REGULATION OF C/EBPBETA1 IN TRANSFORMED MAMMARY EPITHELIAL CELLS AND THE ROLE OF C/EBPBETA1 IN ONCOGENE INDUCED SENESCENCE

By

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To my family - thank you, I love you.

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LIST OF ABBREVIATIONS

AGP/EBP – 1-acid glycoprotein gene/enhancer binding protein

Ampho - amphotropic

ATCC – American type culture collection

ATP – adenosine triphosphate

Bax – B cell lymphoma 2 (Bcl-2) – associated X protein

Bcl-2 – B cell lymphoma 2

Brdu - bromodeoxyuridine

bFGF – basic fibroblast growth factor

Cdk – cydlin dependent kinase

cDNA – copy deoxyribonucleic acid

C/EBP – CCAAT/enhancer binding protein

CMV - cytomegalovirus

Cox-2 – cyclo-oxygenase 2

C-terminal – carboxy-terminal

Daxx – death domain associated protein

DMEM – Dulbecco's modified Eagle medium

DMSO – dimethyl sulfoxide

E1 – SUMO-activating enzyme

E2 – SUMO-conjugating enzyme

E-cadherin – epithelial cadherin

EDTA – ethylenediaminetetraacetic acid

eGFP – enhanced green fluorescent protein

Elk-1 – ets like gene 1

EMEM – Eagle's minimal essential medium

EMSA – electromobility shift assay

EMT – epithelial to mesenchymal transition

Erk – extracellular signal-regulated kinase

FACs – fluorescence activated cell sorting

FBS – fetal bovine serum

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GDP – guanine diphosphate

GEFs – guanine nucleotide exchange factors

GFP – green fluorescent protein

GSK3beta – glycogen synthase kinase-3 beta

GTP – guanine triphosphate

HA - hemagglutinin

HDAC – histone deacetylase

HDFs – human diploid fibroblasts

HMEC – human mammary epithelial cells

HRP – horseradish peroxidase

hTERT – human telomerase reverse transcriptase

IL6 – interleukin 6

IL6-DBP – interleukin 6 DNA binding protein

IRES – internal ribosomal entry site

Jnk – Jun N-terminal kinase

kDa – kilo Dalton

LAP – liver-enriched activator protein

LIP – liver-enriched inhibitory protein

MAPK – mitogen-activated protein kinase

MEF2 – myocyte enhancer factor 2

MEFs – mouse embryonic fibroblasts

MEK – mitogen activated protein kinase/Erk kinase

MMPs - matrix metalloproteinases

NEM – N-ethylmaleimide

Neo - neomycin

NFDM – non-fat dried milk

NF-IL6 – nuclear factor of interleukin 6

NF-M – nuclear factor in myeloid cells

N-terminal – amino-terminal

NURD – nucleosome remodelling and deacetylase

OIS – oncogene-induced senescence

p16INK4A – protein 16 inhibitor of cdk4/cyclin D2

Pax3 – paired box protein 3

PCR – polymerase chain reaction

PBS – phosphate buffered saline

phospho or p - phosphorylated

PI3 kinase – phosphytidl inositol 3 kinase

PLAC1 – placenta-specific 1

PPAR gamma – peroxisome proliferator-activated receptor gamma

pRB – retinoblastoma protein

RIPA - radioimmunoprecipitation

Ras – rat sarcoma protein

RNA – ribonucleic acid

SA-beta-gal – senescence-associated beta-galactosidase

SAHF – senescence-associated heterochromatic foci

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser - serine

siRNA – small interfering ribnucleic acid

SRF – serum response factor

STAT1 – signal transducer and activator of transcription

STE – salt, Tris, EDTA

SUMO – small ubiquitin-like modifier

SV40T/t – simian virus 40 large and small t antigen

SWI/SNF – switch sucrose nonfermentable

TBS-T – Tris-based saline solution plus Tween-20

Thr - threonine

Ubc9 – ubiquitin-like protein SUMO-1 conjugating enzyme

VEGF – vascular endothelial growth factor

CHAPTER I

INTRODUCTION

The American Cancer Society expects that there will be over 1.5 million people diagnosed with cancer in the United States in 2010. They also predict that over half a million Americans will die of cancer this year. Cancer continues to be a major disease in this country; however the American Cancer Society reports that advances in early detection and better treatment options in recent years have significantly improved patient outcomes and survival. Advances in cancer research in the laboratory have allowed biologists to better understand the origins and progression of this disease, allowing for more effective prevention and treatment of cancer. There is a large body of work revealing cancer to be a disease of genomic alterations. Several studies indicate that tumorigenesis is a multistep process in which genetic mutations conferring a growth advantage are acquired throughout progression of the disease (reviewed in Hanahan and Weinberg, 2000). Cancer is generally considered to be a disease of the aging, implicating several rate-limiting events that are necessary to take place (Renan, 1993). Additional support for the idea that carcinogenesis is a multistep process is that pathological analyses reveal lesions that represent intermediate steps in a process through which cells evolve from normal to premalignant states and then to cancer (Foulds, 1954). Taken together, cancer occurs through a succession of genetic changes, each conferring a type of growth advantage, leading to the progression of normal cells into cancer (Foulds, 1954, Nowell, 1976, Hanahan and Weinberg, 2000).

Essential capabilities acquired by cancer cells

Many of the genomic mutations in tumors lead to the acquisition of capabilities allowing for a growth advantage. The capabilites acquired by malignant cells are thought to be similar in most types of cancers. This theory proposed by Hanahan and Weinberg, 2000 is based on current knowledge in the cell biology field that most mammalian cells contain similar molecular machinery that regulates proliferation, differentiation and cell death. They propose that cancer cells have defects in the regulatory circuitry that governs normal cell growth. They suggest that genetic mutations in transformed cells are a manifestation of six necessary alterations in cell physiology that allow for tumorigenesis. These six capabilities acquired by malignant cells, also known as the hallmarks of cancer, are self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These six characteristics are described below in more detail.

The proliferation of normal cells relies on the presence of external growth factors, where these cells are unable to grow if mitogenic signals are absent. In contrast, cancer cells have a greatly reduced dependence on external growth factors. Cancer cells are able to proliferate in the absence of mitogenic stimulation because of an increase in the activity of oncogenes that are able to produce constitutive mitogenic signals (Hanahan and Weinberg, 2000). Growth factor receptors that transduce mitogenic signals or molecules that are involved in the intracellular propogation of mitogenic signaling are frequently activated in cancers to allow for this acquired independence. One example of this is the Ras oncogene. Ras functions downstream of growth factor receptors. After growth factors bind to their receptors, the receptor tyrosine kinases undergo autophosphorylation and subsequently recruit adapter proteins that bind guanine

nucleotide exchange factors (GEFs) (DeNicola and Tuveson, 2009). GEFs catalyze the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) on small GTP-binding proteins, including Ras. Ras bound to GTP is then able to interact with numerous effector proteins to regulate cell proliferation and survival. The best characterized growth pathway through which Ras signals is the Ras-Raf-Mek-Erk mitogen-activated protein kinase (MAPK) pathway. This growth pathway, which activates the next signaling molecule via phosphorylation, eventuatlly signals to the nucleus allowing for the transcription of genes involved in proliferation (Figure 1). Ras is found in its mutant, activated state in approximately 25% of all human tumors (Medema and Bos, 1993). The activated Ras oncogene encodes for a protein that is typically mutated at codons 12, 13, or 61. These mutants are rendered constituitively GTP bound and therefore have an increased affinity for downstream effectors such as Raf (DeNicola and Tuveson, 2009). This allows for the release of a continuous stream of mitogenic signals, thereby promoting cell growth in the absence of external mitogenic factors (McCormick, 1991).

In addition to becoming self-sufficient in growth signals, tumor cells are insensitive to antigrowth signals. In normal tissue, a variety of antiproliferative signals are present to prevent unnecessary proliferation. Antigrowth signals can block proliferation by preventing progression through the cell cycle and forcing cells into the G_0 quiescent state. Therefore, much of the signaling that enables cells to respond to antigrowth factors is associated with the cell cycle, especially the molecules responsible for regulating the transition through the G1 phase (Hanahan and Weinberg, 2000). One such gatekeeper protein is the retinoblastoma protein (pRB) (Kaelin, 1999). pRB plays an important role in determining whether a cell will progress through G1. When pRB is hypophosphorylated, it blocks progression through the cell cycle by sequestering the

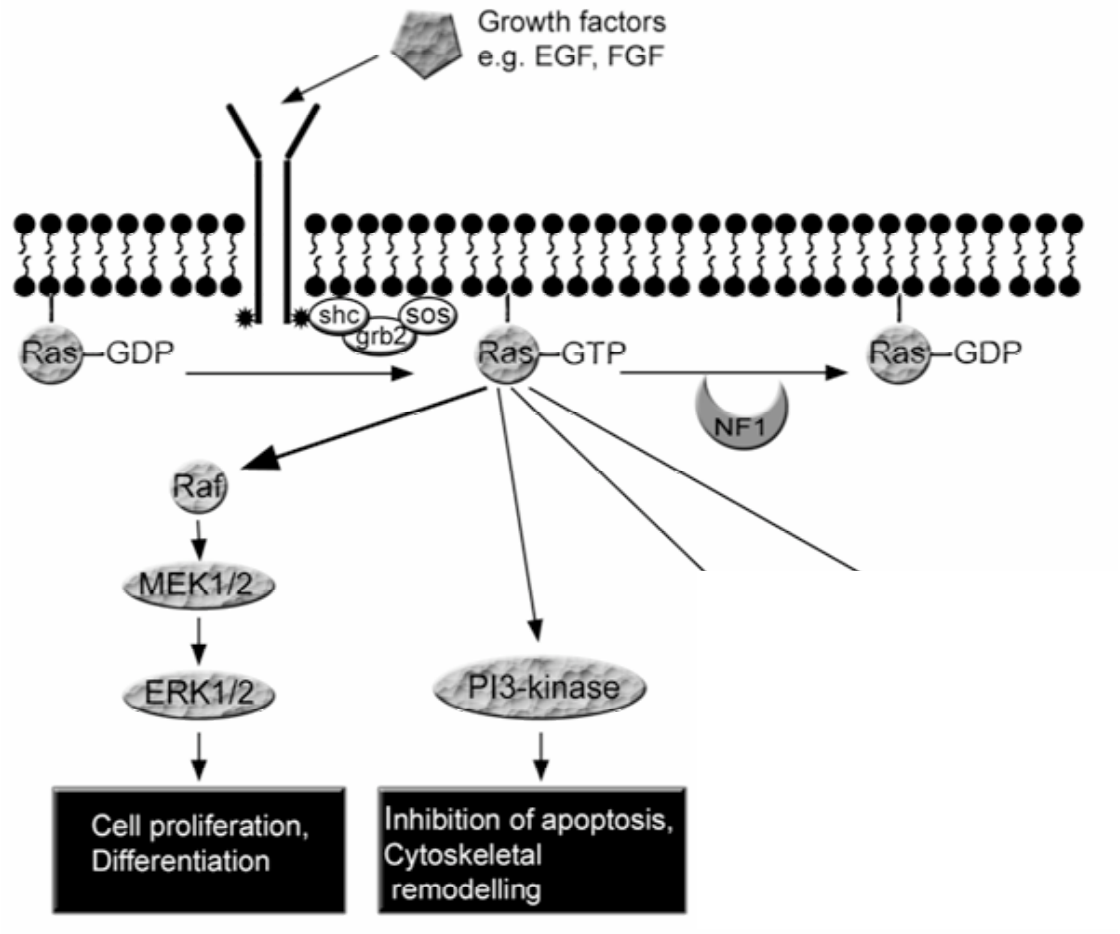

Figure 1. The Ras signaling pathway. Ras is activated when growth factors bind to growth factor receptors. GTP-bound Ras can then activate the Raf-MEK-Erk MAPK pathway, which then leads to the activation of transcription in the nucleus, thus leading to proliferation. Active Ras also signals to the PI3-kinase pathway, allowing for inhibition of apoptosis and cytoskeletal remodeling. This cytoskeletal remodeling contributes to an invasive phenotype. Figure with permission and adapted from Koivunen, 2003.

E2F transcription factors, thereby preventing the transcriptional activation of genes critical for proliferation (Figure 2) (Weinberg, 1995). Disruption of the pRB pathway frees the E2F proteins thus allowing for progression through the cell cycle. Signaling to and from pRB can be disrupted by several mechanisms. The pRB protein itself is inactivated in several cancers (Sellers and Kaelin, 1997). Other cell cycle inhibitors in the pRB signaling pathway such as p16INK4A are frequently inactivated in malignant tumors (Sherr, 2001). p16INK4A binds cyclin dependent kinases (cdks), specifically cdk4 and -6, so that these cdks cannot form complexes with cyclin D (Auerkuri, 2006). Thus p16INK4A prevents cell cycle progression. In addition, proteins that promote progression through the cell cycle, such as the cyclins and cdks are oftentimes activated in cancers (Jacks and Weinberg, 1998).

The ability of cells to increase in number, as in the case of a tumor for example, is determined not only by the rate of proliferation but also by the rate of cell death (Hanahan and Weinberg, 2000). Therefore, it is critical for cancerous cells to be able to evade cell death in order to form a tumor. The most studied form of cell death is a type of programmed cell death called apoptosis. Almost all cell types are capable of undergoing apoptosis, which involves disruption of the cell membrane, extrusion of the cytosol, degradation of the chromosomes, and nuclear fragmentation. The cell remains are then quickly engulfed by nearby cells (Wyllie *et al*., 1980). Cells have "sensors" such as receptors that monitor for abnormal conditions that influence whether a cell should die. One important sensory protein that can induce apoptosis is p53. When the cell encounters a harmful event such as DNA damage, oncogene hyperexpression, or hypoxic conditions, the detrimental signals are directed through p53 which in turn oftentimes signals to the apoptotic machinery (Evan and Littlewood, 1998). Otherwise

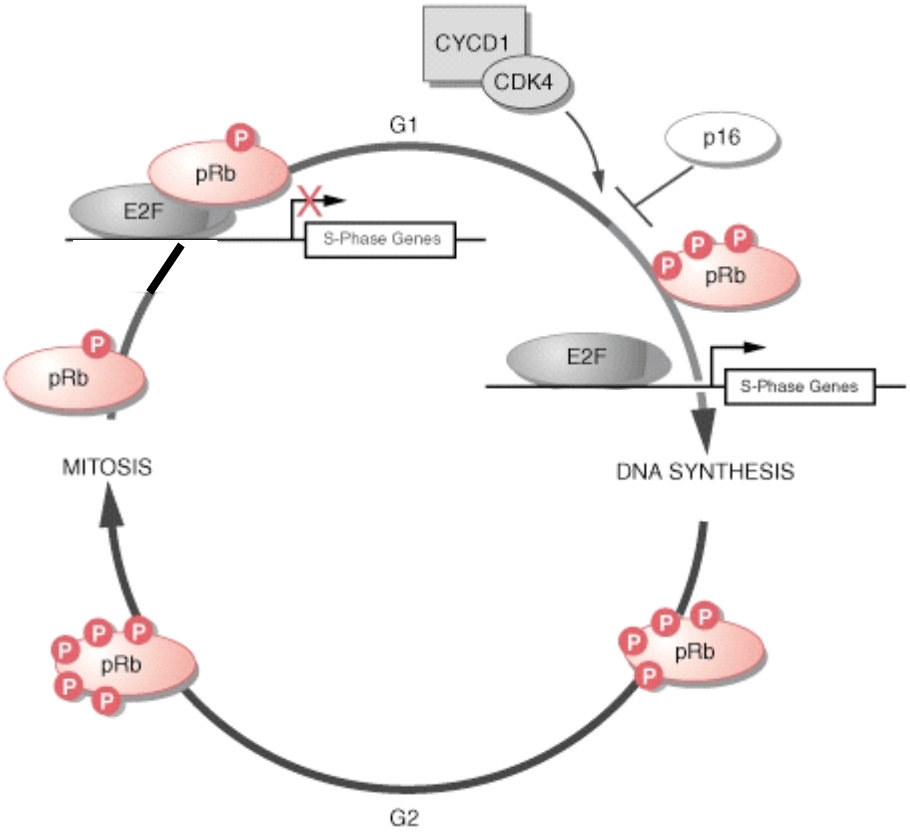

Figure 2. pRB as a gatekeeper of the cell cycle. The pRB protein plays a large role in determining whether a cell will progress through the G1 phase of the cell cycle and thus undergo cell division. When pRB is in a hypophosphorylated state, it prohibits cell cycle progression by preventing the E2F transcription factors from transcriptionally activating genes involved in cell growth, as shown above. However when pRB is phosphorylated by cell cycle proteins such as cyclin D1/cdk4 as demonstrated above, this frees the E2F proteins and allows them to transcriptionally activate S-phase genes, thus promoting progression through the cell cycle. The cell cycle inhibitor p16INK4A inhibits cyclin D/cdk complexes, thus preventing cell cycle progression. (Adapted from Holland and Frei, 2003). http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cmed6&part=A1570

these damaging conditions could lead to tumorigenesis. Other proteins termed "effectors" carry out the cell death process. Sensory proteins that propogate proapoptotic signals frequently signal through Bcl-2 family members such as Bax, Bak, Bid and Bim. These proteins subsequently converge on the mitochondria, which in turn release cycochrome C (Green and Reed, 1998). Cytochrome C then activates caspases, cellular proteases that execute the cell death program through destruction of organelles and subcellular structures (Thornberry and Lazebnik, 1998). p53 can elicit apoptosis by upregulating Bax, which then stimulates the mitochondria to release cytochrome C and leads to subsequent activation of caspases. The p53 pathway is mutated in most cancers, with p53 itself being altered in nearly half of all human tumors (Harris, 1996).

The above three capabilities acquired by tumors – self sufficiency in growth signals, insensitivity to antigrowth signals, and evasion of cell death - lead to an uncoupling of a cell's growth program from environmental signals. However, acquisition of only these three characteristics is not sufficient to ensure expansive tumor growth. Most cell types have an intrinsic program that limits their multiplication potential. Normal cells in culture have a finite replicative potential (Hayflick, 1997). Once these cells have undergone a certain number of doublings, the cells stop growing and become senescent. Replicative senescence occurs due to the shortening of telomeres – the protective ends of chromosomes. Telomeres are composed of several thousand repeats of a short 6 basepair sequence elements at the end of chromosomes. 50-100 basepairs of telomeric DNA are lost from the ends of every chromosome during each cell doubling (reviewed in Hanahan and Weinberg, 2000). This shortening of telemeres during cell division will eventually cause telomeres to lose their ability to protect the ends of DNA. Therefore, after a certain number of cell divisions, signals to induce replicative

senescence are triggered. The maintenance of telemeres is essential for the indefinite proliferation of cells, a phenomenon called cell immortalization. The indefinite proliferation of cells is a critical ability for cancer cells to obtain if a malignant tumor is to form (Hahn and Meyerson, 2001, Harley *et al.* 1994, Shay *et al.*, 2001). Evidence of immortalization is present in most malignancies (Shay and Bacchetti, 1997). Approximately 90% of all cancers demonstrate maintenance of telomeres through activation of telomerase, the enzyme that adds hexanucleotide repeats onto the ends of telomeric DNA (Kim *et al.*, 1994). Activation of telomerase is an important step towards cells achieving immortalization.

Once cells acquire the ability to proliferate indefinitely, they must be able to access oxygen and nutrients provided by the vasculature (Hanahan and Folkman, 1996). Cells must be within 100um of a blood vessel in order to survive. Therefore malignant cells must be able to induce the growth of new blood vessels, a process called angiogenesis. Tumor cells promote angiogenesis through the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and downregulating the expression of antiangiogenic factors such as thrombospondin 1 (Hanahan and Folkman, 1996). These proangiogenic signals attract and stimulate endothelial cells allowing for the construction of capillaries within the tumor. These capillaries are then able to connect with existing blood vessels, thus providing oxygen and nutrients to the tumor cells (Hanahan and Folkman, 1996).

The final acquired capability of a malignant tumor is tissue invasion and metastasis. Tumor cells invade their surrounding tissue, intravasate into nearby blood vessels through which the malignant cells are able to travel to distant sites, extravasate out of the blood vessel to a new site, and finally colonize the new site. It is these distant colonies of tumor cells, called metastases, which cause 90% of cancer deaths (Sporn,

1996). Tumor cells metastasize to areas where nutrients and space are not initially limiting. Alterations in cell-cell adhesion molecules, such as downregulation of the cell adhesion molecule E-cadherin, and proteins that mediate cell interactions with the extracellular matrix such as integrins, promote invasion (Aplin *et al.*, 1998). Additionally upregulation of extracellular proteases that degrade the extracellular matrix such as matrix metalloproteinases (MMPs) are frequently observed in cancer and further promote invasion and metastasis (Coussens and Werb, 1999, Chambers and Matrisian, 1997).

Taken together, the acquisition of the first four acquired capabilites – self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of cell death and limitless replicative potential – is required for the uncoupling of a cell's growth program from environmental cues and permits cells to escape their intrinsic ability to limit their replicative potential. Acqisition of these characteristics is necessary to initiate tumor formation. Once these cells form a tumor that is large enough to require its own blood supply, the cells must be able to induce angiogenesis, the fifth hallmark of cancer, to continue to thrive. Finally, the tumorigenic cells need to acquire the ability to invade and metastasize to distant sites to be truly deadly. The six hallmarks of cancer work in concert to produce malignancies.

Experimentally transforming cells by genetic manipulation

The theory that the above biological characteristics are shared by most tumor types supports the idea that a set of common rules govern the transformation of a variety of human cells. This raises the question of how many (and what) distinct regulatory changes are required to transform a normal cell into a tumor cell (Hahn and Weinberg, 2002)? Based on the theory that there are six capabilites that normal cells must acquire

to become tumorigenic, it was hypothesized that the alteration of proteins that control these six biological functions would be sufficient to achieve transformation. To test this, scientists genetically manipulated normal human cells by introducing specific genes into cultured cells. The genes that were introduced were chosen because they encoded for proteins critically involved in the signaling pathways that control the six capabilities acquired by tumor cells. These functionally altered proteins are also proteins that are typically found to be altered in cancer.

To first achieve immortalization of cultured human cells, it is important to uncouple the cell's growth program from environmental signals. Two major players involved in growth inhibition and cell death are the pRB and p53 tumor suppressor proteins. Additionally, expression of these two proteins is typically altered in cancer. To inactivate these proteins, the simian virus 40 (SV40) early region, which encodes for the large T and small t oncoproteins, was stably expressed in normal human fibroblasts in culture (Shay *et al.*, 1991). SV40 large T sequesters and inactivates pRB and p53. In addition to altering the function of p53 and pRB, normal human fibroblasts require the ability of telomere maintenance to lead to immortalization. To do this, hTERT, the gene which encodes for the catalytic subunit of telomerase, was also introduced into the normal human fibroblasts (Meyerson *et al.*, 1997, Nakamura *et al.*, 1997, Harrigton *et al.*, 1997). The combined introduction of the SV40 early region and hTERT was sufficient to immortalize normal human fibroblasts (Hahn *et al.*, 1999).

To achieve transformation, it is necessary to introduce a factor that would promote independence from mitogenic stimulation, angiogenesis, invasion and metastasis. The activated Ras oncogene is most well-known for its role in propogating mitogenic signaling; however, Ras promotes angiogenesis and invasion through the upregulation of factors involved in these processes such as VEGF and MMPs (Rak *et*

al., 1995, Thant *et al.*, 1999, reviewed in Kranenburg *et al.*, 2004, Campbell *et al.*, 2004). Thus, the activated Ras oncogene was introduced into the human cells immortalized by the SV40 early region and hTERT, and transformation was attained as assessed by anchorage independent growth and tumor formation in immunodeficient nude mice. (Hahn *et al.*, 1999). These results suggest that transformation of cultured normal human fibroblasts can be accomplished by disturbing a small set of intracellular pathways which are involved in the proposed six acquired capabilites of cancer (Figure 3, Hahn and Weinberg, 2002).

