2A, either through loss of the tumor suppressor B subunits or overexpression of cellular inhibitors such as SET*

and CIP2A, results in cell transformation. Therefore PP2A is a good target for cancer therapies.

Appendices

Contribution to Figures:

Figure 2.1. qPCR analysis of SET and CIP2A was provided by Dale Christensen

Figure 2.4. Brittany L. Allen-Petersen performed the MTS assay using primary human breast tumor samples treated with OP449.

Figure 2.5. Amy Farrell performed the ChIP assays. Carl Pelz helped with RNA-seq analysis and created the ranked gene lists.

Figure 2.6. OP449 detection in xenografts and *in vitro* PP2A phosphatase assay was provided by Dale Christensen.

Figure S2.1. RNA-set analysis of SET and CIP2A was provided by Carl Pelz.

Figure S2.4. Carl Pelz helped with RNA-seq analysis and created the ranked gene lists.

Figure S2.5. Amy Farrell performed the xenograft of MDA-MB-231 cells. Pharmacokinetics and stability of OP449 were provided by Dale Christensen.

Figure S3.4. Xiaoyan Wang helped with treating mice with DMBA/TPA

Figure S3.5. Anupriya Agarwal helped with CFU assays and flow cytometry.

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Education

2008-2014 Oregon Health and Science University, OR, U.S.A

PhD candidate in Cancer Biology, GPA: 3.34

2007-2008 University of Wisconsin-Milwaukee, WI, U.S.A.

Bachelor of Science, Major: Biology, 2008, GPA 3.67

Project: Regulation of bacterial bioluminescence, and its metabolic cost, Chuck Wimpee, UWM

2003-2006 Shiraz University, Shiraz, Iran

Major: Cell and Molecular Biology, GPA 15.44/ out of 20

Research Experience

2009-present: Identifying the role of the tumor suppressor PP2A-B56α in a novel mouse model, Rosalie Sears lab, OHSU

Techniques: mouse handling, mouse cell culture, cloning, RNA/DNA/protein extraction, primer design, PCR, sequencing, Immunohistochemistry analysis, flow cytometry

2009-2013: Targeting c-Myc by antagonizing PP2A inhibitors in breast cancer, Rosalie Sears lab, OHSU. Submitted to PNAS- in press.

Techniques: cell and molecular biology, signal transduction, cancer biology, human cell culture, stable and transient knockout, lentivirus production, SDS-PAGE and western blotting, RNA purification, PCR, viability assay, therapeutic analysis of drugs *in vitro* and *in vivo*, RNA-seq analysis, pathway analysis, orthotropic xenograft in mammary gland of NSG mice, IP and IV injection

Honors and Award

- Winner of the Student Oral Presentation Award, OHSU Research Week 2014
- Winner of the Student Oral Presentation Award, OHSU Research Week 2013
- Tartar Trust Fellowship, OHSU, 2011
- Dean's List Recognition, UWM, 2007

Publication

- **Janghorban M**, Farrell AS., Allen-Petersen B L, Pelz C, Daniel CJ**,** Oddo J, Langer EM**,** Christensen DJ, and Sears RC . Targeting Myc by antagonizing PP2A inhibitors in breast cancer. Proc Natl Acad Sci USA. 2014 Jun 9. pii: 201317630.
- Farrell AS, Pelz C, Wang X, Daniel CJ, Wang Z, Su Y, **Janghorban M**, Zhang X, Morgan C, Impey S, Sears RC. Pin1 Regulates the Dynamics of c-Myc DNA Binding to Facilitate Target Gene Regulation and Oncogenesis. Mol Cell Biol. 2013 May 28.
- Zhang X, Farrell AS, Daniel CJ, Arnold H, Scanlan C, Laraway BJ, **Janghorban M**, Lum L, Chen D, Troxell M, Sears R. Mechanistic insight into Myc stabilization in breast cancer involving aberrant Axin1 expression. Proc Natl Acad Sci U S A. 2012 Feb 21;109(8):2790-5. Epub 2011 Aug 1.

Research Presentation

- May 2014. OHSU Research Week. Oral presentation “Negative regulation of c-Myc oncogenic activity through the tumor suppressor PP2A-B56α”. **Mahnaz Janghorban**, Rosalie Sears.
- April 2013. AACR 104th Annual Meeting. Activation of PP2A by SET antagonism destabilizes c-Myc and inhibits breast cancer growth. **Mahnaz Janghorban**, Jessica Oddo, Tyler Risom, Amy Farrell, Michael P Vitek, Rosalie C Sears, Dale J Christensen
- August 2013. Mechanisms and Models of Cancer Symposium- SALK Institute. Poster “Regulation of Skin Homeostasis by the Tumor Suppressor PP2A-B56α”. **Mahnaz Janghorban**, Jody Hooper, Rosalie Sears.
- February 2013. Poster containing some of my work presented at PI 3-Kinase and Interplay with Other Signaling Pathways meeting- Keystone Symposia-“Synergistic enhancement of targeted kinase inhibitors in triple negative breast cancer through phosphatase activation”. Tyler Risom, **Mahnaz Janghorban**, Dale Christensen, Rosalie Sears.
- May 2013. OHSU Research Week. Oral presentation “A Role for The PP2A Inhibitor SET in Breast Cancers”. **Mahnaz Janghorban**, Rosalie Sears.
- July 2012. Protein Phosphatase meeting- FASEB. Poster “Targeting c-Myc by activating PP2A through SET in breast cancers”. **Mahnaz Janghorban**, Amy S. Farrell, Dale Christensen, Rosalie Sears.
- April 2011. Poster containing some of my work presented at AACR 102nd Annual Meeting– “PP2A activation as a strategy for treatment of breast cancer”. Dale J. Christensen, Jessica Oddo, Amy S. Farrell, **Mahnaz Janghorban**, Michael P. Vitek, and Rosalie C. Sears.
- August 2010. Mechanisms and Models of Cancer Symposium- SALK Institute. Poster “Regulation of dynamic c-Myc promoter binding and transcriptional activity by the peptidyl prolyl isomerase Pin1”. Amy S. Farrell, Colin J. Daniel, Xiaoyan Wang, Xiaoli Zhang, **Mahnaz Janghorban**, Rosalie Sears.

Extracurricular Activities

- Founded Iranian Student Association of Portland (ISAP) at Portland State University, Portland, OR; 2010
- Volunteered at Louis Stokes Alliance for Minority Participation and instructed journal club for undergraduate students, Portland State University, Portland, OR; Jan 2012-July 2012