Ras doesn't just promote tumorigenesis – a role for Ras in oncogene-induced senescence

As described above, introduction of activated Ras along with other cooperating alterations into normal human fibroblasts allows for transformation of these cells. Considering that Ras is an oncogene known to propagate mitogenic signaling, it would be expected that introduction of Ras alone into normal human fibroblasts would give these cells a growth advantage. This is not the case, however. When oncogenes such as activated Ras are introduced into normal human fibroblasts, these cells actually stop growing and undergo senescence (Serrano *et al.*, 1997). This growth inhibitory response, known as oncogene-induced senescence (OIS), is a tumor suppressive mechanism inherent in normal cells. This protects cells from the initial steps of tumorigenesis caused by a variety of stimuli including activation of oncogenes, DNA damage, and oxidative stress. Several characteristics define the senescent phenotype. Senescence is a largely irreversible growth inhibitory response characterized in vitro by cells having a large, flat morphology. The cells also frequently express a senescence-associated beta-galactosidase (SA-beta-gal), which is lysosomal in origin. Additionally

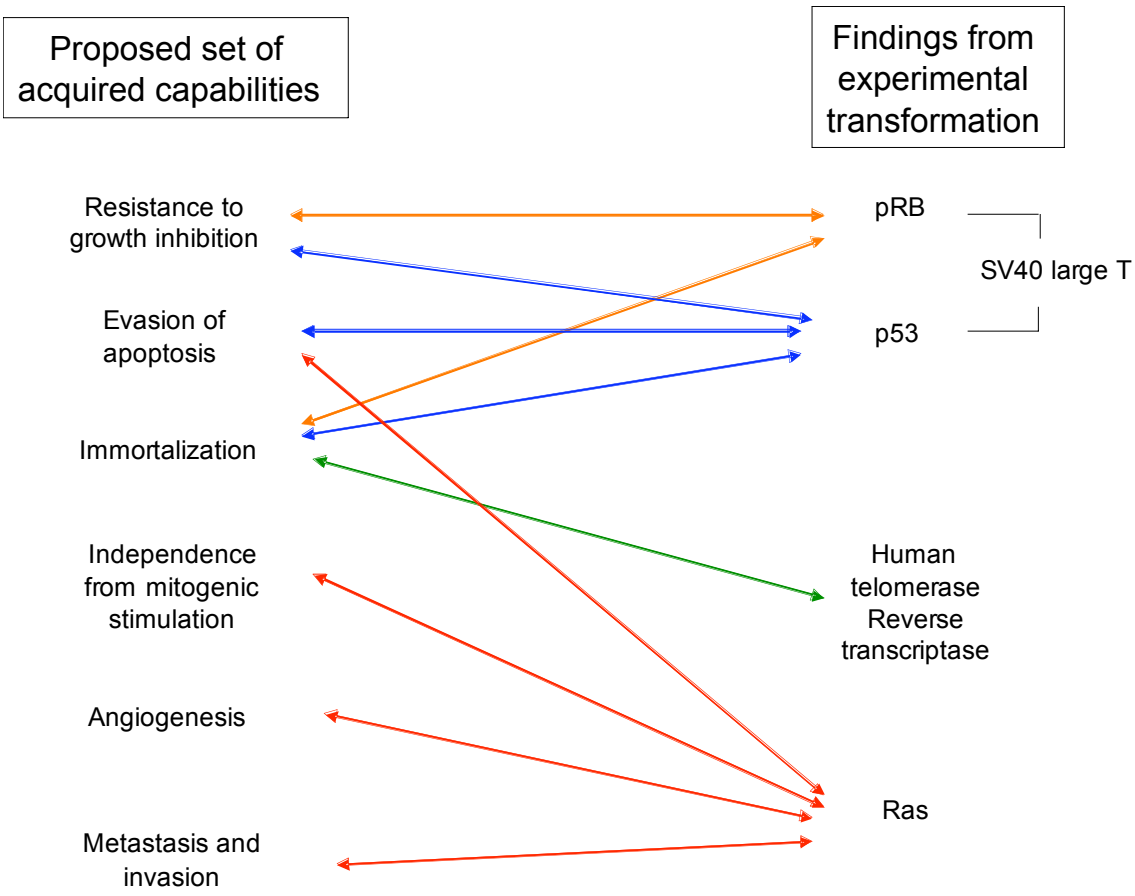

Figure 3. How the factors introduced for experimental transformation of normal human fibroblasts affect the proposed set of acquired capabilites proposed by Hanahan and Weinberg, 2000. With permission and adapted from Hahn and Weinberg, 2002.

the chromatin of senescing cells condenses, forming senescence-associated heterochromatic foci (SAHF). SAHF formation prevents the transcription of genes whose protein products are involved in proliferation, thus allowing for growth inhibition. Until recently, it was thought that OIS may only occur in vitro due to the artificial environment of cell culture; however, accumulating evidence demonstrates that OIS occurs in vivo. For example, benign tumors that contain activating mutations in oncogenes such as Ras and Raf also demonstrate markers of senescence, such as activation of p16 and expression of SA-beta-gal (Michaloglou *et al.*, 2005). These markers of senescence are not observed in advanced tumors. Because of this, it has been suggested that evasion of OIS should be included as the seventh acquired capability required of transformed cells (Mooi and Peeper, 2006).

Mechanistically, Ras induced senescence converges on the pRB and p53 pathways. Introduction of activated Ras into normal human fibroblasts initially leads to a brief period of increased proliferation followed by senescence. This period of increased growth is believed to stress the cell, leading to activation of a stress checkpoint (DeNicola and Tuveson, 2009). p16INK4A becomes activated, which prevents CDK4 and CDK6 from complexing with cyclinD. This allows for pRB to be in its hypophosphorylated form and therefore activated. pRB plays a critical role in SAHF formation. The dense foci of heterochromatin coincide with pRB-dependent heterochromatic repression of genes encoding cyclins and other positive cell cycle regulators (Campisi, 2005). Additionally, many of these repressed genes are activation targets of E2F transcription factors. As mentioned earlier, the E2F transcription factors are unable to transcriptionally activate genes that promote proliferation, because pRB inhibits the ability of the E2Fs to accomplish this. In addition to the activation of pRB, the DNA damage response is activated during Ras induced senescence

(Figure 4). The DNA damage response signals through p53, which in turn leads to the induction of senescence. Activation of oncogenes, DNA damage, and oxidative stress signal through p53, which leads to either the induction of senescence or apoptosis. It is currently unclear what determines whether activation of p53 leads to the induction of senescence or apoptosis. It is interesting that both of these mechanisms are utilized by cells to protect cells from transformation. Apoptosis is a widely accepted mechanism by which cells are protected from transformation because the cell dies and is thus eliminated as an oncogenic threat (Courtois-Cox *et al.*, 2008). It has been proposed that OIS is available as an alternative to oncogene-induced apoptosis, because immediate cell death upon the acquisition of a first oncogenic mutation may not be beneficial in all cells and tissues (Chandeck and Mooi, 2010). Some cell types are long-lived cells and not replacable in large numbers. Loss of these cells would therefore be detrimental to the function of the tissue. Hence, OIS may have evolved as a mechanism to protect cells from transformation while sparing the cells and thus allowing them to continue to be a functional component of their respective tissue (Chandeck and Mooi, 2009).

Ras – induction of senescence versus transformation

Currently it is unclear what regulates Ras induced senescence versus Ras transformation. It has been hypothesized that transformation by Ras can only occur when proteins critical for the senescence response, such as p53 and p16, have already been inactivated in cells (DeNicola and Tuveson, 2009). Another theory is that the level of expression of activated Ras is important in determining whether cells undergo Ras induced senescence or Ras transformation. Ectopic expression of activated Ras in fibroblasts results in hyperactivation of Ras effector pathways, stabilization of p53, and subsequent senescence, whereas physiological expression of activated Ras from its endogenous promoter fails to hyperactivate downstream pathways (Tuveson *et al.,*

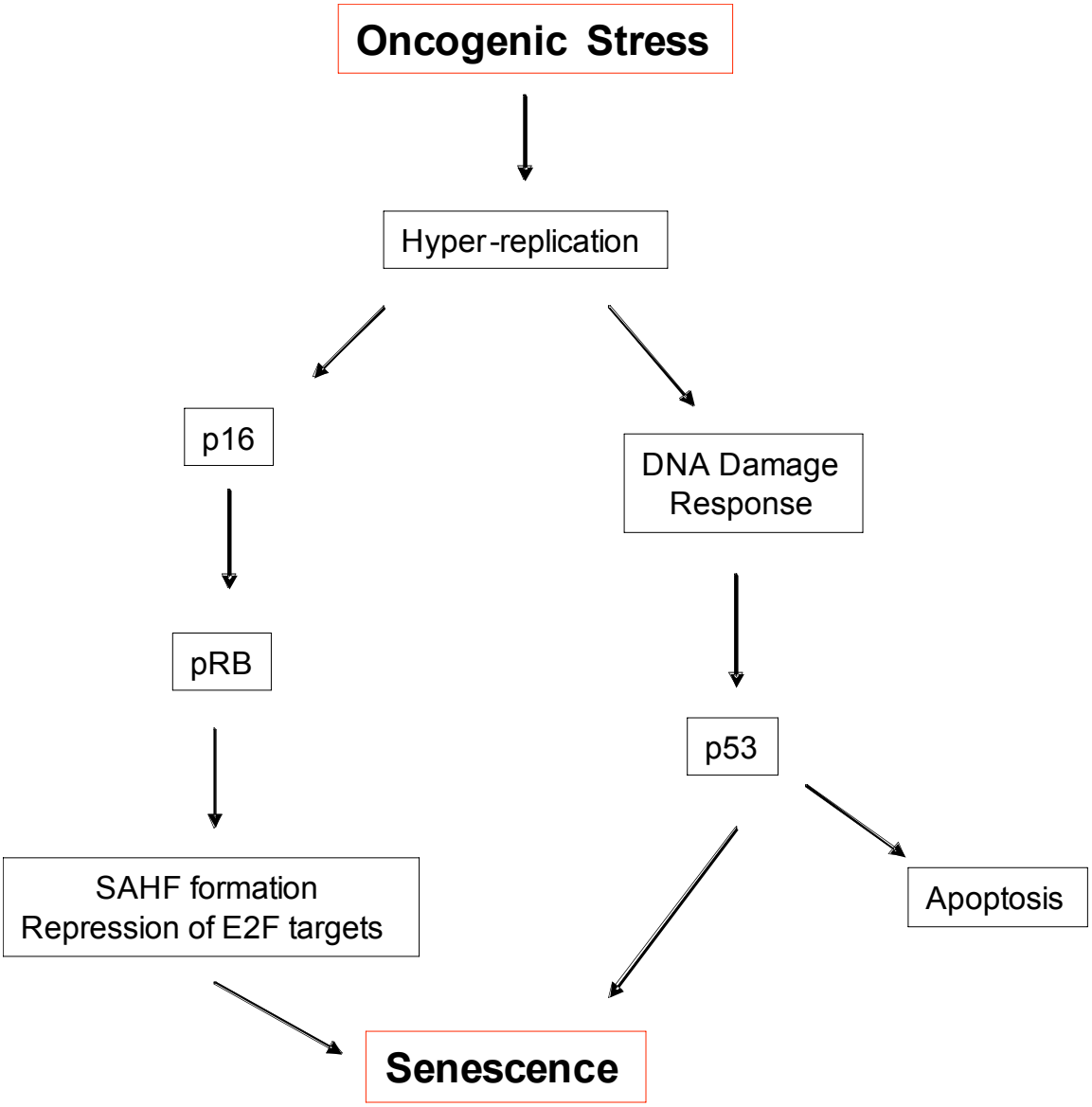

Figure 4. Mechanism of oncogene-induced senescence. Introduction of an oncogenic stress into normal human fibroblasts leads to a brief period of proliferation followed by activation of cell cycle inhibitors and the DNA damage response. This activates pRB and p53 allowing for SAHF formation and thus repression of genes involved in growth and subsequent senescence, or apopotosis.

2004). In addition, this physiological expression of activated Ras eventually leads to immortalization of mouse fibroblasts. In support of these findings, high levels of activated Ras expression in the mouse mammary gland induced a transient period of mammary epithelial cell proliferation followed by induction of senescence, whereas low levels of activated Ras expression that mimicked endogenous levels promoted proliferation and hyperplasia (Sarkisian *et al.*, 2007). However, there is evidence countering the argument that levels of activated oncogenes determine whether cells undergo senescence or transformation, (Cagnol and Chambard, 2009). For example, naevi (moles) in humans are considered an in vivo example of B-Raf-driven senescence because activated B-Raf is expressed at physiological levels under the control of its own promoter. Additionally, a mouse model of inducible tumorigenesis in lung epithelium driven by activated Raf demonstrated that activated Raf expression led to benign, senescent lesions instead of tumorigenesis (Dankort *et al.*, 2007).

It is of critical importance to study the differences in signaling between Ras induced senescence versus transformation, because knowledge of this could give insight into how to therapeutically target activation of Ras induced senescence and/or inhibition of Ras induced transformation in tumor cells. Mechanistic differences are likely responsible for the differential regulation of Ras transformation versus Ras induced senescence. It is widely accepted that OIS signaling converges on the pRB and p53 pathways; however, the signaling upstream of these two tumor supressors has not been completely elucidated (Courtois-Cox *et al.*, 2008). One such protein that may play a role in the differences in signaling between Ras induced senescence versus transformation is the transcription factor CCAAT/Enhancer Binding Protein beta or C/EBPbeta. C/EBPbeta is an essential downstream mediator of both Ras transformation (Zhu *et al.*, 2002) and activated Ras/Raf-induced senescence (Sebastian *et al.*, 2005, Kuilman *et*

al., 2008). Three protein isoforms of C/EBPbeta exist due to alternative translation initiation at three in-frame methionines (Figure 5). It is the production of these three different isoforms that may play a part in determining whether expression of activated Ras leads to transformation or senescence. In this present study, I examine how the full-length isoform of C/EBPbeta, C/EBPbeta1, induces senescence and is negatively regulated during Ras transformation.

CCAAT/Enhancer Binding Protein family

C/EBPbeta is a member of the CCAAT/enhancer binding protein (C/EBP) family of basic leucine zipper transcription factors. These family members contain a C-terminal basic region, which is involved in DNA binding, and a leucine zipper region that is involved in dimerization. Despite these conserved regions, variation is observed in their N-terminal transactivation domains, allowing for functional differences. Six C/EBP family members exist, which includes C/EBPalpha, -beta, -gamma, -delta, -epsilon, and zeta (Lekstrom-Himes and Xanthopoulous, 1998, Ramji and Foka, 2002). Most of the C/EBPs are expressed in and play important roles in the development and function of a variety of tissue types including liver, adipose, intestine, lung, and immune cells (Lekstrom-Himes and Xanthopoulous, 1998).

C/EBPbeta

C/EBPbeta is also known as NF-IL6 (human) (Akira *et al.*, 1990), LAP/LIP (rat and mouse) (Descombes *et al.*, 1990), NF-M (chicken) (Sterneck *et al.*, 1992), AGP/EBP (Chang *et al.*, 1990), IL6-DBP (Poli *et al.*, 1990), and CRP2 (Williams *et al.*, 1991) due to the fact that it was isolated independently by different groups of investigators. Three protein isoforms of C/EBPbeta exist due to alternative translation initiation at three in-frame methionines (Figure 5). In humans, full-length C/EBPbeta1 begins at the first in-

frame ATG, is 346 amino acids long (297 in rat and mouse) and has an apparent molecular weight of 52kDa. The second isoform, C/EBPbeta2, begins at the second in-frame methionine, 23 amino acids (21 in rat and mouse) downstream from the first and appears as a doublet on immunoblots at 45kDa and 48kDa. C/EBPbeta3 begins at the last in-frame ATG at amino acid 198 in humans and has an apparent molecular weight of 20kDa. C/EBPbeta1 and C/EBPbeta2 both contain the C-terminal DNA binding/dimerization domain as well as an N-terminal transactivation domain, allowing them to function as activators of transcription. C/EBPbeta3 lacks the N-terminal transactivation domain and is thus a repressor of transcription (Descombes and Schibler, 1991).

Regulation of expression of the three isoforms of C/EBPbeta has been proposed to be through translational control due to the presence of alternative translation initiation sites. This translational control is through a leaky ribosome scanning mechanism (Descombes and Schibler, 1991). A small upstream open reading frame is important in regulating the translation of the three C/EBPbeta isoforms (Lincoln *et al.,* 1998, Calkhoven *et al.*, 2000, Timchenko *et al.*, 1999). There is further evidence that C/EBPbeta3 can be produced via proteolytic degradation of the longer isoforms (Baer and Johnson, 2000, Welm *et al.*, 1999). C/EBPbeta is widely expressed in many tissues and cell types and numerous genes are regulated by C/EBPbeta including c-FOS (Metz *et al.,* 1991, Sealy *et al.*, 1997), IL-6 (Akira *et al.*, 1990), and COX-2 (Wu *et al.*, 2005). The C/EBPbeta knockout mouse demonstrated the importance of this protein in many tissues by displaying dramatic phenotypic alterations in the immune system (Tanaka *et al.*, 1995, Screpanti *et al.*, 1995), liver (Greenbaum *et al.*, 1998), adipose tissue (Tanaka *et al.*, 1997), skin (Zhu *et al.*, 1999), and mammary glands (Robinson *et al.*, 1998,

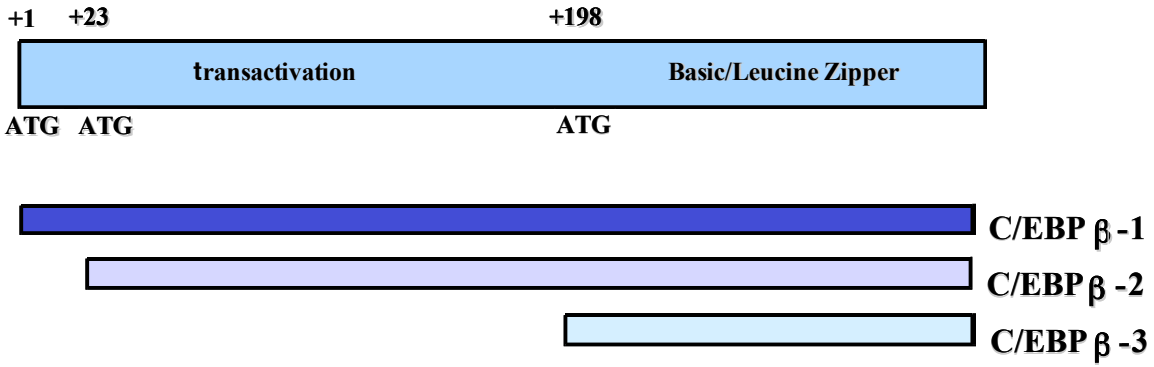

Figure 5. The three isoforms of C/EBPbeta. The relative sizes of the different C/EBPbeta isoforms beginning at the three in-frame ATGs. C/EBPbeta1 and C/EBPbeta2 both contain the N-terminal transactivation domain whereas C/EBPbeta3 does not. All three isoforms contain the C-terminal dimerization/DNA binding domain.

Seagroves *et al.*, 1998). These studies have implicated C/EBPbeta as a key regulator in the proliferation and differentiation of a variety of tissues.

C/EBPbeta in the mammary gland and breast cancer

I have focused on the role of C/EBPbeta in mammary epithelial cells and breast cancer. The *C/EBPbeta* knockout mouse demonstrated that C/EBPbeta plays an essential role in the development of the mammary gland. Development of the mammary gland is a complex and dynamic process that begins embryonically and continues through pregnancy and weaning of the offspring. A rudimentary epithelial ductal tree is formed during embryonic development and remains largely unchanged until puberty. During puberty, estrogen stimulates the growth and development of the ductal tree and lobuloalveolar structures at the ends of the ducts (Sternlicht *et al.*, 2006). Additional proliferation and development of the ducts and lobuloalveoli occur in response to estrogen and progesterone during pregnancy. Following birth of the offspring, functional differentiation is marked by milk production and secretion. After lactation, the mammary epithelial ductal tree returns to a virgin-like state through large-scale cell death, the process of involution.

This process of epithelial cell growth, differentiation, and involution of the mammary gland occurs with each pregnancy (Sternlickt *et al.*, 2006). Most breast cancers originate in the epithelial cells of the mammary gland. Proteins involved in mammary epithelial proliferation, differentiation and cell death are frequently mutated in breast cancers, as mentioned in the first part of this introduction. C/EBPbeta is one such protein that plays a critical role in mammary gland development and whose expression is altered in breast cancers. Transgenic mice that lack the C/EBPbeta gene fail to develop normal mammary glands at puberty or pregnancy and fail to lactate (Robinson *et al.*,

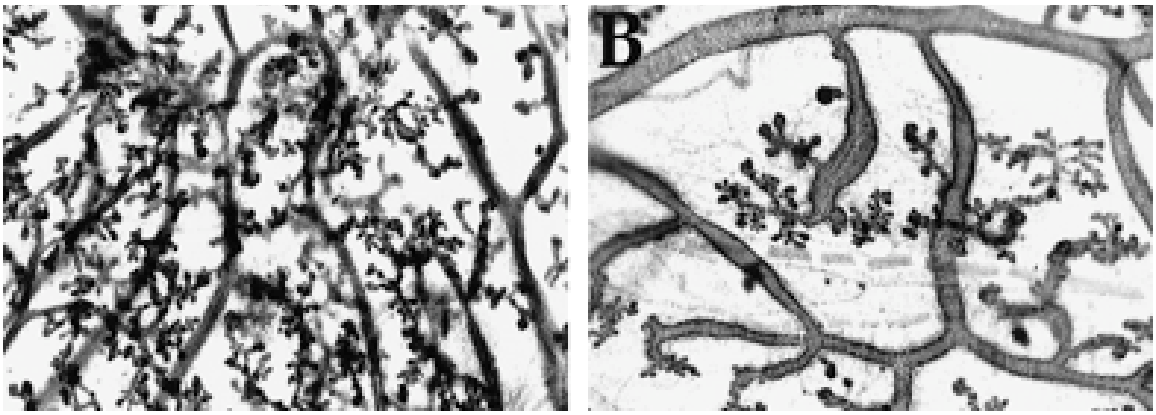

Figure 6. Lobuloalveolar development of the mammary gland is impaired in C/EBPbeta knockout mice upon stimulation with estrogen and progesterone. The thoracic glands from mice treated with estrogen and progesterone were fixed and stained with hematoxylin by whole mount preparation. Treatment with estrogen and progesterone is necessary to mimick pregnancy because C/EBPbeta -/- mice are infertile. The C/EBPbeta heterozygous (C/EBPbeta +/-) mouse mammary glands on the left look very similar to C/EBPbeta wild type mouse mammary glands (not shown). Upon treatment with estrogen and progesterone, mammary glands from C/EBPbeta +/- mice undergo extensive lobuloalveolar development, as shown in the panel on the left. In contrast, mammary glands from C/EBPbeta -/- mice demonstrate a significant decrease in ductal branching and lobuloalveolar development. Large areas of the ductal epithelium do not contain either secondary/tertiary side branches or alveoli. (Adaped from Seagroves *et al.*, 1998.)

1998, Seagroves *et al.*, 1998). Defects in ductal morphogenesis and lobuloalveolar development were observed (Figure 6). Additionally, functional differentiation of the mammary gland was defective as judged by the lack of milk protein production (Robinson *et al.*, 1998, Seagroves *et al.*, 1998). Expression of beta casein and whey acidic protein, two milk-specific proteins, was dramatically reduced. These mice therefore display a dual phenotype: a defect in mammary epithelial proliferation in response to hormonal stimulation at puberty or pregnancy and a defect in mammary epithelial cell differentiation in response to lactation specific hormones.

It was not surprising that the C/EBPbeta knockout mouse failed to lactate, since investigators had previously determined that there are C/EBP consensus sites in the promoter of beta-casein, a milk-specific protein (Doppler *et al.*, 1995). Electromobility shift assay (EMSA) demonstrated that C/EBPbeta bound to these sites (Doppler *et al.*, 1995, Raught *et al.*, 1995). Mutation of the C/EBP sites confirmed that these sites are critical for the production of beta-casein. Additionally, all three isoforms of C/EBPbeta are expressed at relatively high levels during pregnancy in the mouse. However, during lactation C/EBPbeta2 and C/EBPbeta3 levels decrease 20-fold and 100-fold respectively, whereas C/EBPbeta1 levels remain fairly high, decreasing only about 3-fold (Raught *et al.*, 1995). Furthermore, abundant C/EBPbeta1 expression can be detected in secretory mammary epithelial cells exfoliated in human breast milk (Eaton *et al.,* 2001).

C/EBPbeta1 versus C/EBPbeta2

I focused on the functional differences between the different isoforms of C/EBPbeta in mammary epithelial cells and breast cancer. The functional differences between the first two isoforms of C/EBPbeta have not been studied in great detail.

Because C/EBPbeta1 and -2, are so similar in size (only 23 amino acids difference in humans) and because both isoforms are transcriptional activators, some groups consider them to be functionally redundant. Most groups focus on C/EBPbeta2 because of its role in proliferation in a number of cell types as well as evidence that C/EBPbeta1 does not seem to be expressed at very high levels in a number of cultured cell lines. More recent studies are reporting differences in these two isoforms and are indicating that C/EBPbeta1 and -2 both play unique and important roles in the cell. C/EBPbeta1 but not C/EBPbeta2 to cooperates with c-Myb synergistically in co-expression assays to turn on differentiation genes such as mim-1 in myeloid cells. This activation was attributed to the ability of C/EBPbeta1, but not -2, to interact with and recruit the SWI/SNF chromatin remodeling complex (Kowenz-Leutz and Leutz, 1999). The N-terminal amino acids present in C/EBPbeta1 but not -2 were necessary for this recruitment.

Differences exist between the first two transactivator isoforms in mammary epithelial cells and breast cancer also. p52-C/EBPbeta1 is found in normal mammary epithelial cells from reduction mammoplasties, whereas C/EBPbeta2 is not. Moreover, a majority of primary human breast tumors examined had acquired significant levels of C/EBPbeta2 expression (Eaton *et al.,* 2001). C/EBPbeta2 was expressed in the entire panel of breast cancer cell lines studied, while p52-C/EBPbeta1 expression was not observed in any of these breast cancer cell lines. The work in this thesis examines what happens to p52-C/EBPbeta1 during transformation of mammary epithelial cells.

Although C/EBPbeta1 and -2 are both transactivators, their distinct expression profiles in mammary epithelial cells and breast cancer cells suggest that functional differences exist between the two isoforms. Because significant C/EBPbeta2 expression had been acquired in a majority of the primary human breast tumors examined and this

was the only C/EBPbeta activator isoform expressed in cultured breast cancer cell lines, the ability of C/EBPbeta2 to transactivate the promoter of the cyclin D1 gene was examined. Cyclin D1 promotes growth and is commonly upregulated in cancers. Transactivation was observed by C/EBPbeta2 but not C/EBPbeta1 in human mammary epithelial cells and NIH-3T3 cells, demonstrating a functional difference between these two transactivator isoforms (Eaton *et al.,* 2001). Additionally, C/EBPbeta2 but not C/EBPbeta1 can transactivate *PLAC1,* another gene involved in proliferation and upregulated in breast cancer (Koslowski *et al.*, 2009). When C/EBPbeta2 is overexpressed in MCF10A mammary epithelial cells, these MCF10A-C/EBPbeta2 cells exhibit transformed characteristics (Bundy and Sealy, 2003). These cells are anchorage independent and form colonies in soft agar. In addition, over-expression of C/EBPbeta2 causes MCF10As to express markers of epithelial-to-mesenchymal transition and become more invasive, as evidenced by increased invasion in modified Boyden chambers and growth morphology in Matrigel (Bundy and Sealy, 2003). Over-expression of C/EBPbeta1 in MCF10A mammary epithelial cells does not have this effect on the cells.

Taken together, these results indicate that C/EBPbeta1 and C/EBPbeta2 exhibit functional differences in the mammary gland and breast cancer. There is evidence that supports a role for C/EBPbeta in differentiation of the mammary gland. The C/EBPbeta knockout mouse displays a defect in mammary epithelial cell differentiation in response to lactogenic hormones (Robinson *et al.*, 1998, Seagroves *et al.*, 1998). In addition, EMSA experiments demonstrate that C/EBPbeta binds to C/EBP sites in the beta-casein promoter (Doppler *et al.*, 1995, Raught *et al.*, 1995). Evidence indicates that the C/EBPbeta1 isoform specifically plays a role in differentiation of the mammary gland. High levels of C/EBPbeta1 are detected in the mouse mammary gland during lactation

and C/EBPbeta1 is present at high levels in secretory mammary epithelial cells exfoliated in human breast milk (Raught *et al.*, 1998, Eaton *et al.*, 2001). Furthermore, p52-C/EBPbeta1 is expressed in the normal mammary epithelium obtained from reduction mammoplasty and not in breast cancer cell lines (Eaton *et al.,* 2001). p52-C/EBPbeta1 is probably not expressed in breast cancer cells because it is not advantageous for cancer cells to be in a differentiated state.

On the other hand, there is indication that C/EBPbeta2 is the isoform responsible for proliferation and transforming properties in the mammary gland and breast cancer. C/EBPbeta2 is not present in normal mammary tissue obtained from reduction mammoplasty, but expression of this second isoform is acquired in tumor tissue (Eaton *et al.*, 2001). Additionally, C/EBPbeta2 is the major C/EBPbeta isoform present in breast cancer cell lines. Importantly, C/EBPbeta2 transactivates the cyclin D1 promoter in transient cotransfecion experiments (Eaton *et al.,* 2001). Moreover, C/EBPbeta2 has been implicated in anchorage independence, formation of colonies in soft agar, EMT, and invasion (Bundy and Sealy, 2003).

C/EBPbeta3

As mentioned earlier, C/EBPbeta3 lacks the N-terminal transactivation domain and is thus a repressor of transcription (Descombes and Schibler, 1991). Exogenous expression of C/EBPbeta3 in mammary epithelial and breast cancer cells leads to cell death (Bundy *et al.*, 2005, Abreu and Sealy, 2010). In addition, this cell death is preceded by autophagy (Abreu and Sealy, 2010). Autophagy is a process involving the degradation of cellular components in the cytoplasm via lysosomal degradation. Autophagy can play a protective role in stressful conditions such as nutrient depletion; however, extensive degradation of molecules or organelles essential for survival can

lead to autophagy-mediated cell death. The physiological role for C/EBPbeta3-induced autophagic cell death may be during involution of the mammary gland after pregnancy, since this process involves massive cell death (Abreu and Sealy, 2010).

Modification of C/EBPbeta

Cells receive numerous and diverse signals from their external environments, which typically signal into the cell via signal transduction pathways. Transcription factors such as C/EBPbeta integrate information by serving as nuclear effectors of these signaling cascades. These transcription factors are highly regulated by various post-translation modifications including serine/threonine or tyrosine phosphorylation, lysine acetylation, lysine or arginine methylation, ubiquitination, or modification by ubiquitin-like modifiers such as sumoylation. These modifications can regulate the functions of transcription factors including localization, stability, interaction with cofactors, transcriptional activity, and other post-translational modifications (Tootle and Rebay, 2005). C/EBPbeta is modifed by phosphorylation on a number of different serine and threonine residues by various kinases. C/EBPbeta contains a consensus MAPK site centered around Threonine 235, which is the most studied phosphorylation site in this protein (Figure 7). This site is phosphorylated by activated Ras-dependent kinases in vivo and by a partially purified MAPK preparation in vitro (Nakajima *et al.*, 1993). Activation of the Ras pathway leads to the activation of numerous kinases that phosphorylate C/EBPbeta on Threonine 235 (Thr235) including Erk-2 (Hanlon *et al.*, 2001), cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007), and p38 (Engelman *et al.*, 1998, Horie *et al.*, 2007). The current study examines the effect phosphorylation of C/EBPbeta1Thr235 has on the stability of this protein and susceptibility to other post-translational modifications in mammary epithelial and breast cancer cells.

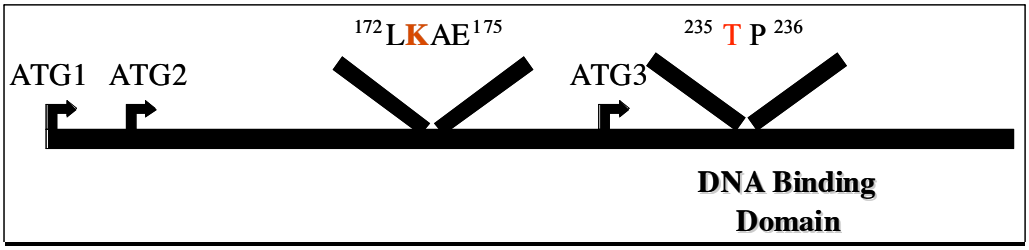

Figure 7. The MAPK phosphorylation site and sumoylation site in C/EBPbeta. The MAPK site is centered around Thr235 in C/EBPbeta and the consensus sumoylation site is centered around Lys173.

C/EBPbeta1 is also modified by the post-translational modification SUMO-2/3 (Small Ubiquitin-like MOdifier) (Figure 7, Eaton and Sealy, 2003). Four members of the SUMO (Small Ubiquitin-like MOdifier) family have been identified, SUMO-1, -2, -3, and -4, all of which share homology with ubiquitin (Geiss-Friedlander and Melchior, 2007). SUMO-2 and SUMO-3 only differ from one another by three amino acid residues and are viewed as being functionally identical. SUMO-2/3 are 50% identical to SUMO-1 (Johnson, 2004). Much less is known about SUMO-4 than the first three members of the SUMO family. SUMO proteins are a group of polypeptides that conjugate to the lysine residue within the target four amino acid consensus sequence: large, hydrophobic amino acid, lysine, alanine, glutamate. SUMO is conjugated to target proteins in much the same manner as ubiquitin. A SUMO-activating enzyme (E1) carries out an ATP-dependent activation of the SUMO carboxy terminus and then transfers the activated SUMO to the SUMO-conjugating enzyme (E2), also known as Ubc9. SUMO is then transferred from Ubc9 to the target, oftentimes with the assistance of one of several SUMO E3 ligases (Johnson, 2004, Geiss-Friedlander and Melchior, 2007).

The SUMO peptides are about 11kDa but they appear larger on SDS-PAGE gels and can add as much as 20kDa to the apparent molecular weight of substrates (Johnson, 2004). SUMO-2/3 are able to form chains on target proteins because the SUMO-2/3 peptide contains a SUMO consensus site. It is thought that most of the SUMO-1 in cells is conjugated to proteins whereas free pools exist of the more abundant SUMO-2/3 (Hay, 2005). SUMO-2/3 is believed to be utilized when cells are exposed to a variety of stresses, including genotoxic, replicative and heat stresses. The bulk of SUMO substrates that have been identified are involved in chromatin organization, transcription, and RNA metabolism (Hay, 2005).

Sumoylation is involved in maintenance of genome integrity, localization, inhibiting ubiquitination, and regulation of transcription, among other cellular functions (Johnson, 2004). Sumoylation of transcription factors frequently causes transcriptional repression. This transcriptional repression is oftentimes due to sumoylation leading to an alteration in binding partners. The sumoylated protein interacts with transcriptional co-repressors such as histone deacetylases (HDACs), Daxx, members of the NURD co-repressor complex, and Polycomb group proteins (Gill, 2005). Elk-1 (Yang and Sharrocks, 2004), PPAR-gamma (Pascual *et al.*, 2005) and Pax3 (Hollenbach *et al.*, 1999) are examples where sumoylation of these transcription factors led to their association with transcriptional co-repressors and consequently repression of target genes. The current study examines how C/EBPbeta1 is regulated by sumoylation in breast cancer cells.

Purpose of this study

Oftentimes in the treatment of cancer, the goal of molecular therapy is to target a specific protein. If the particular protein promotes tumorigenesis, then the objective would be to downregulate or inhibit the actions of that protein. On the other hand, if the protein is a tumor suppressor, then the goal would be to reactivate or re-express the protein of interest. In the case of C/EBPbeta, targeting of this transcription factor is complicated by the fact that three isoforms of this protein exist and are functionally distinct. C/EBPbeta3 promotes cell death, a tumor suppressive function, whereas C/EBPbeta2 promotes proliferation and other transforming characteristics. Therefore, cancer therapy targeting C/EBPbeta would have to target a specific isoform of C/EBPbeta to achieve the desired effect. It is important to determine the function of each of the three isoforms of C/EBPbeta. This dissertation examines the function of C/EBPbeta1 in normal cells and how C/EBPbeta1 is negatively regulated during

transformation. This work further contributes to clarifying the functional differences between the three isoforms of C/EBPbeta.

Introduction of activated Ras into primary cells leads to senescence, whereas activated Ras expression in immortalized cells frequently leads to transformation. It is important to determine the different molecular players necessary for Ras transformation versus Ras-induced senescence, as these could be critical targets for cancer therapy. C/EBPbeta is a transcription factor that is a downstream effector of Ras signaling during Ras transformation and Ras-induced senescence. A potential mechanism for the dual functionalities displayed by Ras may be that Ras is signaling to different isoforms of C/EBPbeta in different cell types. The first goal of this study was to determine which transactivator isoform of C/EBPbeta induces senescence. Since we observed that p52-C/EBPbeta1 is not present in breast cancer cells, cells that escape senescence, and we know that C/EBPbeta2 promotes growth and transforming properties in MCF10A mammary epithelial cells, we hypothesized that full length C/EBPbeta1 would be the isoform responsible for the induction of senescence. We show that C/EBPbeta1 induces IL6 and subsequently senescence to a greater extent than C/EBPbeta2.

The second aim of this study was to determine what happens to p52-C/EBPbeta1 during transformation, since p52-C/EBPbeta1 is present in normal cells but not breast cancer cell lines. To examine this, I utilized the MCF10A mammary epithelilal cell line that is immortalized but non-transformed. Introduction of activated Ras(V12) into these cells transforms this cell line. Therefore I had a system available in which I could transform normal mammary epithelial cells simply by the introduction of activated Ras(V12). I demonstrate that when activated Ras(V12) is introduced into MCF10A cells, Ras(V12) leads to the phosphorylation of p52-C/EBPbeta1 on Thr235 by cdk2 and subsequent degradation of p52-C/EBPbeta1. These results indicate that C/EBPbeta1 is

negatively regulated by activated Ras(V12) in immortalized MCF10A cells and that this full length isoform of C/EBPbeta is able to induce senescence in normal cells by inducing IL6. Therefore degradation of C/EBPbeta1 by Ras activation may represent a mechanism to bypass oncogene-induced senescence.

I further demonstrate that degradation is not the only mechanism by which Ras negatively regulates C/EBPbeta1 during transformation. An antibody specific to C/EBPbeta1 recognizes higher molecular weight bands in breast cancer cell lines. Knowing that C/EBPbeta1 can be modified by the post-translational modification sumoylation, thus increasing the apparent molecular weight of C/EBPbeta1 via SDS-PAGE, we hypothesized that these higher molecular weight bands were sumoylated C/EBPbeta1. Sumoylation frequently represses the transcriptional abilities of target proteins, so we hypothesized that breast cancer cells sumoylate C/EBPbeta1 so that it can no longer induce IL6 expression and thus not induce senescence, a tumor suppressive mechanism. I demonstrate C/EBPbeta1 is sumoylated in breast cancer cells and that phosphorylation of C/EBPbeta1 on Thr235 enhances this sumoylation. Taken together, degradation or sumoylation of C/EBPbeta1 by Ras activation may represent mechanisms to bypass OIS. Therefore we conclude C/EBPbeta1 plays a unique role and is distinctly regulated in normal and transformed cells when compared to the other two isoforms of C/EBPbeta.

CHAPTER II

C/EBPBETA1 AND ONCOGENE-INDUCED SENESCENCE

Introduction

Oncogene-induced senescence (OIS) is a phenomenon that protects normal cells from becoming tumorigenic. When a potentially tumor-initiating oncogene is activated, such as Ras or Raf, cells have protective mechanisms such as OIS to avert the oncogenic threat. Sometimes though these tumor suppressive mechanisms are not successful and transformation of the cell occurs. It is still unclear why these means by which normal cells are protected from transformation occasionally fail. Studying the signaling differences between oncogene-induced transformation and OIS will give insight into potential targets for cancer therapy.

The transcription factor C/EBPbeta is an essential downstream mediator of both activated Ras/Raf-induced senescence (Sebastian *et al.*, 2005, Kuilman *et al.*, 2008) and Ras transformation (Zhu *et al.*, 2002). The current study focuses on the role of C/EBPbeta in OIS. The production of multiple isoforms of C/EBPbeta, an essential downstream mediator of Ras signaling, may be a potential mechanism by which this transcription factor could regulate the functional differences observed upon expression of activated Ras. Activation of interleukin 6 (IL6) by C/EBPbeta is essential for activated Raf-induced senescence (Kuilman *et al.*, 2008). We examine which transactivator isoform of C/EBPbeta is able to more effectively induce IL6 expression and thus senescence in the human diploid fibroblast (HDF) cell line WI-38. Since C/EBPbeta1 is expressed in normal mammary epithelial cells capable of undergoing Ras-induced senescence and this first isoform is negatively regulated in breast cancer cells in which

activation of the Ras pathway contributes to transformation and in which senescence is evaded (Eaton *et al.*, 2001), we hypothesized that C/EBPbeta1 is the isoform that Ras signals through to induce IL6 and senescence. In contrast, MCF10A mammary epithelial cells that overexpress C/EBPbeta2 exhibit transforming characteristics and breast cancer cells express high levels of this second isoform of C/EBPbeta. Because of this, we hypothesized that C/EBPbeta1 would be able to more effectively induce IL6 and senescence. We show that full length C/EBPbeta1 upregulates IL6 over 6-fold when introduced into WI-38 cells, a line commonly used to study senescence. Additionally we show that C/EBPbeta1 induces senescence in these cells. Introduction of C/EBPbeta2 into WI-38 cells resulted in only a 2.3 fold increase in IL6 expression. This second isoform was able to induce senescence, but to a lesser extent than C/EBPbeta1.

Materials and Methods

Reagents

Unless otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibody directed against the C-terminus of C/EBPbeta was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and was used at 1:5,000. anti-T7 tag monoclonal antibody was obtained from Novagen (Madison, WI, USA) and used at a dilution of 1:10,000. The N-terminal C/EBPbeta1-specific antibody was made as described in Eaton *et al.*, 2001 and was used at 1:2,000. This rabbit polyclonal antibody was raised to the first 16 amino acids specific to the first isoform of C/EBPbeta. We used an anti-beta-tubulin antibody (Sigma T7816) at 1:10,000 as a loading control. Anti-p16INK4A (Santa Cruz) was used at a dilution of 1:2000. The anti-

rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Promega (Madison, WI, USA).

Cell lines

Tissue culture media was obtained from Life Technologies, Inc. (Carlsbad, CA, USA). Unless otherwise indicated, all tissue culture supplements were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The WI-38 normal human diploid fibroblasts (a gift from the Dr. Hal Moses lab, Vanderbilt University) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT, USA), 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). The phoenix-ampho packaging cell line was obtained from the ATCC with the permission of GP Nolan (Stanford University, Palo Alto, CA, USA) and has been previously described (Grignani *et al.*, 1998). The packaging cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Inc., Logan, UT, USA), 1mM sodium pyruvate, 2mM L-glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). All cells were grown at 37 degrees Celsius in a humidified atmosphere containing 5% carbon dioxide.

Cloning of recombinant retroviral constructs and virus preparation

Generation of LZRS-T7-C/EBPbeta1-IRES-eGFP was as follows: IRES-eGFP-LZRS and T7-C/EBPbeta1-pcDNA3.1-His A were generated as described previously (Bundy and Sealy, 2003; Eaton and Sealy, 2003). T7-C/EBPbeta1-pcDNA3.1 (which contains a perfect Kozak sequence around the first ATG and a mutated second ATG) was digested with EcoRI and NotI and incubated with DNA Polymerase I. The resulting T7-C/EBPbeta1 insert was ligated into LZRS-IRES-eGFP, which had been digested with

EcoRI and incubated with DNA Polymerase I. This generated LZRS-T7-C/EBPbeta1-IRES-eGFP. LZRS-T7-C/EBPbeta2-IRES-eGFP was generated similar to described in Bundy and Sealy, 2003. The only difference being T7-C/EBPbeta2-IRES-eGFP-LZRS was digested with EcoRI, incubated with DNA Polymerase, and re-ligated. Recombinant amphotropic retroviral stock generation and retroviral infection were performed as described in Bundy and Sealy, 2003. The pBABE-Ras(V12)-puromycin retroviral construct was a kind gift from Dr. Scott Lowe, Cold Spring Harbor. Cells were infected once and selected with 1ug/mL puromycin (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of cell lysates and immunoblot analysis

Cell lysates were prepared from 100 mm dishes of 90% confluent WI-38 cells. Cells were scraped into cold 1xPBS containing 10uM sodium vanadate. Cells were spun down in eppendorf tubes for 10 minutes at 840 x g. Cell pellets were then resuspended in 100uL cold STE (10mM Tris pH 8.0, 1mM EDTA, 100mM sodium chloride) containing protease and phosphotase inhibitors (10uM sodium vanadate,10mM sodium molybdate, 10mM beta-glycerophosphate, 1ug/mL aprotinin, 1ug/mL leupeptin, 1ug/mL pepstatin, and 1mM phenylmethylsulfonyl fluoride) and then vortexed. 125uL 2x SDS sample buffer was then added followed by boiling of the samples for 5 minutes. Samples were stored at -70 degrees Celsius until ran on SDS-PAGE. Relative protein concentrations were determined using the Protein Assay Reagent (BioRad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Equal amounts of protein were loaded onto 10% or 12% SDS-PAGE and separated by electrophoresis. The proteins were transferred to an Immobilon P filter and the blots were processed as described previously (Eaton *et al.*, 2001). After the nonspecific binding sites were blocked, the blots were inclubated with primary antibody (C-terminal C/EBPbeta at a 1:5 000 dilution; T7 at 1:10 000, N-terminal C/EBPbeta at 1:2 000) in TBS-T (100mM Tris pH 7.5, 150

mM NaCl, and 0.05% Tween-20) containing 0.5% nonfat dried milk (NFDM) for 1 hour at room temperature. The blots were washed with three successeive changes of TBS-T containing 0.5% NFDM at room temperature for 20 minutes and incubated with a HRP-conjugated goat anti-rabbit (1:5 000 or 1:2 000 dilution) or a HRP-conjugated goat anti-mouse antibody (1:10 000 dilution) as described above for an additional hour. The blots were then washed with three successive changes of TBS-T solution for 15 minutes and the signal was detected by chemiluminescence using SuperSignal West Pico reagent (Pierce, Rockford, IL, USA) and autoradiography with Kodak X-OMAT film (Rochester, NY, USA).

Colony Formation Assay

800 cells were plated on 100mm tissue culture plates. After 10 days the media was removed from the cells, the cells were rinsed one time with 10mL of 1x PBS, and then fixed with 10mL of 95% ethanol for 15 min. at room temperature. The ethanol was then removed and the cells were stained with 10mL of Gills hematoxylin (Sigma Cat. #GHS332-1L) for 1 hour at room temperature. The cells were then rinsed with 10mL of water three times. The plates were then allowed to dry and the number of stained colonies were counted.

Brdu Incorporation Assay

13000 cells were seeded per well in an 8-well chamber slide 6 days post-infection. 24 hours post-plating the cells were pulsed with 10ug/mL Brdu (Sigma) in the culture medium for 24 hours. Cells were then fixed in formalin for 5 minutes. The Invitrogen streptavidin-biotin system for Brdu staining kit was then followed per manufacturer's instructions. The dark nuclear stain indicates replicating cells in which Brdu was incorporated. Hematoxylin was used as the counterstain.

Real time PCR

Total RNA was isolated using the RNeasy Mini kit and RNase-Free DNase kit (Qiagen, Maryland, USA). cDNA was synthesized with the high capacity cDNA reverse transcription kit according to the manufactures instructions (Applied Biosystems, Foster City, CA, USA). Taqman real-time PCR was then performed to determine the relative levels of targets, using GAPDH as the internal control. The IL6 probe used was Hs00985639_m1 (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a total volume of 20µl using a real-time PCR instrument (StepOnePlus, Applied Biosystems, Foster City, CA, USA).

Senescence associated beta-galactosidase assay

60% confluent WI-38 cells in 60mm plates were fixed and stained with the senescence beta-galactosidase staining kit as per manufacturers instructions (Cell Signaling Technology, Beverly, MA, USA)

Results

C/EBPbeta1 is not degraded upon activated Ras introduction in WI-38 normal human diploid fibroblast cells

Recently, C/EBPbeta has been shown to be essential for activated Ras- and Raf-induced senescence in MEFs and normal human diploid fibroblasts, respectively (Sebastian *et al.*, 2005, Kuilman *et al.*, 2008). We decided to determine which transactivator isoform of C/EBPbeta is responsible for the induction of senescence. First we wanted to examine the expression of C/EBPbeta1 in the normal human diploid fibroblasts WI-38 cells, cells commonly used to study senescence, and senescing WI-38-Ras cells. p52-C/EBPbeta1 is not detected on immunoblots of breast cancer cells

that have the Ras pathway activated (Eaton *et al.*, 2001). In addition, I demonstrate in Chapter 3 of this work that introduction of activated Ras(V12) into the immortalized but non-transformed MCF10A mammary epithelial cell line leads to transformation of these cells accompanied by degradation of p52-C/EBPbeta1 expression. Therefore it is first important to determine whether p52-C/EBPbeta1 is expressed when activated Ras expression leads to senescence in WI-38 cells. Figure 8 is an immunoblot with an antibody specifc for the N-terminal 23 amino acids present only in C/EBPbeta1. This immunoblot demonstrates that activated Ras(V12) does not lead to the degradation of C/EBPbeta1 in the normal WI-38 cells after 6 days or 3 weeks (Figure 8, compare lanes 2 and 3 with lane 1).

C/EBPbeta1 expression in the human fibroblast cell line WI-38 is incompatible with growth

Next, I wanted to test whether overexpression of C/EBPbeta1 was compatible with growth in the normal fibroblasts. WI-38 cells were infected with a retroviral vector expressing T7-tagged C/EBPbeta1, LZRS-T7-C/EBPbeta1-IRES-eGFP. C/EBPbeta1 is the only transactivator C/EBPbeta isoform produced by this retrovirus because the second ATG necessary to translate C/EBPbeta2 has been mutated. These cells were not sorted, so a heterogenous population of cells existed: GFP negative cells that did not express T7-C/EBPbeta1 and GFP positive cells that did express T7-C/EBPbeta1. The percentage of GFP positive (and therefore T7-C/EBPbeta1 positive) cells were counted every two days for 10 days. The percentage of WI-38-C/EBPbeta1 cells rapidly decreased as compared to WI-38 cells infected with LZRS-eGFP (Figure 9). This indicates that T7-C/EBPbeta1 expression in the WI-38 cells is incompatible with growth and potentially causing these cells to senesce.

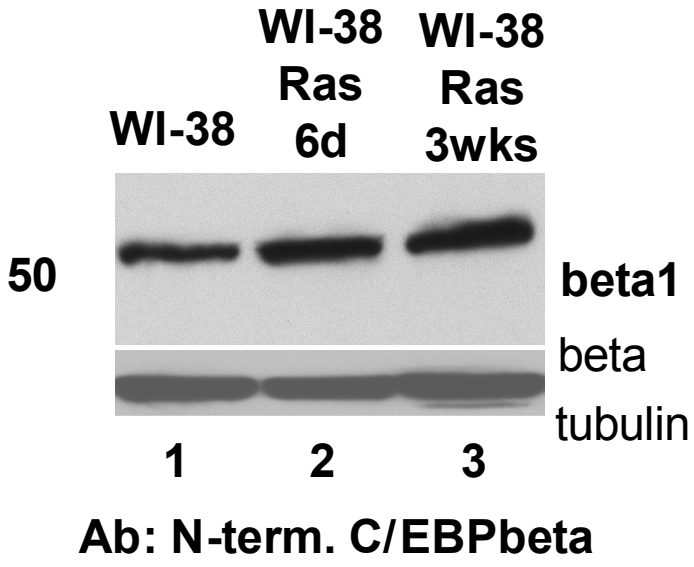

Figure 8. C/EBPbeta1 is not degraded by introduction of activated Ras into WI-38 normal fibroblasts. WI-38 cells were infected with pBABE-Ras(V12)-puromycin and selected with puromycin for 6 days or 3 weeks. Evidence for Ras(V12) expression in these cells includes their senescent phenotype and induction of IL6 (Figures 12 and 14). Lane 1 is uninfected WI-38 cells, lane 2 is WI-38-Ras(V12) cells 6 days post-infection, and lane 3 is WI-38-Ras(V12) cells 3 weeks post-infection. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. Immunoblot analysis was performed with an antibody specific to C/EBPbeta1 as described in Eaton *et al*. (2001). Immunoblot analysis for beta-tubulin was performed as a loading control (Sigma T7816). (beta = C/EBPbeta, d = days, wks = weeks)

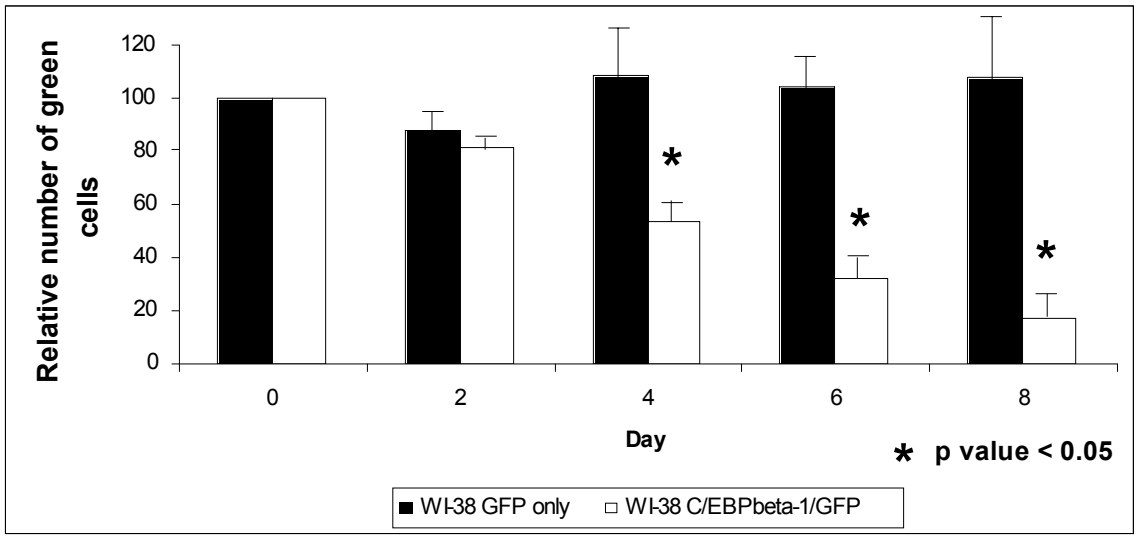

Figure 9. Exogenous expression of T7-C/EBPbeta1 in WI-38 cells is incompatible with growth. WI-38 human diploid fibroblasts were infected with LZRS-T7-C/EBPbeta1-IRES-eGFP. Cells were not sorted for GFP positivity, therefore a heterogeneous population of cells existed: GFP positive cells that expressed T7-C/EBPbeta1 and GFP negative cells that did not express T7-C/EBPbeta1. The percentage of GFP positive (and therefore T7-C/EBPbeta1 positive) cells were counted every two days for 8 days and the relative number of green cells is represented by the white bar. This is compared to WI-38 cells infected with LZRS GFP and not sorted, represented by the black bar. This experiment was repeated three times. Error bars indicate standard deviation. Statistical analysis was performed using the student t test and gave a statistically significant p value of < 0.05 for days 4, 6, and 8.

Next, we performed a colony formation assay using uninfected WI-38 cells or WI-38 cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP or LZRS-eGFP. Infected cells were then sorted by fluoresecence activated cell sorting (FACS) using green fluorescent protein (GFP) as a marker. Immediately after sorting, 800 cells were plated in a 100 mm dish and allowed to grow for 10 days. The cells were then fixed and stained with hematoxylin. As shown in Figure 10, there were significantly fewer WI-38-T7-C/EBPbeta1 colonies compared to controls ($p=0.007$), indicating T7-C/EBPbeta1 expression in WI-38 cells inhibits growth.

To confirm these findings, we performed a bromodeoxyuridine (Brdu) assay using uninfected WI-38 cells, or sorted WI-38 cells expressing T7-C/EBPbeta1 and GFP or GFP only. When cells are treated with Brdu, the Brdu incorporates into replicating cells. Therefore replicating cells stain positive for Brdu whereas cells that are not growing do not stain positive for Brdu. Immediately after sorting the infected cells using GFP as a marker, 13000 cells were seeded per well in an 8-well chamber slide 6 days post-infection. 24 hours post-plating the cells were pulsed with Brdu in the culture medium for 24 hours. Cells were then fixed and stained for Brdu. The dark nuclear stain indicates replicating cells in which Brdu was incorporated. Hematoxylin was used as the counterstain. The WI-38-T7-C/EBPbeta1 cells have significantly less Brdu staining than controls ($p=0.027$), confirming the results from Figures 9 and 10 that C/EBPbeta1 expression in WI-38 cells inhibits their proliferation (Figure 11).

C/EBPbeta1 induces expression of IL6 in WI-38 normal fibroblasts

Activated Raf (a downstream mediator of Ras signaling)-induced senescence in human diploid fibroblasts is due to activation of an inflammatory transcriptome (Kuilman *et al.,* 2008). Activated Raf-

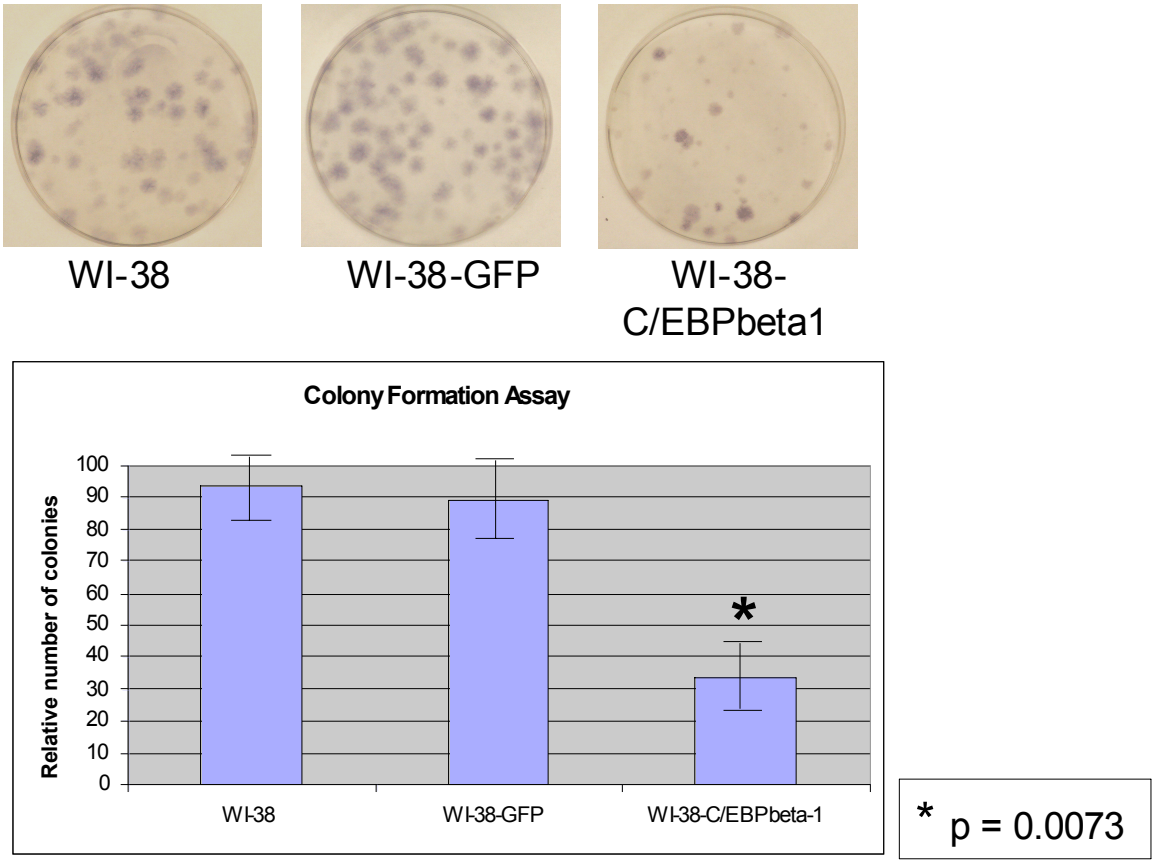

Figure 10. Exogenous expression of T7-C/EBPbeta1 in WI-38 cells inhibits growth as demonstrated by colony formation assay. WI-38 human diploid fibroblasts were infected with LZRS-GFP or LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS using GFP as a marker. 800 cells were plated and after 10 days the cells were fixed with 95% ethanol and stained with Gills hematoxylin (Sigma). The number of stained colonies were counted. T7-C/EBPbeta1 expression was verified by immunoblot (data not shown). c. Quantitative comparison of the colony formation assay. This experiment was repeated three times with the standard deviation represented by error bars. Quantitative comparison using the student t test indicates that there is a statistically significant difference in colony number in the T7-C/EBPbeta1-expressing WI-38 cells versus uninfected or GFP only. *p = 0.007.

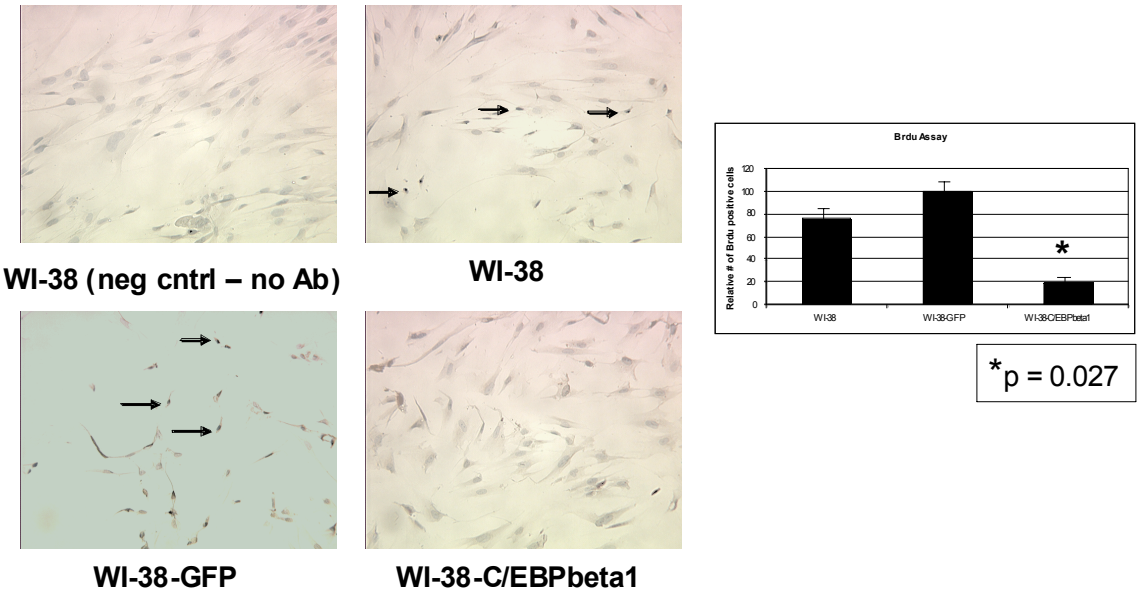

Figure 11. Exogenous expression of C/EBPbeta1 in WI-38 cells inhibits their growth as demonstrated by Brdu incorporation assay. WI-38 human diploid fibroblasts were infected with LZRS-GFP or LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS using GFP as a marker. 13000 cells were seeded per well in an 8-well chamber slide 6 days post-infection. 24 hours post-plating the cells were pulsed with 10ug/mL Brdu (Sigma) for 24 hours. Cells were then fixed in formalin for 5 minutes. The Invitrogen streptavidin-biotin system for Brdu staining kit was then followed per manufacturer's instructions. The dark nuclear stain indicates replicating cells in which Brdu was incorporated (examples indicated by arrows). Hematoxylin was used as the counterstain.

induced senescence was dependent on activation of IL6. They went on to show that activation of IL6 was dependent on C/EBPbeta. C/EBPbeta has long been known to transcriptionally activate IL6, as another name for C/EBPbeta is nuclear factor of IL6 (NF-IL6). Thus we decided to examine if a specific transactivator isoform of C/EBPbeta was inducing IL6 expression. WI-38 cells were infected with LZRS-T7-C/EBPbeta1-IRES-eGFP, LZRS-T7-C/EBPbeta2-IRES-eGFP, LZRS-GFP, or pBABE-Ras(V12)-puromycin and RNA was prepared from these cells six days post-infection. cDNA was prepared from the RNA and real time PCR was performed using an IL6 primer. Real time qPCR results indicate that the T7-C/EBPbeta1-expressing WI-38 cells and the positive control Ras(V12)-expressing cells highly upregulate IL6 at the RNA level by 6.6 fold and 8 fold, respectively (Figure 12). T7-C/EBPbeta2 only induced IL6 expression 2.3 fold compared to uninfected cells. Thus C/EBPbeta1 is the primary transactivator isoform of C/EBPbeta responsible for the induction of IL6.

Figure 13 demonstrates that approximately equal levels of T7-C/EBPbeta1 and T7-C/EBPbeta2 protein were expressed in the WI-38 cells (Figure 13, compare lanes 2 and 3). Cell lysates were prepared from WI-38 cells or WI-38 cells that were infected with either LZRS-T7-C/EBPbeta1-IRES-eGFP or LZRS-T7-C/EBPbeta2-IRES-eGFP. Immunoblot with the C-terminal C/EBPbeta antibody demonstrates that both transactivator isoforms of C/EBPbeta are expressed in WI-38 cells (Figure 13, lane 1). The exogenously expressed T7-C/EBPbeta2 does migrate slower than endogenous C/EBPbeta2 because rat C/EBPbeta2 is being used which is smaller than the human form (Figure 13, lane 3).

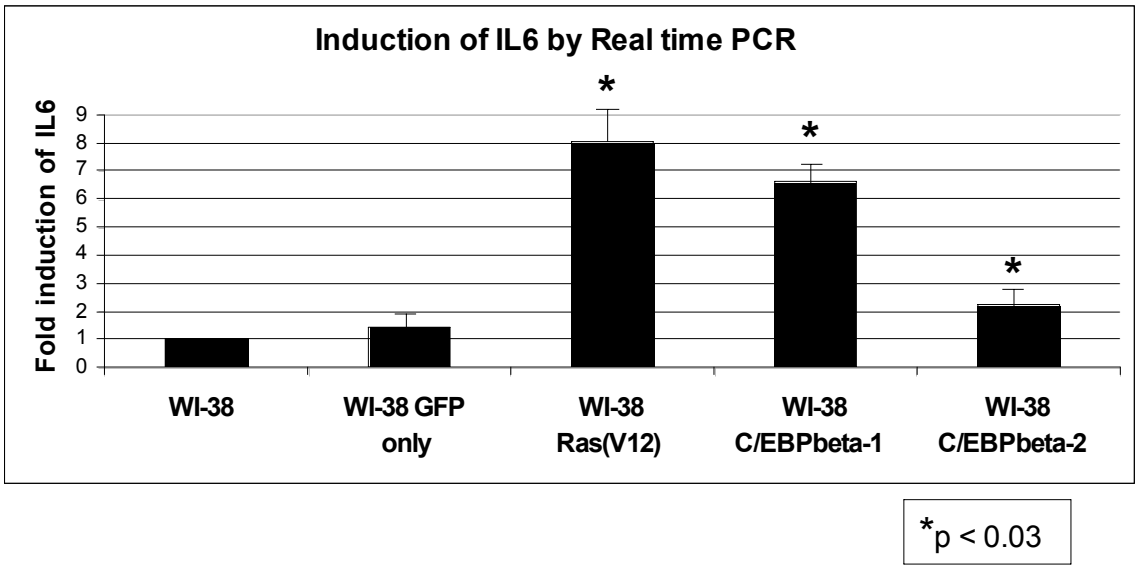

Figure 12. C/EBPbeta1 induces IL6 expression in WI-38 human fibroblasts. WI-38 cells were infected with LZRS-GFP, pBABE Ras(V12)-puromycin, LZRS-T7-C/EBPbeta1-IRES-eGFP, or LZRS-T7-C/EBPbeta2-IRES-eGFP. Six days post-infection RNA was prepared. Total RNA was isolated using the RNeasy Mini kit and RNase-Free DNase kit (Qiagen). cDNA was synthesized with the high capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems). Taqman real-time PCR was performed to determine the relative levels of targets, using GAPDH as the internal control and an IL6 primer (Hs00985639_m1, Applied Biosystems). Results are presented as fold induction of IL6 as compared to uninfected WI-38. This assay was repeated three times with standard deviation represented by the error bars. p values were calculated using the student t test. ** $p < 0.02$, * $p < 0.03$.

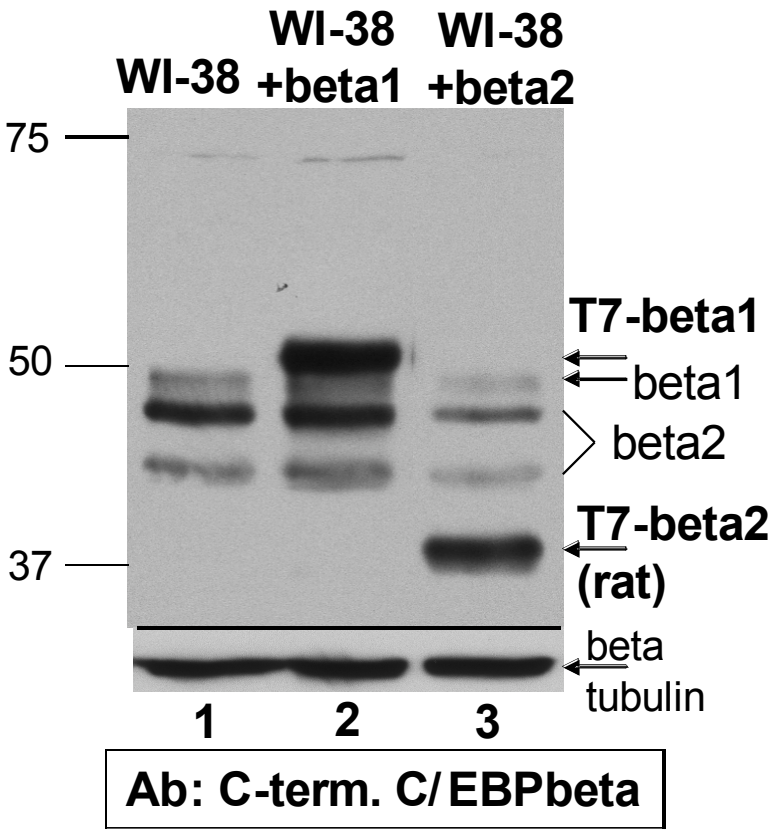

Figure 13. Approximately equal levels of T7-C/EBPbeta1 and T7-C/EBPbeta2 protein are expressed in the WI-38 cells. Immunoblot analysis of T7-C/EBPbeta1 versus T7-C/EBPbeta2 protein levels in infected WI-38 cells six days post-infection. Cell lysates from WI-38 cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP or LZRS-T7-C/EBPbeta2-IRES-eGFP were analyzed via 10% SDS-PAGE. Lane 1 is uninfected WI-38 cells, lane 2 is WI-38-T7-C/EBPbeta1 cells, and lane 3 WI-38-T7-C/EBPbeta2 cells. Equal amounts of total protein were loaded in each lane. Immunoblot analysis was performed with an anti-C/EBPbeta C-terminal antibody (Santa Cruz C-19) at 1:5000. Arrows indicate the particular isoforms of C/EBPbeta. T7-beta indicates the exogenously expressed protein. C/EBPbeta2 is smaller than the endogenous because the T7-tagged rat protein is being expressed, which is smaller than human. Immunoblot analysis for beta-tubulin was performed as a loading control (Sigma T7816). (beta = C/EBPbeta)

C/EBPbeta1, and to a lesser extent C/EBPbeta2, induce senescence in WI-38 normal human fibroblasts

Given the fact that C/EBPbeta1 induces IL6 to a much greater level than C/EBPbeta2, we proceeded to determine if a specific transactivator isoform of C/EBPbeta could cause senescence more effectively in the normal fibroblasts. WI-38 cells were infected with LZRS-T7-C/EBPbeta1-IRES-eGFP, LZRS-T7-C/EBPbeta2-IRES-eGFP, LZRS-GFP, or pBABE-Ras(V12)-puromycin were fixed and stained for senescence-associated beta-galactosidase six days post-infection. Five fields per plate were imaged with a light microscope with representative photomicrographs displayed in Figure 14. On average over 53% of the WI-38 cells that expressed T7-C/EBPbeta1 and over 55% of the positive control WI-38-Ras(V12) cells stained a bright blue, compared to 34.5% of the T7-C/EBPbeta2 expressing cells. Less than 20% of control uninfected or cells infected with LZRS GFP stained a faint blue. This experiment was repeated three times. Quantitative comparison indicates that there is a statistically significant difference in blue staining between the T7-C/EBPbeta1 and Ras(V12) expressing WI-38 cells versus uninfected, resulting in p values 0.005 and 0.003, respectively (Figure 15). This is consistent with the disappearance of T7-C/EBPbeta1/GFP expressing cells from the population noted earlier. Although the T7-C/EBPbeta2 cells induced senescence to a lesser extent than T7-C/EBPbeta1, the percentage of blue cells was still statistically significant as compared to the uninfected WI-38 cells. 35.4% of the T7-C/EBPbeta2-WI-38 cells stained blue giving a p value of 0.03. This level of senescence induction by C/EBPbeta2 is surprising given the small (2.3 fold) induction of IL6 (Figure 12). Therefore C/EBPbeta2 is likely upregulating other factors involved in senescence, as other cytokines are upregulated and essential for senescence (Kuiman *et al.,* 2008).

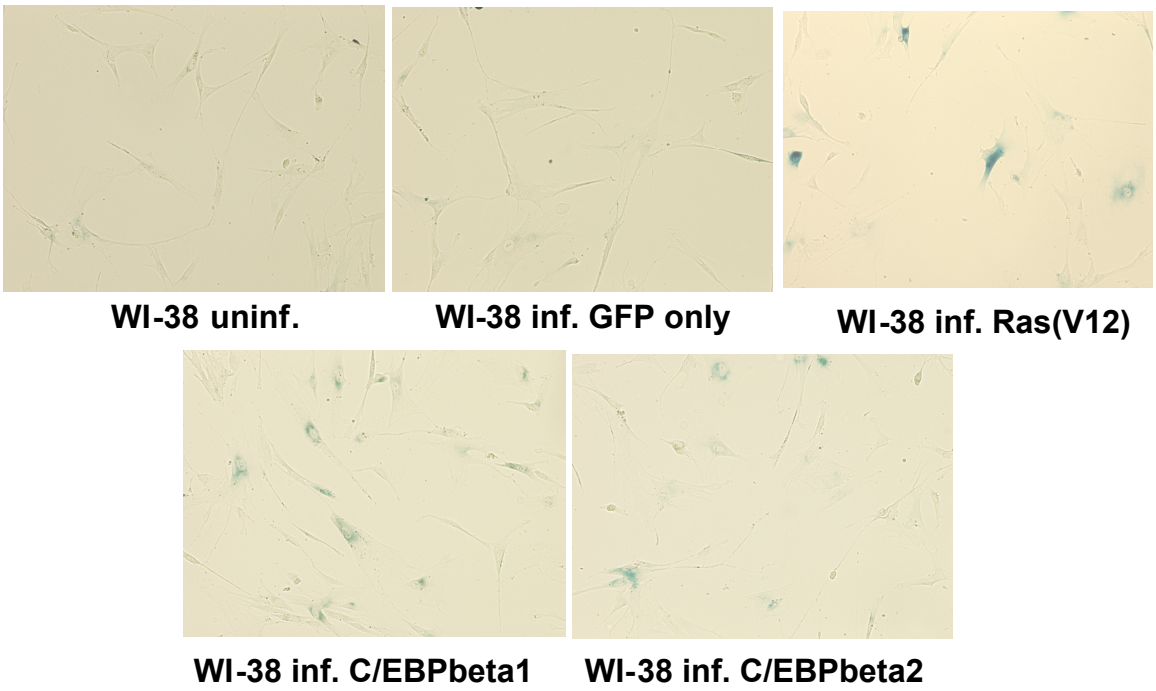

Figure 14. C/EBPbeta1, and to a lesser extent C/EBPbeta2, induce senescence in WI-38 human fibroblasts. WI-38 cells were infected with LZRS-GFP, LZRS-T7-C/EBPbeta1-IRES-eGFP, LZRS-T7-C/EBPbeta2-IRES-eGFP, or pBABE Ras(V12)-puromycin. Six days post-infection 60% confluent cells were fixed in 60mm plates and stained for with the senescence associated beta-galactosidase kit as per manufacturer's instructions (Cell Signaling Technology). Five fields per plate were imaged with a light microscope with representative photomicrographs displayed.

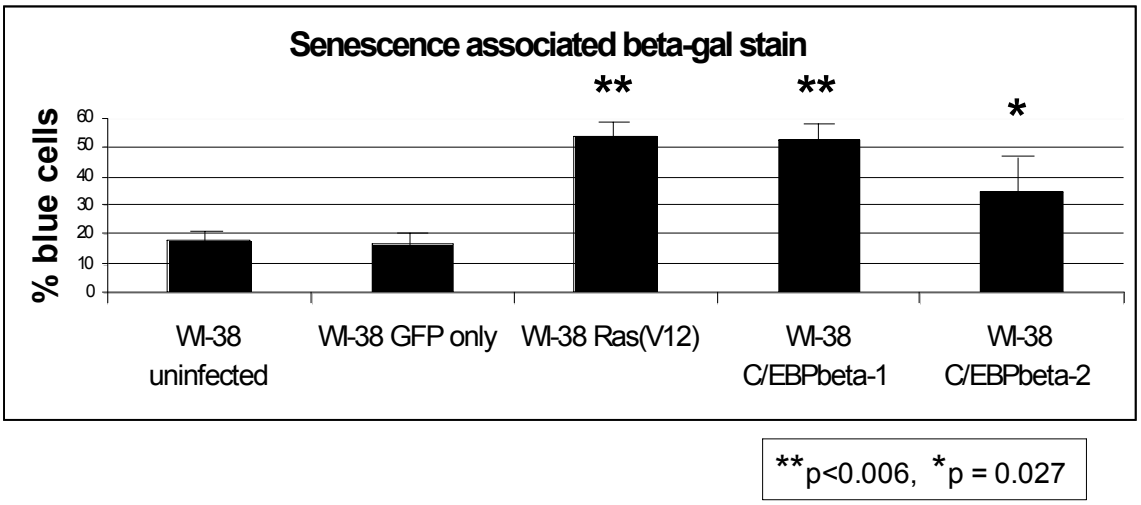

Figure 15. Quantitative evidence that C/EBPbeta1, and to a lesser extent C/EBPbeta2, induce senescence in WI-38 human normal fibroblasts. Quantitative comparison of senescence associated beta-galactosidase stain. This experiment was repeated three times with standard deviation represented by error bars. Quantitative comparison using the student t test indicates that there is a statistically significant difference in blue staining between the Ras(V12), T7-C/EBPbeta1, and T7-C/EBPbeta2 expressing WI-38 cells versus uninfected. ** $p < 0.006$, * $p < 0.03$.

Figure 13 indicates that approximately equal levels of T7-C/EBPbeta1 and T7-C/EBPbeta2 protein were expressed in the WI-38 cells.

To confirm these findings, p16INK4A protein levels were examined, as p16INK4A is upregulated during senescence. WI-38 cells were uninfected, infected with LZRS-T7-C/EBPbeta1-IRES-eGFP, LZRS-T7-C/EBPbeta2-IRES-eGFP or LZRS-GFP, cell lysates were prepared 6 days post-infection and immunoblot analysis was performed for p16INK4A expression. Figure 16 indicates that C/EBPbeta1 and C/EBPbeta2 expression in WI-38 cells induces p16INK4A expression, verifying the induction of senescence. This is consistent with a previous report indicating that C/EBPbeta signals through p16/Rb to induce senescence (Sebastian *et al.*, 2005).

Discussion

Although introduction of activated Ras into many immortalized cell lines leads to transformation, it is known that introduction of Ras into normal primary cells has a different effect. Normal fibroblasts, such as WI-38 human diploid fibroblasts, undergo senescence when forced to express activated oncogenes such as activated Ras or Raf. This is a tumor suppressive mechanism that is present in normal cells, and there is mounting evidence indicating that senescence inhibits tumorigenesis in vivo (reviewed in Mooi and Peeper, 2006, Prieur and Peeper, 2008). Sebastian *et al.* demonstrated that C/EBPbeta is necessary for activated Ras-induced senescence in mouse embryonic fibroblasts (MEFs), and this occurred in a pRB-dependent, p53-independent manner. Recently Kuilman *et al.* has shown that C/EBPbeta is required for activated Raf-induced senescence in human diploid fibroblasts.

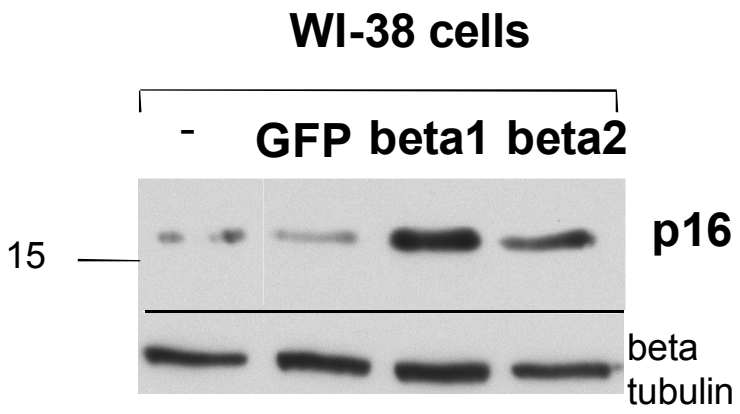

Figure 16. C/EBPbeta1 and C/EBPbeta2 induce p16INK4A expression in WI-38 human fibroblasts. WI-38 cells were infected with LZRS-GFP, LZRS-T7-C/EBPbeta1-IRES-eGFP, or LZRS-T7-C/EBPbeta2-IRES-eGFP. Six days post-infection cell lysates were made and run on a 10% SDS-PAGE. Equal amounts of protein were loaded in each lane. Immunoblot analysis was carried out with an anti-p16INK4A antibody (Santa Cruz). Immunoblot analysis for beta-tubulin was performed as a loading control (Sigma T7816).

In the present study I set out to determine which transactivator isoform of C/EBPbeta is responsible for oncogene-induced senescence, as there is increasing evidence for significant functional differences between the transactivator isoforms of C/EBPbeta (Bundy *et al.*, 2005, Eaton *et al.*, 2001, Eaton and Sealy, 2003, Kowentz-Leutz and Leutz, 1999). First I demonstrate that C/EBPbeta1 is not degraded in senescing WI-38-Ras cells (Figure 8). The mechanism by which C/EBPbeta induced senescence was through induction of IL6 (Kuilman *et al.,* 2008). We show that it is the full length isoform, C/EBPbeta1, that is able to robustly induce IL6 expression. Accordingly, we demonstrate that C/EBPbeta1 is the strongest inducer of senescence in WI-38 fibroblasts. In further support of our findings, Kuilman *et al.* used a C/EBPbeta construct in which the start sites for C/EBPbeta2 and -3 were mutated such that only C/EBPbeta1 could be translated. They demonstrated that expression of this construct in primary fibroblasts led to cell cycle arrest and induction of IL6, consistent with our results. C/EBPbeta2 can induce senescence to a lesser extent. It is interesting that C/EBPbeta2 expression in the WI-38 normal fibroblasts does induce senescence. C/EBPbeta2 is likely upregulating the expression of other factors involved in OIS, as other cytokines are upregulated in OIS (Kuilman *et al.,* 2008). Also, C/EBPbeta1 is expressed in the WI-38 cells, and the C/EBPbeta2 may be heterodimerizing with the endogenous C/EBPbeta1 to induce IL6 to low levels and thus low levels of senescence. Taken together, it is likely that activated Ras/Raf is working through C/EBPbeta1 to induce IL6 and subsequently lead to senescence in normal cells.

The production of multiple isoforms of C/EBPbeta is a possible explanation for which this transcription factor could regulate the functional differences observed upon expression of activated Ras. Overexpression of C/EBPbeta2 in MCF10A cells leads to the acquisition of tumorigenic characteristics (Bundy and Sealy, 2003), similarly to

activated Ras expression in these cells. Therefore, Ras may be signaling to this second isoform of C/EBPbeta during transformation. Unfortunately it is not possible to knockdown each specific isoform of C/EBPbeta via siRNA technology and determine the role each isoform is playing in cells, due to the fact that all three isoforms are translated from a single mRNA molecule. We do know however, that knockdown of all three isoforms of C/EBPbeta via siRNA is incompatible with survival of mammary epithelial cells and breast cancer cells (unpublished data). Importantly, one study has examined the specific role of C/EBPbeta2 in activated macrophages by generating a knock-in mouse in which the second in frame methionine necessary for translation of C/EBPbeta2 was replaced with an alanine (Uematsu *et al.*, 2007). This abolished expression of C/EBPbeta2 in these mice. The induction of C/EBPbeta target genes, such as IL6, was examined in this study. The authors demonstrated that activated macrophages in the mice unable to express C/EBPbeta2 were still able to induce IL6 expression, consistent with our current findings that C/EBPbeta2 is not the isoform responsible for the induction of IL6 in normal fibroblasts.

C/EBPbeta plays many functional roles in the cell, even roles that seem conflicting such as cell survival, cell death, proliferation, senescence, and transformation. This may be explained in part by the presence of the different isoforms of C/EBPbeta and their functional differences. Future studies on C/EBPbeta will need to take into consideration that the different isoforms of this transcription factor are likely playing functionally distinct roles. It will be important to determine the details of the signaling between Ras and C/EBPbeta in normal cells to elucidate mechanisms by which this critical signaling that induces OIS can be triggered or maintained to suppress tumorigenesis.

In this current study, we demonstrate a functional role for C/EBPbeta1 inducing IL6 and subsequently senescence, a tumor suppressive mechanism, in normal fibroblasts. C/EBPbeta is essential for activated Ras- and Raf- induced senescence (Sebastian *et al.*, 2005, Kuilman *et al.*, 2008), and it is likely that activated Ras and Raf signal through this first, full length isoform of C/EBPbeta to induce senescence. Since C/EBPbeta1 plays a role in senescence, a tumor suppressive mechanism, it is not surprising that p52-C/EBPbeta1 is not expressed in breast cancer cells as demonstrated in Eaton *et al.*, 2001. Therefore in the following two chapters I examine how p52-C/EBPbeta1 is negatively regulated by Ras during transformation of mammary epithelial cells.

CHAPTER III

DEGRADATION OF C/EBPBETA1 BY ACTIVATED RAS IN MAMMARY EPITHELIAL AND WI-38 CELLS

Introduction

Ras signaling leads to proliferation and cell survival and is activated in many cancers. Aberrant function of the Ras pathway is very common in breast cancers (reviewed in Malaney and Daly, 2001, Dunn *et al.*, 2005). Introduction of activated Ras(V12) into most immortalized cell lines, including the immortalized MCF10A human mammary epithelial cell (HMEC) line, leads to transformation. However, when Ras(V12) is expressed in primary cells, these cells senesce instead of undergoing transformation. This oncogene-induced senescence (OIS) displayed by primary cells represents a tumor suppressive mechanism inherent in these cells. Research is ongoing to determine the proteins and pathways involved in Ras transformation and Ras-induced senescence. It is important to determine the different molecular players necessary for Ras transformation versus Ras-induced senescence, as these could be critical targets for cancer therapy.

C/EBPbeta is a transcription factor that is a downstream effector of Ras signaling during Ras transformation and Ras-induced senescence (Zhu *et al.*, 2002, Sebastian *et al.*, 2005, Kuilman *et al.*, 2008). v-Ha-ras transgenic mice deficient for C/EBPbeta had smaller and fewer skin tumors than v-Ha-ras mice that expressed C/EBPbeta (Zhu *et al.,* 2002). C/EBPbeta also cooperates with Ras(V12) in transformation of NIH-3T3 cells. Since C/EBPbeta is an essential downstream mediator of Ras signaling, the production of multiple isoforms of C/EBPbeta may be a potential mechanism to explain the functional differences observed upon expression of Ras(V12).

C/EBPbeta2 is expressed at high levels in breast cancer cells and is capable of transforming the MCF10A mammary epithelial cell line very similarly to how activated Ras transforms these cells, suggesting that Ras signals through C/EBPbeta2 during transformation. C/EBPbeta1 on the other hand is expressed in normal mammary epithelial cells but not breast cancer cells (Eaton *et al.*, 2001). In the previous chapter we demonstrate that C/EBPbeta1 is expressed in normal fibroblasts and is the isoform that can effectively induce IL6 expression and senescence in these cells. This data suggests that Ras signals through this full length isoform of C/EBPbeta during Ras-induced senescence. This second chapter focuses on how Ras negatively regulates C/EBPbeta1 during transformation of mammary epithelial cells.

Ras signaling frequently regulates its downstream effectors through phosphorylation. C/EBPbeta is phosphorylated in response to activated Ras signaling, and this phosphorylation is oftentimes critical for the Ras-induced phenotype observed. A residue in C/EBPbeta that is frequently phosphorylated upon introduction of activated Ras is threonine 235 (Thr235) in humans (Thr189 in the rat, Thr188 in the mouse). Activated Ras expression in NIH-3T3s enhances phosphorylation of Thr189 (and Ser64) in C/EBPbeta by cyclin dependent kinase 2 (cdk2) (Shuman *et al.,* 2004). Additionally, this site in C/EBPbeta2 is necessary for the Ras-stimulated interaction of C/EBPbeta2 and serum response factor (SRF) for subsequent transactivation of the serum response element by C/EBPbeta2 in NIH-3T3 cells and is phosphorylated by Erk-2 (Hanlon *et al.*, 2001).

As mentioned earlier, transformed cell lines typically do not express p52-C/EBPbeta1. However, the immortal but non-transformed HMEC line, MCF10A, expresses all three C/EBPbeta isoforms. In this current study, we demonstrate that when activated Ras(V12) is introduced into MCF10A cells, transformation of these cells

is accompanied by loss of expression of p52-C/EBPbeta1. Immunoblot analysis of MCF10A and MCF10A-Ras(V12) cells expressing T7-tagged C/EBPbeta1 reveals a band at 52kDa in the MCF10A-C/EBPbeta1 cells whereas the MCF10A-Ras-C/EBPbeta1 cells display bands at 31kDa and 23kDa, indicative of degradation. We show that treatment of the MCF10A-Ras-C/EBPbeta1 cells with proteasomal inhibitors stabilizes expression of C/EBPbeta1 in these cells, and that degradation of C/EBPbeta1 is likely ubiquitin-mediated. Treatment of the MCF10A-Ras-C/EBPbeta1 cells with the cdk inhibitor roscovitine leads to stabilization of p52-C/EBPbeta1. cdk2 phosphorylates C/EBPbeta on Thr235 (Shuman *et al.*, 2004, Li *et al.*, 2007). We demonstrate that mutation of Thr235 to alanine in C/EBPbeta1 is sufficient to restore the stability of p52-C/EBPbeta1 expression in the MCF10A-Ras cells. In addition, we show that senescing WI-38-Ras cells do not have activated cdk2, which is likely why these cells do not degrade C/EBPbeta1. To confirm that transformation of cells other than MCF10As by Ras(V12) leads to loss of C/EBPbeta1, we demonstrate that transformation of WI-38 cells via the introduction of hTERT, SV40T/t, and Ras(V12) lead to the loss of p52-C/EBPbeta1. Additionally these transformed cells have activated cdk2.

Materials and Methods

Reagents

Unless otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibody directed against C/EBPbeta was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at a dilution of 1:5,000. The anti-T7 tag monoclonal antibody was obtained from Novagen (Madison, WI, USA) and used at 1:10,000. The N-terminal C/EBPbeta1-specific antibody was made as described in Eaton *et al.*, 2001 or was purchased from Abcam (18F8) and was used at 1:2,000.

The anti-Ubiquitin antibody (Enzo Life Sciences FK2) was used at a dilution of 1:2,000. The anti-pErk, anti-Erk, anti-cdk2 (all Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-pcdk2 (Cell Signaling Technology, Beverly, MA, USA) antibodies were used at 1:2000. We used an anti-beta-tubulin antibody (Sigma T7816) at 1:10,000 as a loading control. The anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Promega (Madison, WI, USA). The following kinase inhibitors were resuspended in DMSO: the MEK inhibitor UO126 (Cell Signaling Technology, Beverly, MA, USA) was used at a concentration of 25uM, cdk inhibitor roscovitine (LC Laboratories, Woburn, MA) at 50uM, JNK inhibitor SP600125 at 50uM, p38 inhibitor at 20uM, and the GSK3 inhibitor TDZD-8 at 25uM. The proteasome inhibitor MG132 (Calbiochem, San Diego, CA, USA) was resuspended in DMSO and used at a concentration of 50uM.

Cell lines

Tissue culture media was obtained from Life Technologies, Inc. (Carlsbad, CA, USA). Unless otherwise indicated, all tissue culture supplements were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MCF10A human mammary epithelial cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in a 1:1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle medium (DMEM) containing 2.5 mM L-glutamine and supplemented with 5% horse serum, 10ug/mL insulin, 0.5 ug/mL hydrocortisone, 20ng/mL epidermal growth factor, 100 ng/mL cholera toxin (Calbiochem Novabiochem, San Diego, CA, USA), 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). The phoenix-ampho packaging cell line was obtained from the ATCC with the permission of GP Nolan (Stanford University, Palo Alto, CA, USA) and has been previously described (Grignani *et al.*, 1998). The packaging cells were maintained in DMEM supplemented with 10%

heat-inactivated FBS (HyClone Laboratories, Inc., Logan, UT, USA), 1mM sodium pyruvate, 2mM L-glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). All cells were grown at 37 degrees Celsius in a humidified atmosphere containing 5% carbon dioxide.

Cloning of recombinant retroviral constructs and virus preparation

Generation of LZRS-T7-C/EBPbeta1-IRES-eGFP was generated as follows: the IRES eGFP LZRS construct was generated by replacing the beta-galactosidase (LacZ) coding sequence present in the previously described hybrid EBV/retroviral contstruct pLZRS-LacZ (Kinsella and Nolan, 1996) with the 1346 base pair internal ribosome entry site (IRES) and enhanced GFP coding sequences excised from pIRES2-EGFP expression vector (Clontech, Palo Alto, CA, USA) at the EcoRI and NotI restriction sites. T7-C/EBPbeta1-pcDNA3.1-His A was generated as described previously (Eaton and Sealy, 2003), which contains a perfect Kozak sequence around the first in-frame ATG and a mutated second in-frame ATG so that the only transactivator isoform of C/EBPbeta produced is the full length. T7-C/EBPbeta1-pcDNA3.1 was digested with EcoRI and NotI and incubated with DNA Polymerase I to generate blunt ends. The resulting T7-C/EBPbeta1 insert was then ligated into LZRS IRES eGFP, which had been digested with EcoRI and BamHI and incubated with DNA Polymerase I to generate blunt ends. This successfully generated LZRS-T7-C/EBPbeta1-IRES-eGFP. LZRS-T7-C/EBPbeta1T235A-IRES-eGFP was generated as follows: the IRES eGFP LZRS construct was made as described above. NF-IL6 T235A CMV, a generous gift from the Akira laboratory, was digested with MscI and BbsI to drop out a fragment of C/EBPbeta that contained the T235A mutation. Our C/EBPbeta1 pRSET-A construct, described in Eaton et. al., was also digested with MscI and BbsI. The T235A fragment was then ligated into C/EBPbeta1 pRSET-A to create C/EBPbeta1 T235A pRSET-A. C/EBPbeta1

T235A pRSET-A was then digested with HindIII, incubated with DNA Polymerase to create a blunt end, and then digested with BamHI to allow for the isolation of C/EBPbeta1 T235A. pcDNA3.1-His A was then digested with BamHI and EcoRV. The C/EBPbeta1 T235A insert was then ligated into pcDNA3.1-His A to create C/EBPbeta1 T235A pcDNA3.1-His A. C/EBPbeta1 T235A pcDNA3.1-His A was then digested with HindIII and XbaI and incubated with DNA Polymerase to isolate T7-C/EBPbeta1 T235A. LZRS IRES eGFP was digested with EcoRI and incubated with DNA Polymerase to create blunt ends. T7-C/EBPbeta1 T235A was then ligated into LZRS IRES eGFP to create LZRS-T7-C/EBPbeta1-IRES-eGFP. LZRS-T7-C/EBPbeta2-IRES-eGFP was generated similar to described in Bundy and Sealy, 2003. The only difference being that T7-C/EBPbeta2 IRES eGFP LZRS was digested with EcoRI, incubated with DNA Polymerase to create blunt ends, and re-ligated. Recombinant amphotropic retroviral stock generation and retroviral infection were performed as described in Bundy and Sealy, 2003. Cells were infected three times before cell sorting. Briefly, growth medium from the cells was removed and replaced with virus-containing media along with 4ng/mL polybrene. After 6-8 hours the virus-containing media was supplemented with growth media overnight. The next day virus-containing media was removed and replaced with growth medium. Infected cell lines were sorted using fluorescence activated cell sorting (FACS) by Cathy Alford at the Veterans Affairs Hospital in Nashville, TN. The pBABE-Ras(V12)-puromycin retroviral construct was a kind gift from Dr. Scott Lowe, Cold Spring Harbor. Cells were infected once with this retroviral vector and selected with 1ug/mL puromycin (Sigma Chemical Co., St. Louis, MO, USA). The pBABE-hTERT-hygromycin and pBABE-SV40T/t-neomycin (also known as pBABE-large T genomic-neo) were purchased from Addgene. Cells were infected once and selected with 300ug/mL hygromycin B and 50ug/mL geneticin, respectively.

Preparation of immunoprecipitations, cell lysates and immunoblot analysis

Immunoprecipitations were performed as described previously (Eaton and Sealy, 2003). Briefly, cells were harvested in cold 1x PBS containing 0.1mM sodium vanadate, spun at 840 x g for 10 minutes, and resuspended on ice in cold radioimmunoprecipiation buffer (RIPA - 20mM Tris pH 7.5, 50mM sodium chloride, 0.5% Triton X-100, 0.5% sodium desoxycholate, 0.1% SDS, 1mM EDTA) containing protease and phosphotase inhibitors (10uM sodium vanadate,10mM sodium molybdate, 10mM beta-glycerophosphate, 1ug/mL aprotinin, 1ug/mL leupeptin, 1ug/mL pepstatin, and 1mM phenylmethylsulfonyl fluoride) as well as 50uM MG132 and 5mM N-ethylmaleimide (NEM). Cell extracts were sonicated for 15 seconds, clarified, and added to T7 monoclonal antibody beads (Covance, Berkley CA USA). The beads had been washed with RIPA buffer containing the inhibitors. After rocking the cell extracts with the beads for 15 minutes at 4 degrees Celsius, the beads were collected at 840 x g and washed three times in RIPA plus inhibitors. 50uL 2x Laemmli SDS sample buffer (95:5 sample buffer:beta-mercaptoethanol) was added and the samples were boiled for 5 minutes. Samples were stored at -70 degrees Celsius until analyzed via SDS-PAGE. Cell lysates were prepared from 100 mm dishes of 90% confluent MCF10A or MCF10A-Ras cells as described previously in Chapter 2. Relative protein concentrations were determined using the Protein Assay Reagent (BioRad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Equal amounts of protein were loaded onto 8% or 10% SDS-PAGE and separated by electrophoresis. The proteins were transferred to an Immobilon P filter and the blots were processed as described previously (Eaton *et al.*, 2001). After the nonspecific binding sites were blocked, the blots were inclubated with primary antibody in TBS-T (100mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 0.5% nonfat dried milk (NFDM) for 1 hour at room temperature (phospho-

specific antibodies were blocked and incubated in 2% bovine serum albumin instead of NFDM). The blots were washed with three successeive changes of TBS-T containing 0.5% NFDM at room temperature for 20 minutes and incubated with a HRP-conjugated goat anti-rabbit or a HRP-conjugated goat anti-mouse antibody as described above for an additional hour. The blots were then washed with three successive changes of TBS-T solution for 15 minutes and the signal was detected by chemiluminescence using SuperSignal West Pico reagent (Pierce, Rockford, IL, USA) and autoradiography with Kodak X-OMAT film (Rochester, NY, USA).

Soft agar colony formation assay

Complete media and 2% agar (Difco, Sparks, MD, USA) were mixed 2:1 to give a 0.7% agar solution. This mixture was pipetted into 60mm dishes to form the bottom layer. 5mL of media containing $1x10^5$ cells was mixed with 2.5mL 1% agar and added as the top layer. Cells were fed with media and 1% agar at a 2:1 ratio.

***In vitro* phosphorylation**

2.5ug of purified rat C/EBPbeta1 (Lap1) was incubated with 100uM ATP and 10mM magnesium chloride in the presence or absence of 2ug of purified, active cdk2/cyclin A2 (Signalchem) at 30 degrees Celsius for 1 hour. SDS sample buffer was then added to the samples and they were boiled for 5 minutes. Samples were stored at -70 degrees Celsius until they were subjected to SDS-PAGE.

Results

Activated Ras(V12) negatively regulates C/EBPbeta1 by leading to the degradation of C/EBPbeta1 in the immortalized MCF10A mammary epithelial cell line

Introduction of activated Ras(V12) into many immortalized cell lines leads to transformation of the cells. We introduced activated Ras(V12) into the MCF10A immortalized yet nontransformed mammary epithelial cell line and examined whether activated Ras expression transformed these cells via a soft agar colong formation assay. Formation of colonies in soft agar indicates transformation *in vitro*. The highly invasive MDA231 breast cancer cell line was used as a positive control while uninfected MCF10A cells were used as a negative control. The graph in Figure 17 shows that the MCF10A-Ras cells form significantly more colonies in soft agar than the MCF10A cells ($p = 0.014$). The inset micrographs above the bar graph demonstrate colonies formed by the MDA231 and MCF10A-Ras cells grown in soft agar whereas the MCF10A cells did not form colonies.

Next we wanted to confirm that we were expressing activated Ras in the MCF10A-Ras cells. To do this, we examined phospho-Erk (pErk) expression in the MCF10A-Ras, MCF10A (negative control), and MDA231 (positive control) cell lines. Erk is a downstream effector of activated Ras, so an increase in activated (or phospho) Erk would indicate expression of activated Ras. Figure 18 demonstrates that the MDA231 and MCF10A-Ras cells express high levels of pErk, whereas the MCF10A cells contain much lower levels of activated pErk. Total Erk is shown as a control demonstrating that the increase in pErk is not due to an increase in total Erk. GAPDH was used as the loading control.

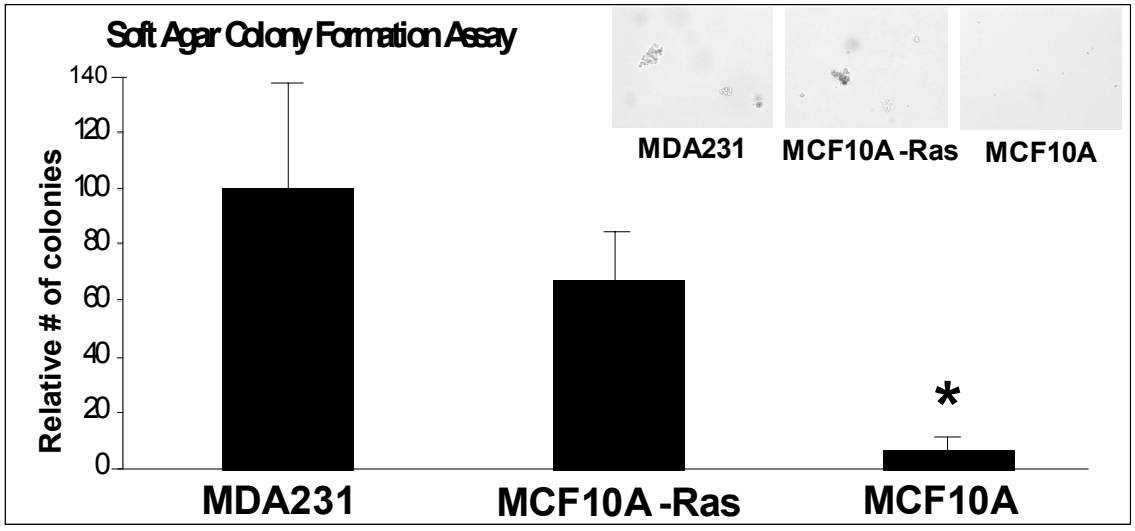

Figure 17. MCF10A-Ras and MDA231s are transformed as demonstrated by soft agar colony formation assay. MCF10A-Ras and MDA231s have significantly more colonies compared to MCF10As (p=0.014 and 0.046, respectively).

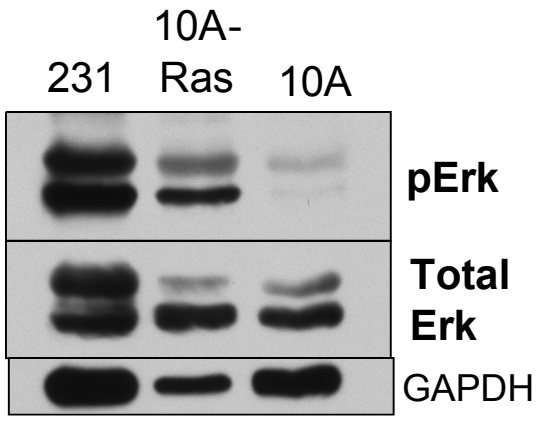

Figure 18. MCF10A-Ras and MDA231s demonstrate activation of the Ras pathway. MCF10A-Ras and MDA231s have increased phospho-Erk, a downstream effector of Ras.

Expression of all three isoforms of C/EBPbeta can be observed via immunoblot of cell lysates from MCF10A cells. (Figure 19, lane 1; note - the expression levels of the isoforms of C/EBPbeta in MCF10A cells can be somewhat variable depending on the passage.) Examination of the isoforms of C/EBPbeta expressed in the MCF10A-Ras cells indicates that there is a striking loss of C/EBPbeta1 expression in these Ras(V12) transformed cells (Figure 19, lane 2). This loss of C/EBPbeta1 is accompanied by an increase in C/EBPbeta2 and C/EBPbeta3 expression.

After observing that introduction of activated Ras into MCF10A cells leads to the loss of endogenous C/EBPbeta1 expression, we infected MCF10A and MCF10A-Ras cells with LZRS-T7-C/EBPbeta1-IRES-eGFP. Infected cells were then sorted by fluoresecence activated cell sorting (FACS) using green fluorescent protein (GFP) as a marker. Immediately after sorting, p52 T7-C/EBPbeta1 was expressed in both the MCF10A and MCF10A-Ras cells (Figure 21). However, T7-C/EBPbeta1 expression three weeks post-sorting is shown in Figure 20 and 21. The MCF10A-C/EBPbeta1 cells still expressed p52 T7-C/EBPbeta1 (Figure 20, Lanes 1 and 3), but the MCF10A-Ras-C/EBPbeta1 cells largely lost p52 T7-C/EBPbeta1 expression (Figure 20, Lanes 2 and 4). A 31kDa band was observed in the MCF10A-Ras-C/EBPbeta1 cells in the anti-T7 tag immunoblot, indicative of proteolysis (Figure 20, Lane 2). This was confirmed by the presence of 31kDa and 23kDa bands in the anti-C-terminal C/EBPbeta immunoblot (Figure 20, Lane 3). These smaller molecular weight bands were not present in the MCF10A-C/EBPbeta1 cells (Figure 20, Lanes 1 and 3).

The loss of p52 T7-C/EBPbeta1 and the appearance of bands at 31kDa and 23kDa in the MCF10A-Ras cells indicate that C/EBPbeta1 is being degraded in these cells. We wanted to determine whether C/EBPbeta1 is being proteolyzed by the proteasome in the MCF10A-Ras cells. We treated the MCF10A-Ras-C/EBPbeta1 cells

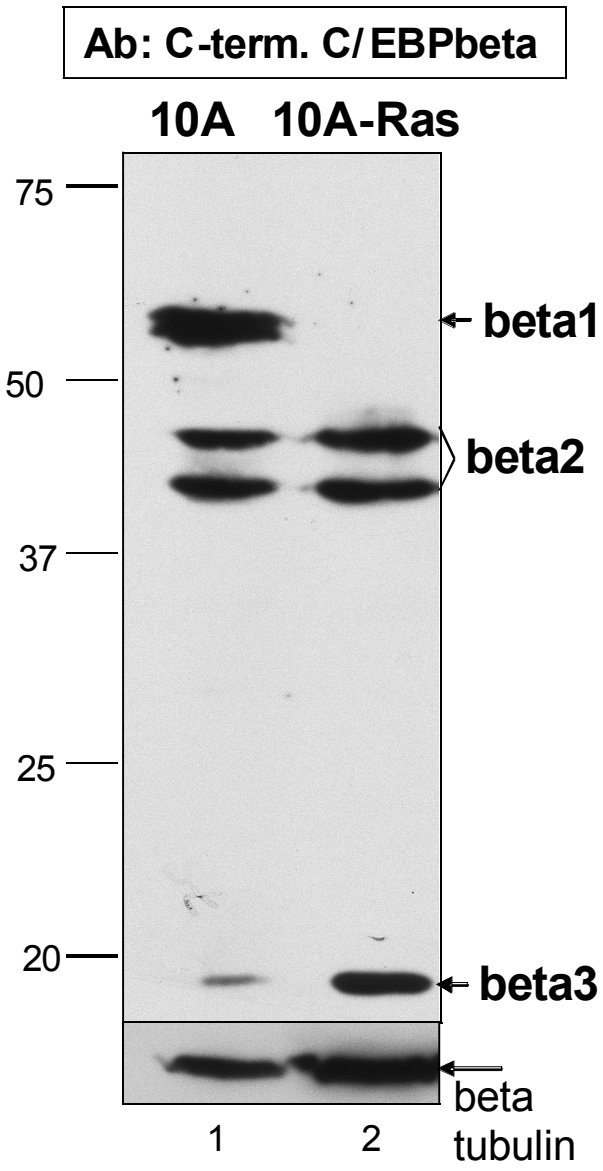

Figure 19. Activated Ras(V12) negatively regulates C/EBPbeta1 expression in the immortalized MCF10A mammary epithelial cell line a. Immunoblot analysis of endogenous C/EBPbeta expression in MCF10A cells (lane 1) and MCF10A that express activated Ras(V12) (MCF10A-Ras) cells (lane 2). Equal amounts of total protein were loaded in each lane of a 12% SDS-PAGE. The different isoforms of C/EBPbeta are indicated with arrows. Bars indicate the mobility's of standard molecular weight markers, in kilo- Daltons (kDa), in all figures. Immunoblotting was performed with a C-terminal C/EBPbeta antibody (Santa Cruz C-19). (10A = MCF10A) The molecular weight markers used in this figure are the same as those used in previous papers which identify C/EBPbeta1 as a 55kDa protein. The remaining figures use a different molecular weight marker that shows C/EBPbeta1 having an apparent molecular weight of 52kDa. Beta tubulin (Sigma) is used as a loading control.

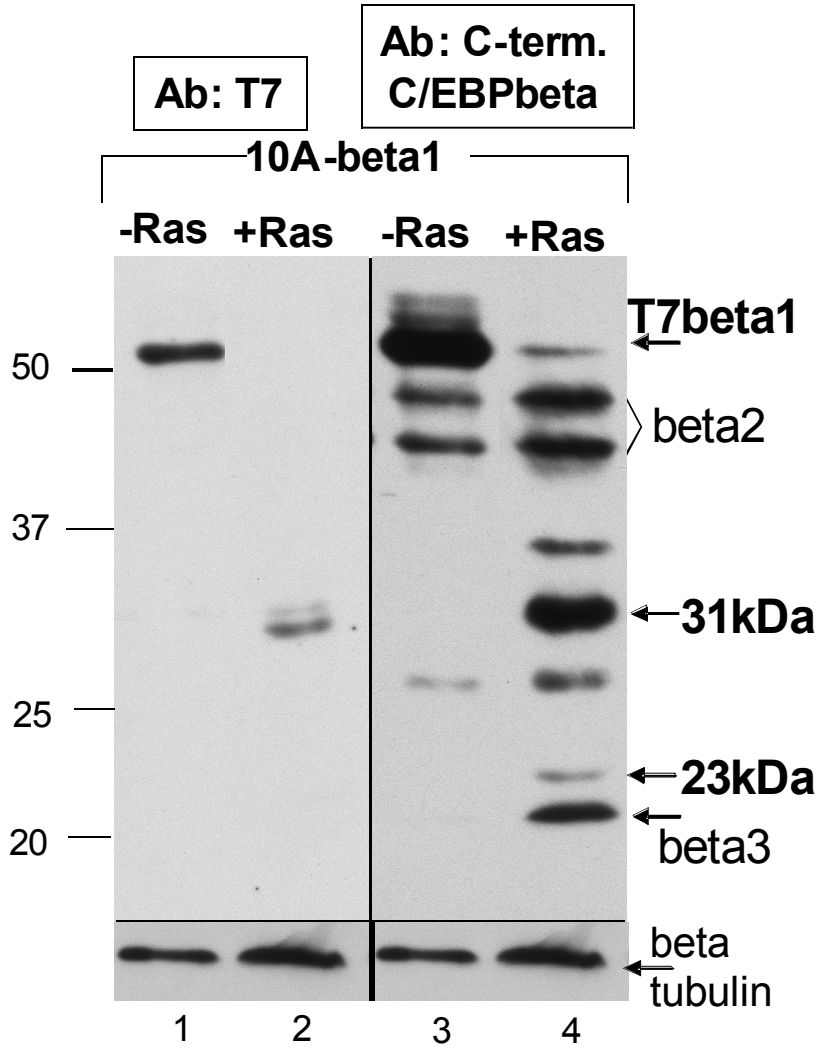

Figure 20. Activated Ras(V12) negatively regulates T7-C/EBPbeta1 expression in MCF10A cells. MCF10A and MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS for GFP positive cells. C/EBPbeta1 is the only transactivator C/EBPbeta isoform produced by this retrovirus because the second ATG necessary to translate C/EBPbeta2 has been mutated. In addition, a perfect Kozak sequence was made around the first ATG. This immunoblot is three weeks after sorting. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. Immunoblot analysis of lanes 1 and 2 were performed with a T7 tag mouse monoclonal antibody (Novagen) at 1:10000 whereas lanes 3 and 4 are with an anti-C/EBPbeta C-terminal antibody (Santa Cruz C-19) at 1:5000. Arrows indicate full length p52-T7-C/EBPbeta1 along with smaller degradation products of T7-C/EBPbeta1. Immunoblot analysis with the beta-tubulin antibody is shown as a loading control (Sigma T7816 at 1:10000) (beta = C/EBPbeta, 10A = MCF10A)

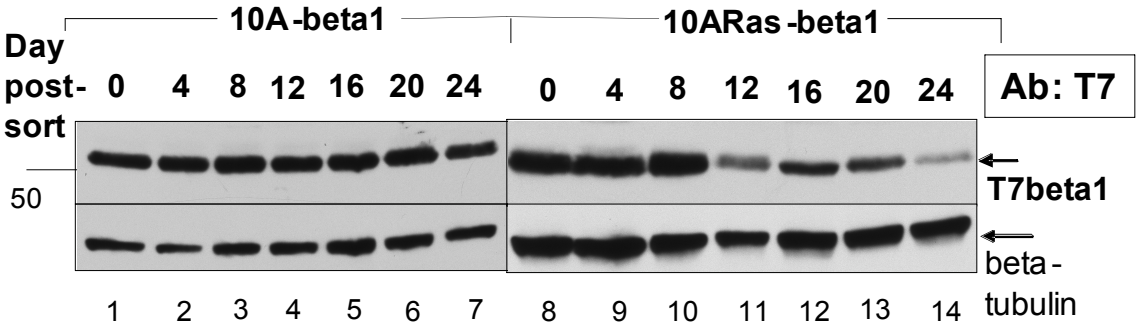

Figure 21. Timecourse of activated Ras(V12) negatively regulating C/EBPbeta1 expression in MCF10As. MCF10A (lanes 1-7) and MCF10A-Ras (lanes 8-14) cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS for GFP positive cells. Cell lysates were prepared every four days for 24 days. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. Immunoblot analysis was performed with the T7-tag antibody (Novagen) at a dilution of 1:10000. Arrows indicate full length p52-T7-C/EBPbeta. Immunoblot analysis with the beta-tubulin antibody is shown as a loading control (Sigma T7816 at 1:10000) (beta = C/EBPbeta, 10A = MCF10A)

with MG132, a potent and selective reversible inhibitor of the proteasome. Treatment of the MCF10A-Ras-C/EBPbeta1 cells with MG132 for 4 or 8 hours stabilized expression of p52 T7-CEBPbeta-1 (Figure 22, compare lanes 3 and 4 to lane 2). A similar effect was observed upon treatment of the MCF10A-Ras-C/EBPbeta1 cells with lactacystin (Figure 23), another potent and selective irreversible inhibitor of proteasome.

It was then important to determine if endogenous p52-C/EBPbeta1 could be stabilized in MCF10A-Ras cells by treating the cells with MG132. MCF10A-Ras cells were treated with MG132 for 8 hours and cell lysates were prepared. Figure 24 demonstrates that treatment of MCF10A-Ras cells with MG132 stabilizes endogenous C/EBPbeta1, confirming that endogenous C/EBPbeta1 is being degraded by the proteasome in MCF10A-Ras cells.

We then wanted to determine whether degradation of C/EBPbeta1 is ubiquitin-mediated. MCF10A-Ras-C/EBPbeta1 cells were treated with MG132 and immunoprecipitated with T7-tag antibody beads. Immunoblot analysis with a Ubiquitin antibody demonstrated that MCF10A-Ras-C/EBPbeta1 cells treated with MG132 exhibit increased reactivity with this antibody compared to controls (Figure 25, lane 5 versus lanes 3 and 4). Immunoblot analysis of the treated cells with the C/EBPbeta antibody demonstrates a ladder of higher molecular weight bands that coincide with the molecular weights of bands in the ubiquitin immunoblot (Figure 25, lanes 2 and 5, see arrows), indicating ubiquitination of C/EBPbeta1.

Most breast cancer cell lines exhibit activation of the Ras pathway through activation of upstream receptors, upregulation of Ras itself, or through activation of downstream signaling molecules. Therefore we expressed T7-C/EBPbeta1 in the breast cancer cell lines MDA231 and MDA435 in which the Ras pathway is activated.

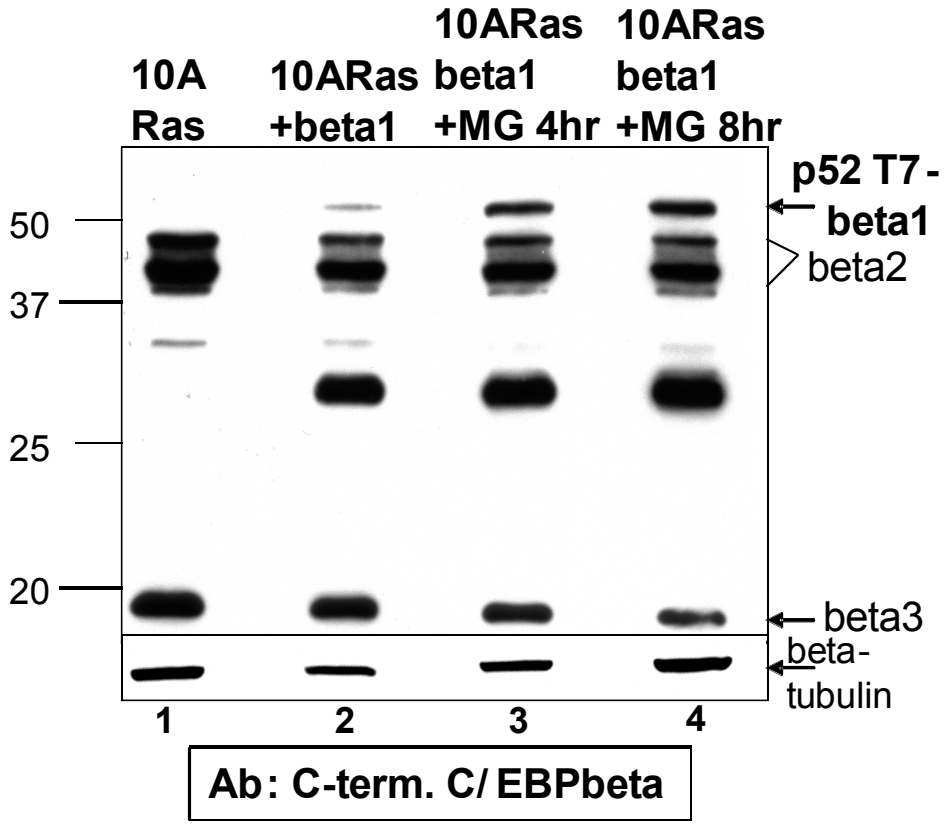

Figure 22. T7-C/EBPbeta1 is protealyzed by the proteasome in MCF10A-Ras cells. MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS for GFP positive cells. This immunoblot is three weeks post-sorting. Lane 1 is uninfected MCF10A-Ras cells, lane 2 is MCF10A-Ras-C/EBPbeta1 cells, lane 3 is MCF10A-Ras-C/EBPbeta1 treated with 50uM MG132 (re-suspended in DMSO; Calbiochem) for 4 hours, and lane 4 is MCF10A-Ras-C/EBPbeta1 cells treated with 50uM MG132 for 8 hours. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. The Santa Cruz C-19 C-terminal C/EBPbeta antibody was used for immunoblot analysis at 1:5000. Beta-tubulin was used as the loading control. (Sigma T7816). Arrows indicate the different C/EBPbeta isoforms. (10A = MCF10A, beta = C/EBPbeta, MG = MG132)

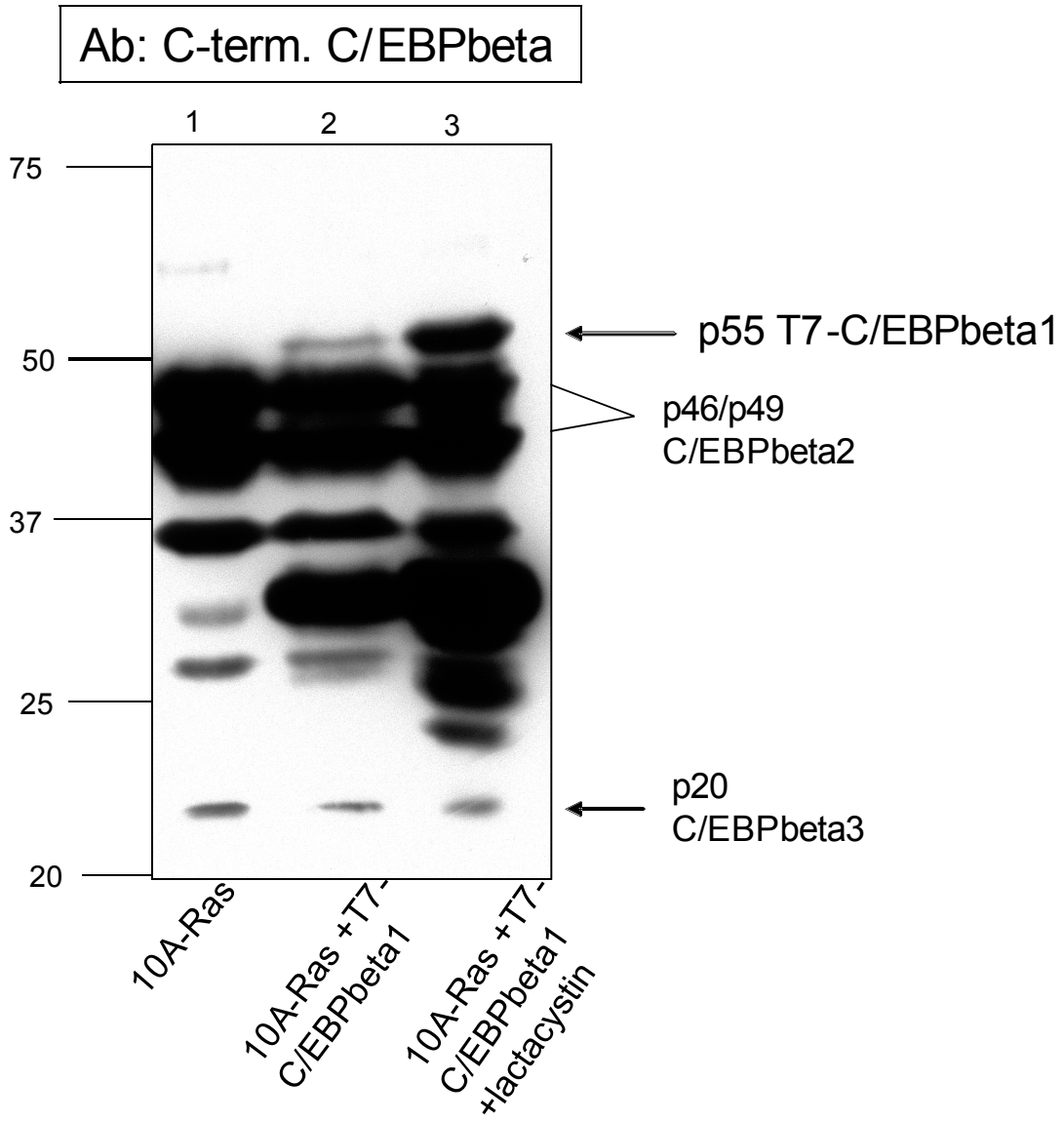

Figure 23. C/EBPbeta1 is protealyzed by the proteasome in cells in which the Ras pathway is activated. MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS for GFP positive cells. This immunoblot is three weeks post-sorting. Lane 1 is uninfected MCF10A-Ras cells, lane 2 is MCF10A-Ras-C/EBPbeta1 cells, and lane 3 is MCF10A-Ras-C/EBPbeta1 treated with 50uM lactacystin for 8 hours. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. The Santa Cruz C-19 C-terminal C/EBPbeta antibody was used for immunoblot analysis at 1:5000. Arrows indicate the different C/EBPbeta isoforms.

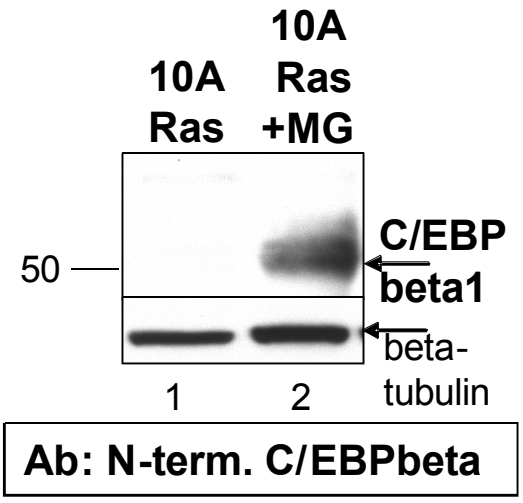

Figure 24. Endogenous C/EBPbeta1 is protealyzed by the proteasome in MCF10A-Ras cells. Cell lysates were prepared from MCF10A-Ras cells untreated (lane 1) or treated with 50uM MG132 for 8 hours (lane 2). Equal amounts of total protein were loaded into each lane of a 10% SDS-PAGE. An antibody specific for the N-terminal 21 amino acids specific to C/EBPbeta1 (Abcam 18F8) was used for immunoblot analysis (1:2000). Beta-tubulin was used as the loading control for all of the above immunoblots (Sigma T7816).

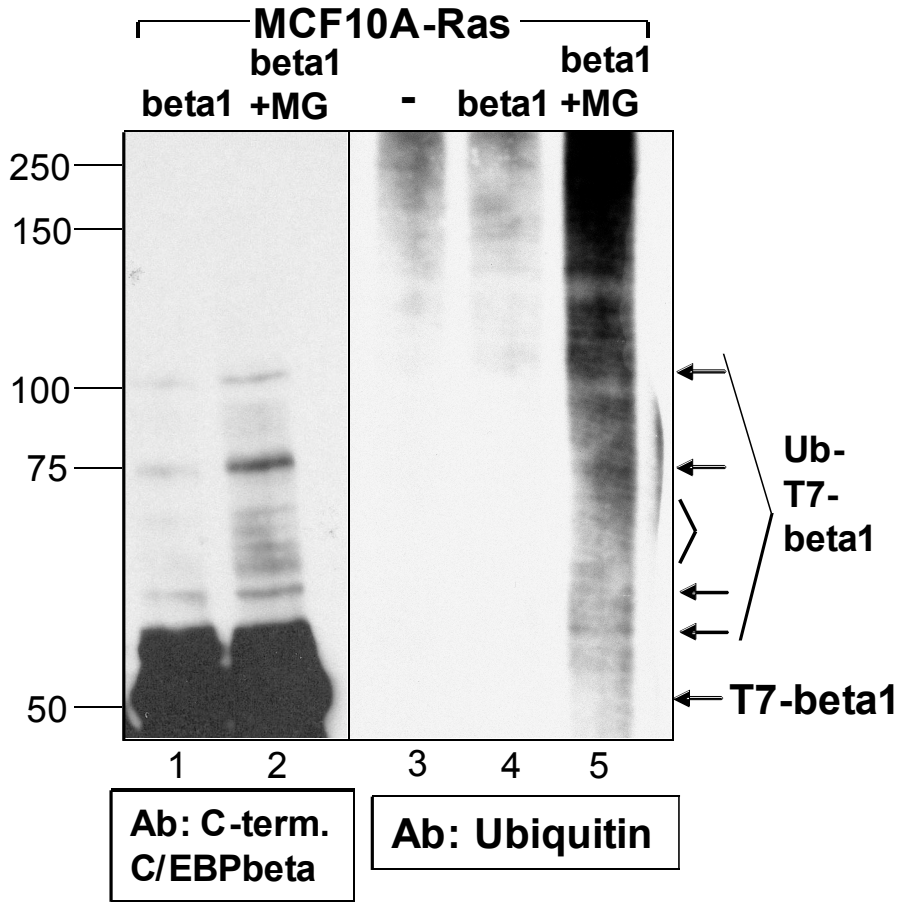

Figure 25. Proteolysis of C/EBPbeta1 is likely ubiquitin-mediated. MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACS for GFP positive cells. Lane 3 is uninfected MCF10A-Ras cells. Cells were untreated (lanes 1, 3 and 4) or treated with 50uM MG132 for 8 hours and 5mM N-ethylmaleimide for 30 minutes (lanes 2 and 5). Lanes 1 and 2 are cell lysates whereas lanes 3-5 are immunoprecipitations with T7-tag antibody beads (Novagen). Immunoprecipitations were performed as described previously (Eaton and Sealy, 2003) with the following exceptions: the immunoprecipitations were for 15 minutes and 50uM MG132 and 5mM N-ethylmaleimide were included in the immunoprecipitation buffer. Immunoblot analysis was performed with the Santa Cruz C-19 C-terminal C/EBPbeta antibody (lanes 1 and 2) or anti-Ubiquitin antibody (Enzo Life Sciences FK2). (10A = MCF10A, beta = C/EBPbeta, MG = MG132)

Expression of T7-C/EBPbeta1 in the highly invasive MDA231s and MDA435s decreases over time, similar to the MCF10A-Ras-C/EBPbeta1 cells, and p52 T7-C/EBPbeta1 was stabilized when MDA231-C/EBPbeta1 and MDA435-C/EBPbeta1 cells were exposed to the proteasomal inhibitor MG132 for 8 hours (Figure 26, compare lanes 3 and 4, and Figure 27, compare lanes 4 and 5 with lanes 2 and 3).

Treatment with the cdk inhibitor roscovitine or mutation of C/EBPbeta1 Thr235 to alanine stablizes expression of T7-C/EBPbeta1 in MCF10A-Ras cells

Ras signaling activates a number of phosphorylation cascades that leads to the phosphorylation of C/EBPbeta. Therefore, expression of activated Ras in the MCF10A cells may be leading to the phosphorylation of C/EBPbeta1, which is subsequently causing C/EBPbeta1 to be degraded by the proteasome. To further investigate the mechanism by which Ras negatively regulates C/EBPbeta1, MCF10A-Ras-C/EBPbeta1 cells were treated with a panel of kinase inhibitors that inhibit kinases known to phosphorylate C/EBPbeta. The panel of kinase inhibitors included a mitogen activated protein kinase/Erk kinase (MEK) inhibitor (MEK is the upstream activator of Erk), cdk inhibitor, jun N-terminal kinase (JNK) inhibitor, p38 inhibitor, and glycogen synthase kinase 3 beta (GSK3beta) inhibitor. Cell lysates were prepared from the treated cells and expression of C/EBPbeta1 was analyzed via immunoblot. Treatment with the cdk inhibitor roscovitine stabilized expression of p52 T7-C/EBPbeta1 (Figure 28, compare lanes 2 and 4). C/EBPbeta is phosphorylated on Thr235 by cdk 2 (Shuman *et al.*, 2004, Li *et al.*, 2007). This phosphorylation can be inhibited by treatment with roscovitine (Shuman *et al.*, 2004, Li *et al.*, 2007). Treatment with the cdk inhibitor roscovitine was the only treatment that stabilized expression of p52 T7-C/EBPbeta1 in the MCF10A-Ras

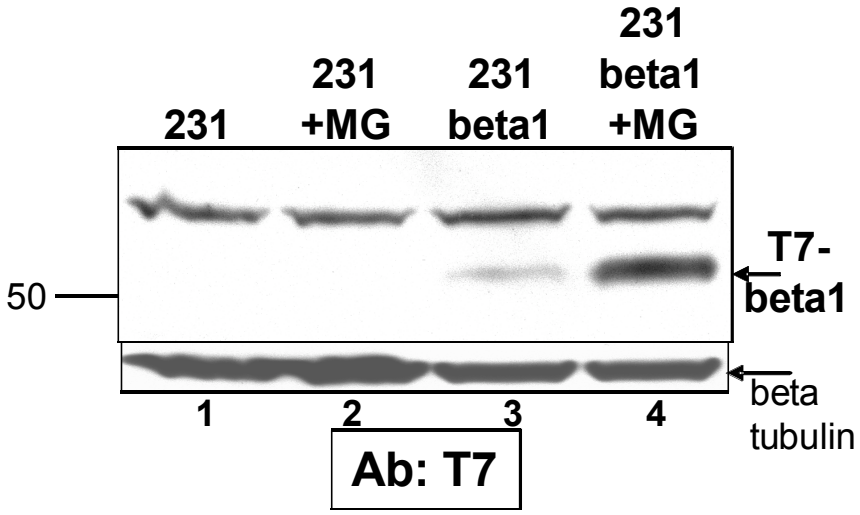

Figure 26. T7-C/EBPbeta1 is protealyzed by the proteasome in breast cancer cells in which the Ras pathway is activated. MDA231 breast cancer cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACs for GFP positive cells. Lane 1 is uninfected MDA231 cells, lane 2 is uninfected MDA231 cells treated with 50uM MG132, lane 3 is MDA231-C/EBPbeta1, and lane 4 is MDA231-C/EBPbeta1 cells treated with 50uM MG132 for 8 hours. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. Immunoblot analysis was performed with a T7 tag mouse monoclonal antibody (Novagen) at 1:10000. The arrow indicates p52-T7-C/EBPbeta1. Beta-tubulin was used as the loading control for all of the above immunoblots (Sigma T7816).

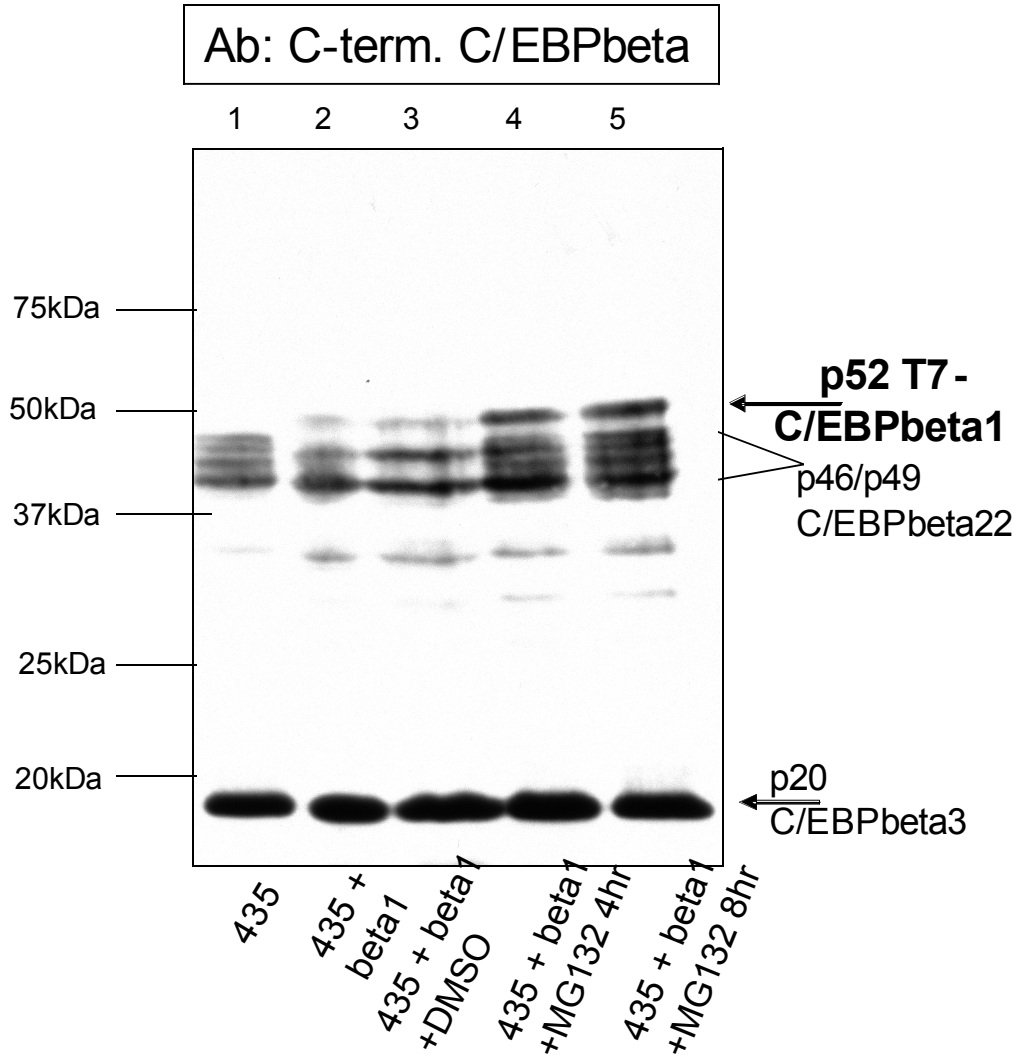

Figure 27. T7-C/EBPbeta1 is protealyzed by the proteasome in MDA435 breast cancer cells in which the Ras pathway is activated. MDA435 breast cancer cells were infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by FACs for GFP positive cells. Lane 1 is uninfected MDA435 cells, lane 2 is MDA435-C/EBPbeta1 cells, lane 3 is MDA435-C/EBPbeta1 cells treated with vehicle control, lane 4 is MDA435-C/EBPbeta1 cells treated with 50uM MG132 for 4 hours, and lane 5 is MDA435-C/EBPbeta1 cells treated with 50uM MG132 for 8 hours. Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE. Immunoblot analysis was performed with the C-terminal C/EBPbeta antibody (Santa Cruz) at 1:5,000. The arrows indicate the isoforms of C/EBPbeta.

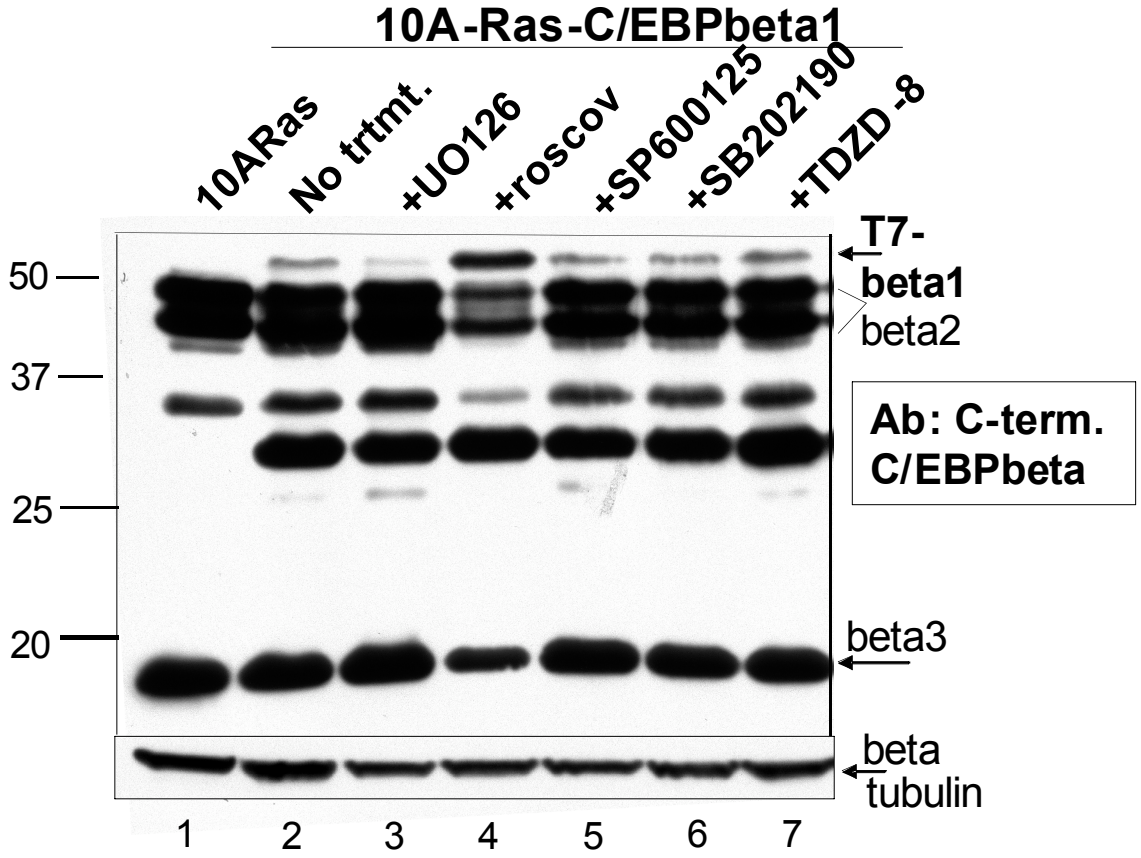

Figure 28. Treatment with the cdk inhibitor roscovitine stablizes expression of T7-C/EBPbeta1 in MCF10A-Ras cells a. MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP and sorted by flow cytometry for GFP positive cells. Lane 1 is uninfected MCF10A-Ras cells and lanes 2-7 are MCF10A-Ras-C/EBPbeta1 cells three weeks post-sorting, treated with various kinase inhibitors. Lane 2 is untreated; lane 3 is treated with 25uM of the MEK inhibitor UO126, lane 4 is treated with 50uM of the cdk inhibitor roscovitine, lane 5 is treated with 50uM of the JNK inhibitor SP600125, lane 6 with 20uM p38 inhibitor SB202190, and lane 7 with 25uM GSK3 inhibitor TZDZ-8. All treatments were for 12 hours. Equal amounts of total cell protein were loaded and analyzed by SDS 10% polyacrylamide gel elecrophoresis. Immunoblotting was performed with an anti-C/EBPbeta C-terminal antibody (Santa Cruz C-19). Arrows indicate the particular isoforms of C/EBPbeta.

cells. Treatment of MDA231-C/EBPbeta1 cells with roscovitine also stabilized expression of p52 T7-C/EBPbeta1 (data not shown).

We then wanted to demonstrate that cdk2 could phosphorylate full length C/EBPbeta1, because previous studies examining phosphorylation of C/EBPbeta on Thr235 by cdk2 did not look at the specific isoform of C/EBPbeta being modified. To examine this, an *in vitro* phosphorylation assay was performed. Purified rat C/EBPbeta1 (Lap1 – which is slightly smaller than human C/EBPbeta1) was incubated with purified, active cdk2/cyclin A2 and ATP. Figure 29 is an immunoblot using the anti-phosphoThr235-C/EBPbeta specific antibody demonstrating that cdk2 phosphorylates C/EBPbeta1 on Thr235, *in vitro.* The bottom immunoblot is with the anti-T7 tag antibody showing that approximately equal amounts of protein was loaded in both lanes.

It was then important to demonstrate that cdk2 is activated in MCF10A-Ras and MDA231 cells, since active cdk2 is leading the phosphorylation and subsequent degradation of C/EBPbeta1 in these cell lines. Immunoblot analysis with an anti-phosphoT160 cdk2 antibody in Figure 30 demonstrates that there is an increase in phosphorylation and thus activation of cdk2 in the MCF10A-Ras and MDA231 cells when compared to MCF10A cells.

To confirm that phosphorylation by cdk2 on Thr235 of C/EBPbeta1 was leading to the degradation of C/EBPbeta1 by the proteasome, Thr235 was mutated to an alanine (T235A) so that this residue could no longer be phosphorylated. An LZRS-T7-C/EBPbeta1T235A-IRES-eGFP retroviral vector was then constructed and the resulting retrovirus was then used to infect MCF10A-Ras cells. These cells were then sorted by FACS using GFP as a marker for infected cells. After three weeks in culture, cell lysates were prepared and immunoblot analysis was performed to analyze the stability of p52

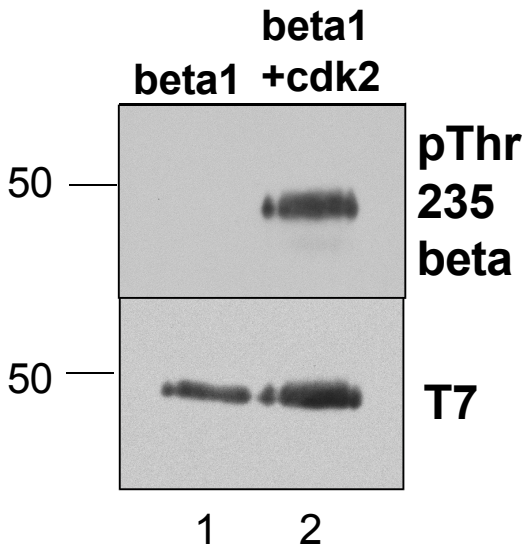

Figure 29. Purified C/EBPbeta1 is phosphorylated on Thr235 by cdk2, *in vitro*. Purified rat C/EBPbeta1 (Lap1, which is smaller than human C/EBPbeta1) was *in vitro* phosphorylated with purified, active cdk2/cyclin A2 (Signalchem). Equal amounts of C/EBPbeta1 alone (lane 1) or *in vitro* phosphorylated C/EBPbeta1 (lane 2) were run on a 10% SDS-PAGE. Immunoblotting was performed with an anti-phosphoThr235 C/EBPbeta antibody (Cell Signaling) at 1:2000 or anti-T7 antibody (Novagen) at 1:10000 dilution. (10A = MCF10A, beta = C/EBPbeta)

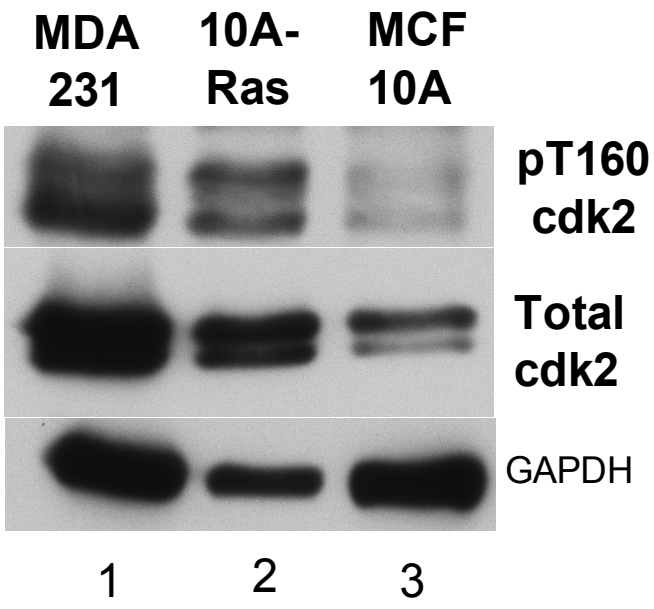

Figure 30. cdk2 is activated in the MCF10A-Ras and MDA231 cells when compared to MCF10As. Cell lysates from MDA231 breast cancer cells are in lane 1, MCF10A-Ras in lane 2, and MCF10A in lane 3. The top immunoblot is with the phosphoT160 cdk2 antibody, the middle panel is with an anti-cdk2 antibody and the bottom panel is the GAPDH loading control.

T7-C/EBPbeta1 T235A. Mutation of Thr235 to alanine in C/EBPbeta1, thus preventing phosphorylation of this residue by cdk2, stabilized the expression of p52 T7-C/EBPbeta1 (Figure 31, compare lanes 3 and 6) as compared to T7-C/EBPbeta1 that did not contain this point mutation (Figure 31, compare lanes 2 and 5).

Since C/EBPbeta1 plays a role in OIS, a tumor suppressive mechanism (Chapter 2), it is important to determine whether T7-C/EBPbeta1 and/or T7-C/EBPbeta1T235A have an effect on the transformed phenotype of the MCF10A-Ras cells. To examine this MCF10A-Ras-C/EBPbeta1 and MCF10A-Ras-C/EBPbeta1T235A cells were subjected to a soft agar colony formation assay. Formation of colonies in soft agar indicates transformation of cells *in vitro.* 100,000 cells were allowed to grow in a soft agar/complete growth media mixture. After 20 days, 15 random fields of each plate was imaged under a light microscope at 10x magnification. The number of colonies per field was counted and the average for each cell type was calculated. Figure 32 demonstrates that T7-C/EBPbeta1 and T7-C/EBPbeta1T235A expression did not have an effect on the transformed phenotype of MCF10A-Ras cells, as these cells' ability to form colonies in soft agar was unaltered. The MDA231 breast cancer cell line was used as a positive control. T7-C/EBPbeta1 and T7-C/EBPbeta1T235A are unable to induce senescence in MCF10A cells in part because these cells have the p14ARF/p15INK4B/p16INK4A locus deleted (Iavarone and Massague, 1997). p16INK4A is an important player in OIS, and C/EBPbeta-induced senescence signals through this tumor suppressor during OIS (Sebastian *et al.*, 2005). Additionally, C/EBPbeta induces p15INK4B during OIS (Kuilman *et al.*, 2008).

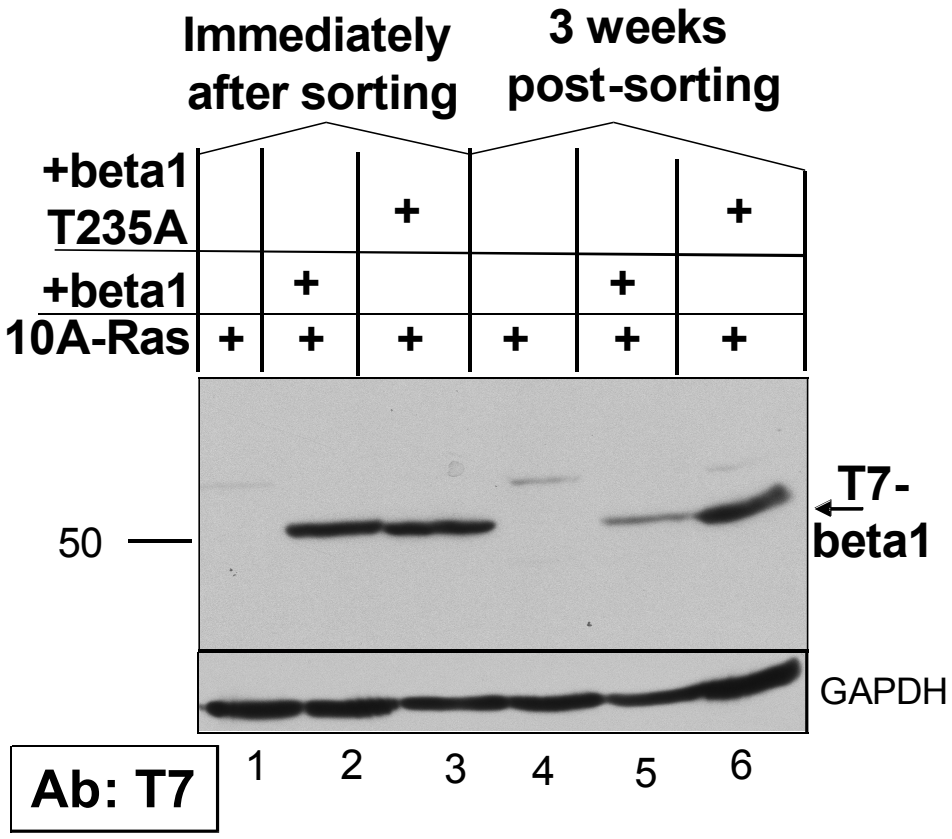

Figure 31. Mutation of C/EBPbeta1Thr235 to alanine stabilizes expression of C/EBPbeta1 in MCF10A-Ras cells. MCF10A-Ras cells infected with LZRS-T7-C/EBPbeta1-IRES-eGFP or LZRS-T7-C/EBPbeta1T235A-IRES-eGFP. Lanes 1 and 4 are uninfected MCF10A-Ras cells, lanes 2 and 5 are MCF10A-Ras-C/EBPbeta1 cells immediately after sorting and three weeks post-sorting respectively, and lanes 3 and 6 are MCF10A-Ras-C/EBPbeta1 T235A cells immediately after sorting and three weeks post-sorting, respectively. Equal amounts of total cell protein were loaded and analyzed via 10% SDS-PAGE. Immunoblotting was performed with an anti-T7 tag mouse monoclonal antibody (Novagen). The arrow indicates p52 T7-C/EBPbeta1. (10A = MCF10A)

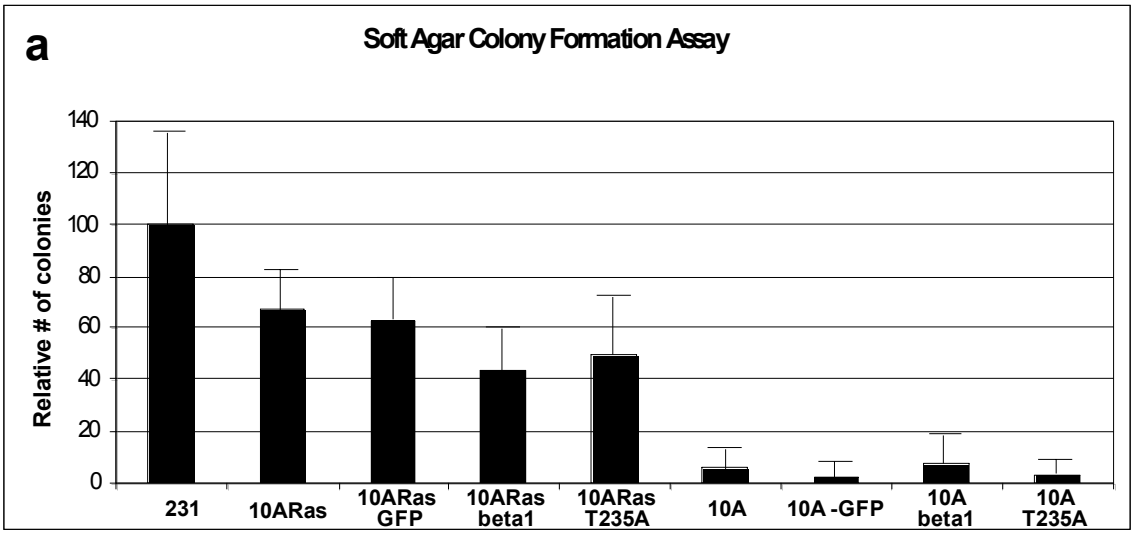

Figure 32. T7-C/EBPbeta1 and T7-C/EBPbeta1T235A expression does not have an effect on colony formation of MCF10A and MCF10A-Ras cells in soft agar. a. Quantification of soft agar colony formation assay. There is no significant difference in relative colony number when T7-C/EBPbeta1 or T7-C/EBPbeta1T235A is expressed in MCF10As or MCF10A-Ras.

Examination of endogenous C/EBPbeta1 expression and cdk2 activity in WI-38 cells, senescing WI-38-Ras cells, and transformed WI-38 cells

After observing that phosphorylation of C/EBPbeta1 on Thr235 by cdk2 leads to degradation of C/EBPbeta1 in transformed mammary epithelial cells, and knowing that C/EBPbeta1 is not degraded in senescing WI-38-Ras cells (Chapter 2), we wanted to verify that cdk2 is not activated in the WI-38-Ras cells. To do this, cell lysates from WI-38-Ras cells were prepared and an immunoblot with an anti-phosphoT160 cdk2 antibody was performed. The results in Figure 33 demonstrate that cdk2 is not activated in the WI-38-Ras cells, which is likely why C/EBPbeta1 is not degraded in these cells.

It is well documented that WI-38 normal fibroblasts can be transformed by the introduction of a small number of oncogenes. Introduction of hTERT, SV40 large and small T (T/t), and activated Ras into normal fibroblasts is sufficient for their transformation (Hahn *et al.*, 1999). Therefore, we decided to examine p52-C/EBPbeta1 expression in transformed WI-38 cells expressing these three genes to determine if transformation of fibroblasts by Ras would lead to loss of p52-C/EBPbeta1 expression, similar to what we observe in mammary epithelial cells transformed by Ras. To produce the WI-38-hTERT-SV40T/t-Ras cells, serial infections were performed followed by the appropriate selection. The WI-38 cells were first infected with pBABE-hTERT-hygromycin and grown in selection media containing hygromycin. After the cells finished selecting and a homogeneous population of WI-38-hTERT cells was obtained, we infected these cells with pBABE-SV40T/t-neomycin and cultured the cells in media containing genetacin. Finally the WI-38-hTERT-SV40T/t cells were infected with pBABE-Ras(V12)-puromycin and grown in selection media containing puromycin. The resulting cells were transformed WI-38-hTERT-SV40T/t-Ras cells. Immunoblot analysis of these cell lines was perfomed with our C/EBPbeta1-specific antibody. Interestingly,

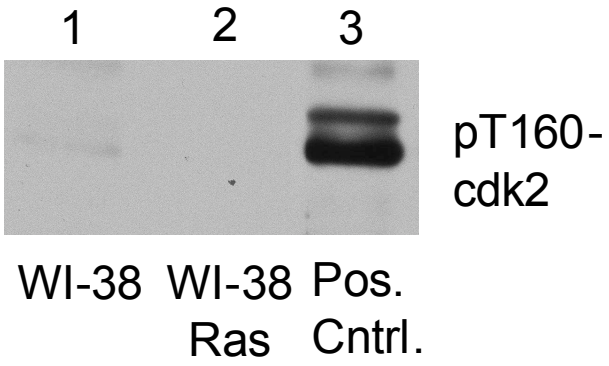

Figure 33. cdk2 is not activated in senescing WI-38-Ras cells. Cell lysates of uninfected WI-38 cells (lane 1) or WI-38 cells infected with pBABE-Ras(V12)-puromycin (lane 2) and selected with puromycin for 6 days were run on a 10% SDS-PAGE. Immunoblot analysys is with an anti-phosphoT160 cdk2 antibody (Cell Signaling). Lane 3 is positive control WI-38-hTERT-SV40T/t-Ras cells.

we do see loss of p52-C/EBPbeta1 in the transformed WI-38-hTERT-SV40T/t-Ras cells (Figure 34). p52-C/EBPbeta1 is expressed in the WI-38-hTERT and the immortalized WI-38-hTERT-SV40T/t cells. This confirms our observations with the transformed mammary epithelial cells (MCF10A-Ras) that transformation by Ras leads to the loss of p52-C/EBPbeta1.

Finally, we wanted to confirm that cdk2 is activated in the transformed WI-38-hTERT-SV40T/t-Ras cells which lack p52-C/EBPbeta1. We performed an immunoblot with the anti-phosphoT160 cdk2 antibody. As shown in Figure 35, cdk2 is activated in the transformed WI-38-hTERT-SV40T/t-Ras cells. However, cdk2 is also activated in the immortalized but non-transformed WI-38-hTERT-SV40T/t cells in which p52-C/EBPbeta is not degraded. This indicates that phosphorylation of C/EBPbeta1 on Thr235 by cdk2 is necessary but not sufficient for loss of p52-C/EBPbeta1.

Discussion

Introduction of activated Ras into the immortalized mammary epithelial cell line MCF10A transforms these cells. We demonstrate here that introduction of activated Ras(V12) into MCF10As and subsequent transformation is accompanied by loss of expression of C/EBPbeta1. We go on to show that when exogenous T7-C/EBPbeta1 is expressed in MCF10A and MCF10A-Ras cells, the MCF10A-C/EBPbeta1 cells maintain expression of p52 T7-C/EBPbeta1, whereas the MCF10A-Ras cells forced to express T7-C/EBPbeta1 quickly degrade p52 T7-C/EBPbeta1. Stability of C/EBPbeta1 expression may be regulated by ubiquitin-dependent degradation. It has been previously demonstrated that p52-C/EBPbeta1 is expressed in normal mammary epithelial tissue and normal mammary epithelial cells grown in culture, but is not expressed in breast cancer cells (Eaton *et al.*, 2001). It is known that the Ras pathway

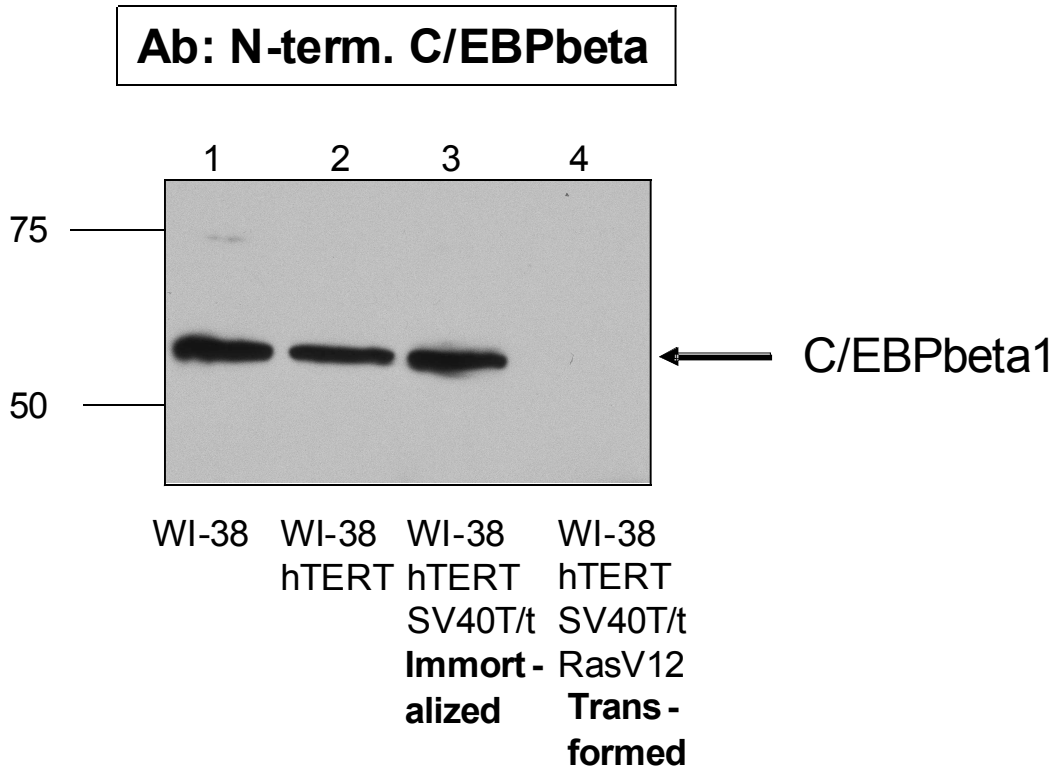

Figure 34. p52-C/EBPbeta1 is not expressed in transformed WI-38-hTERT-SV40T/t-Ras(V12) cells. WI-38 cells were uninfected (lane 1), infected with pBABE-hTERT-hygromycin (lane 2), pBABE-hTERT-hygromycin and pBABE-SV40T/t-neomycin (lane 3), or pBABE-hTERT-hygromycin, pBABE-SV40T/t-neo and pBABE-Ras(V12)-puromycin (lane 4) and selected with appropriate selection reagent. Cell lysates were prepared from confluent plates and run on a 10% SDS-PAGE. Immunoblot analysis was performed with an antibody specific for C/EBPbeta1.

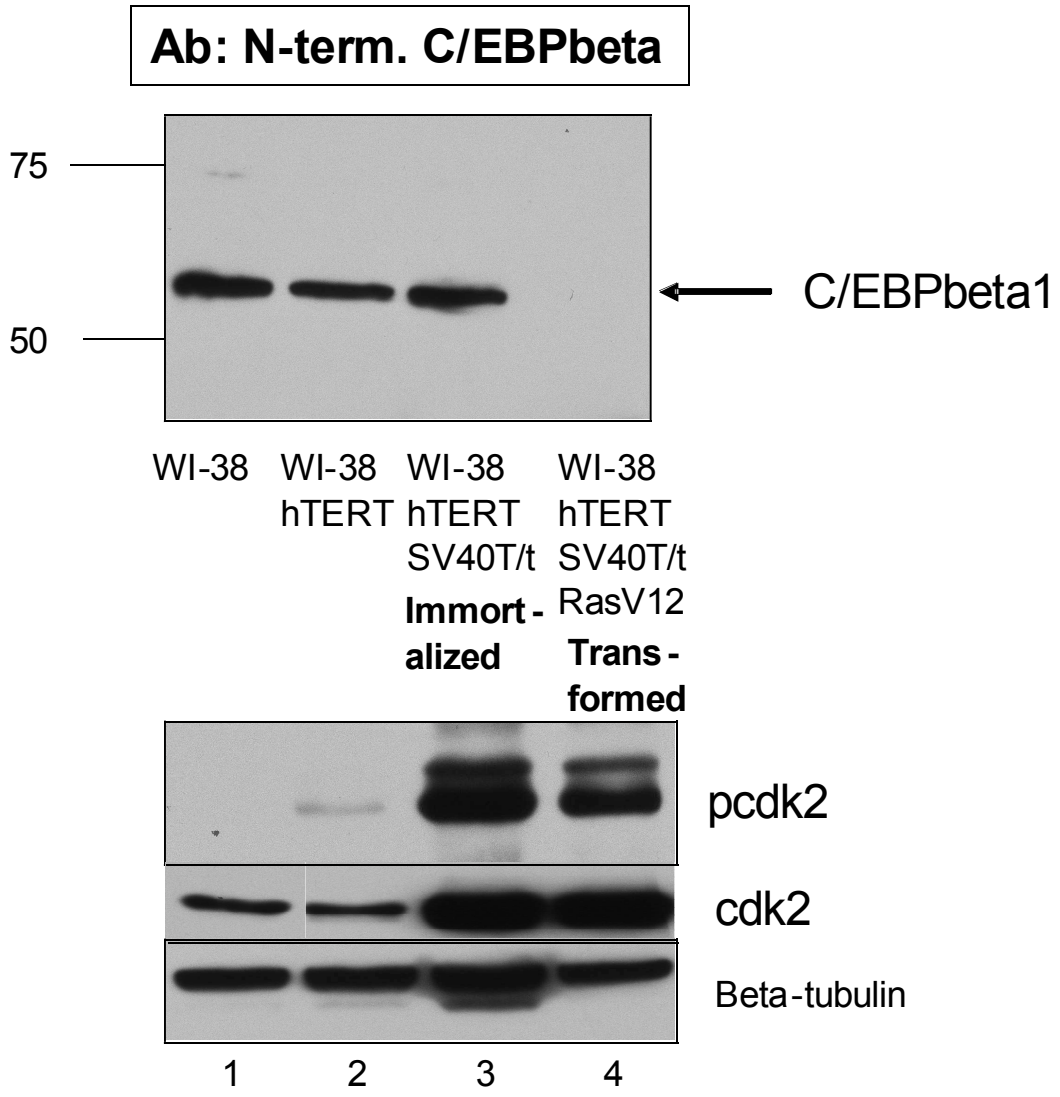

Figure 35. Transformed WI-38-hTERT-SV40T/t-Ras cells that do not express p52-C/EBPbeta1 have activated cdk2. WI-38 cells were uninfected (lane 1), infected with pBABE-hTERT-hygromycin (lane 2), pBABE-hTERT-hygromycin and pBABE-SV40T/t-neomycin (lane 3), or pBABE-hTERT-hygromycin, pBABE-SV40T/t-neo and pBABE-Ras(V12)-puromycin (lane 4) and selected with appropriate selection reagent. Cell lysates were prepared from confluent plates and run on a 10% SDS-PAGE. In the top immunoblot, immunoblot analysis was performed with an antibody specific for C/EBPbeta1. In the bottom immunoblot, the top panel is with anti-phosphoT160 cdk2 (Cell Signaling), the middle panel with anti-cdk2 (Santa Cruz), and the bottom panel is the anti-beta-tubulin loading control (Sigma).

is activated in most breast cancers, either through mutation/upregulation of upstream receptors such as ErbB2, upregulation of the Ras protein itself, or through mutation/upregulation of downstream signaling molecules such as the mitogen activated protein kinases (MAPKs) (Malaney and Daly, 2001). Expression of T7-C/EBPbeta1 in the breast cancer cell lines MDA231 and MDA435 decreased over time, similar to T7-C/EBPbeta1 expression in MCF10A-Ras cells. In addition, treatment of MDA231- and MDA435-C/EBPbeta1 cells with a proteasomal inhibitor stabilized the expression of p52-T7-C/EBPbeta1. Therefore it is likely that C/EBPbeta1 is negatively regulated in breast cancer through activation of the Ras pathway.

Ras signaling oftentimes regulates downstream effectors via phosphorylation. C/EBPbeta is phosphorylated by a number of kinases in the Ras pathway including Erk-2, cdk2, and p38. Therefore we treated the MCF10A-Ras-C/EBPbeta1 cells with a panel of kinase inhibitors to determine if inhibiting phosphorylation could stabilize expression of p52-C/EBPbeta1. We found that treatment with the cdk inhibitor roscovitine stabilized expression of C/EBPbeta1. Ras activation activates cdk2 in a variety of ways such as downregulation of inhibitors of cdk2 such as $p27^{Kip1}$ and $p21^{WAF1/Cip1}$, stabilization of binding partner cyclin E, and potential phosphorylation and thus activation of cdk2 itself (reviewed in Musgrove, 2006). C/EBPbeta can be phosphorylated on Thr235 by cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007). This phosphorylation is specific to the cdk family member cdk2, and that when cells are treated with roscovitine there is a decrease in phosphorylation of Thr235. Additionally, knockdown of cdk2 leads to a decrease in phosphorylation of C/EBPbeta at Thr235 (Li *et al.*, 2007). The non-phosphorylatable C/EBPbeta1T235A could be stably expressed in MCF10A-Ras cells as compared to wild type C/EBPbeta1. Therefore, activation of the Ras pathway in mammary epithelial cells negatively regulates C/EBPbeta1 by promoting phosphorylation of C/EBPbeta1 on

Thr235 by cdk2 and subsequently leading to the degradation of C/EBPbeta1. Loss of T7-C/EBPbeta1 does take several days. This may be due to an initial compensatory mechanism by the cell leading to increased synthesis of C/EBPbeta1 and/or a requirement for expression of a specific ubiquitin E3 ligase necessary for the degradation of C/EBPbeta1.

C/EBPbeta1 induces senescence in normal fibroblasts such as WI-38 cells. C/EBPbeta is necessary for Ras-induced senescence and the C/EBPbeta1 isoform is not degraded in senescing WI-38 cells (Sebastian *et al.*, 2005, Chapter 2). Therefore, we examined whether cdk2 was activated in the WI-38-Ras cells, since cdk2 phosphorylates C/EBPbeta1 on Thr235 and leads to the degradation of C/EBPbeta1 in MCF10A-Ras cells. We found that cdk2 is not activated in WI-38-Ras cells, which is likely why C/EBPbeta1 is not degraded in these cells. To confirm that transformation of cells other than MCF10As by Ras(V12) leads to loss of C/EBPbeta1, we transformed the WI-38 cells by introducing hTERT, SV40T/t, and Ras(V12). Introduction of hTERT and SV40T/t immortalizes the WI-38 cells, but is not sufficient to transform these cells (Hahn *et al.*, 1999). We demonstrate that the transformed WI-38-hTERT-SV40T/t-Ras cells indeed lose expression of p52-C/EBPbeta1. Examination of activated cdk2 in the engineered cells revealed that the transformed WI-38-hTERT-SV40T/t-Ras cells have phosphorylated (and thus activated) cdk2. However, the immortalized but non-transformed WI-38-hTERT-SV40T/t cells also have activated cdk2. These cells express p52-C/EBPbeta1 also. Therefore, phosphorylation of p52-C/EBPbeta1 by activated cdk2 is necessary but not sufficient for degradation of p52-C/EBPbeta1. Addition or removal of other post-translational modifications of p52-C/EBPbeta1, such as deacetylation, may be required for its degradation. Also, expression or appropriate

localization of a necessary ubiquitin E3 ligase may be required for ubiquitination and subsequent degradation of C/EBPbeta1.

Degradation of C/EBPbeta1 by Ras may be a mechanism by which cells bypass oncogene-induced senescence. Loss of expression of this full length isoform of C/EBPbeta may be necessary for the escape from senescence and a step closer to transformation. Further support of this lies in the expression profile of C/EBPbeta1 in normal versus transformed cells. C/EBPbeta1 is present in normal cells, and is not degraded when Ras is introduced and induces senescence in normal cells. Moreover, this first isoform of C/EBPbeta is negatively regulated by Ras during Ras transformation of the MCF10A immortalized mammary epithelial cell line. Additionally, p52-C/EBPbeta1 expression is not observed in breast cancer cells in which the Ras pathway is activated. The expression pattern of C/EBPbeta1 in normal versus transformed cells supports our current findings that C/EBPbeta1 plays an important role in senescence and is negatively regulated during transformation. It is not surprising that a protein that plays a critical role in oncogene-induced senescence, a tumor suppressive mechanism, be down-regulated during transformation. Our study sheds light on how Ras signaling is altered in Ras(V12)-induced senescence versus transformation through the regulation of C/EBPbeta1; however, further investigation of what dictates this transition is necessary to more completely understand this critical switch.

p52-C/EBPbeta1 is expressed in normal mammary epithelial cells but not breast cancer cells (Eaton *et al.*, 2001). This is likely because it is advantageous for breast cancer cells to negatively regulate factors such as C/EBPbeta1 that are involved in tumor suppressive mechanisms such as OIS (Chapter 2). The current chapter examines how transformation of mammary epithelial cells by activated Ras negatively regulates p52-C/EBPbeta1 through phosphorylation of C/EBPbeta1 on Thr235 by cdk2 and

subsequent proteasomal degradation. This is not the only mechanism by which C/EBPbeta1 is regulated in breast cancer cells. The following chapter investigates another mechanism in which C/EBPbeta1 is regulated in breast cancer cells, modification of C/EBPbeta1 by sumoylation.

CHAPTER IV

SUMOYLATION OF C/EBPBETA1 IN BREAST CANCER CELLS

Introduction

The post-translational modification sumoylation regulates the function of a growing list of proteins that have roles in a variety of cell processes. Because of this, deregulation of the SUMO pathway has been observed in numerous diseases including neurodegenerative disorders (Kim and Baek, 2009), diabetes (Wang and She, 2008), and cancer (Sarge and Park-Sarge, 2009). C/EBPbeta contains a SUMO consensus site within its sequence centered around lysine 173 (Eaton and Sealy, 2003). Interestingly, C/EBPbeta1 is the only transactivator isoform of C/EBPbeta that is sumoylated by SUMO-2/3 in Cos-7 cells even though both C/EBPbeta1 and C/EBPbeta2 contain the SUMO consensus sequence around lysine 173 (Eaton and Sealy, 2003). It has been demonstrated that C/EBPbeta1 can be sumoylated on lysine 173 and the first 23 amino acids unique to C/EBPbeta1 are necessary for efficient sumoylation. Mutation of this target lysine 173 to an alanine did not effect sub-nuclear localization of C/EBPbeta1 (Eaton and Sealy, 2003).

Sumoylation of transcription factors frequently causes transcriptional repression due to sumoylation leading to an alteration in binding partners. Sumoylated proteins interact with transcriptional co-repressors such as histone deacetylases (HDACs), Daxx, or members of the NURD co-repressor complex, thus leading to transcriptional repression by sumoylated protein (Gill, 2005). Although expression of the 52kDa form of C/EBPbeta1 is not observed in breast cancer cell lines, sumoylated C/EBPbeta1 would migrate more slowly via SDS-PAGE resulting in higher molecular weight bands. Since

sumoylation oftentimes leads to transcriptional repression of target proteins, negative regulation of C/EBPbeta1 by sumoylation would give cancer cells a growth advantage since C/EBPbeta1 plays a role in oncogene-induced senescence, a tumor suppressive mechanism.

Additionally, phosphorylation of target proteins oftentimes enhances sumoylation. Examples of this include phosphorylation and subsequent enhancement of sumoylation of STAT1 (Vanhatupa *et al.*, 2008), PPAR-gamma (Yamashita *et al.*, 2004), MEF2 (Kang *et al.,* 2006, Gregoire *et al.,* 2006), and Estrogen-related receptor alpha-1 (Vu *et al.*, 2007). Phosphorylation cascades known to phosphorylate C/EBPbeta are activated in breast cancer cells. For example, the Ras pathway is activated in most breast cancer cells via activation of upstream receptors, activation of Ras itself, or activation of downstream kinases (Malaney and Daly, 2001). Activation of the Ras pathway leads to the activation of numerous kinases that phosphorylate C/EBPbeta on Threonine 235 (Thr235) including Erk-2 (Hanlon *et al.*, 2001), cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007), and p38 (Engelman *et al.*, 1998, Horie *et al.*, 2007). Phosphorylation of C/EBPbeta1 on Thr235 in transformed cells may enhance sumoylation, thus repressing the transcriptional ability of C/EBPbeta1 to induce IL6 and allowing for evasion of senescence. In the current study we demonstrate that an antibody specific to C/EBPbeta1 recognizes higher molecular weight bands in a panel of breast cancer cell lines. When C/EBPbeta1 is exogenously expressed in breast cancer cells, sumoylation of C/EBPbeta1 is evident. Importantly we show that the higher molecular weight bands in breast cancer cell lines recognized by the C/EBPbeta1-specific antibody is sumoylated C/EBPbeta1. Additionally, phosphorylation of purified C/EBPbeta1 by Erk-2 enhances sumoylation, in vitro, and sumoylated C/EBPbeta1 is phosphorylated on

Thr235. Finally, mutation of C/EBPbeta1Thr235 to alanine, which prevents phosphorylation of this residue, leads to a decrease in sumoylation of C/EBPbeta1.

Materials and Methods

Reagents

Unless otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibody directed against C/EBPbeta was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-T7 tag monoclonal antibody was obtained from Novagen (Madison, WI, USA) and the anti-C/EBPbeta1-specific antibody raised to the 21 N-terminal amino acids present only in C/EBPbeta1 is Abcam 18F8. The C-terminal C/EBPbeta antibody used in Figure 1c is the Abcam 47A1 antibody. The anti-HA tag antibody The SUMO-2/3 antibodies The anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Promega (Madison, WI, USA). The proteasome inhibitor MG132 (Calbiochem, San Diego, CA, USA) was resuspended in DMSO and used at a concentration of 50uM. N-ethyl maleimide was resuspended in DMSO and used at a concentration of 5mM. T7 tag antibody beads (Novagen).

Cell lines

Tissue culture media was obtained from Life Technologies, Inc. (Carlsbad, CA, USA). Unless otherwise indicated, all tissue culture supplements were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MCF10A human mammary epithelial cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in a 1:1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle medium (DMEM)

containing 2.5 mM L-glutamine and supplemented with 5% horse serum, 10ug/mL insulin, 0.5 ug/mL hydrocortisone, 20ng/mL epidermal growth factor, 100 ng/mL cholera toxin (Calbiochem Novabiochem, San Diego, CA, USA), 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). The human breast cancer cell lines MDA-MB-231, MDA-MB-468, HCC1954, SKBR3, BT474, MDA435, and T47D were obtained from the ATCC (Manassas, VA) and were maintained in Iscove's Modified Eagle media supplemented with 10% fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT, USA), 10 µg/ml bovine insulin, 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). Cos-7 cells were a gift from Dr Steve Hann, Vanderbilt University and were maintained in DMEM plus 10% FBS (HyClone Laboratories, Logan, UT, USA). The phoenix-ampho packaging cell line was obtained from the ATCC with the permission of GP Nolan (Stanford University, Palo Alto, CA, USA) and has been previously described (Grignani *et al.*, 1998). The packaging cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Inc., Logan, UT, USA), 1mM sodium pyruvate, 2mM L-glutamine, 100 U/mL penicillin, and 100 ug/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA). All cells were grown at 37 degrees Celsius in a humidified atmosphere containing 5% carbon dioxide.

Cloning of recombinant constructs and virus preparation

T7-C/EBPbeta1-pcDNA3.1-His A was generated as described in Eaton and Sealy, 2003. C/EBPbeta1 is the only transactivator isoform produced from this construct due to mutation of the second in-frame ATG. Additionally, a perfect Kozak sequence was made centered around the first ATG. Generation of LZRS-T7-C/EBPbeta1-IRES-eGFP and T7-C/EBPbeta1T235A-pcDNA3.1-His A was as previously described (Atwood and Sealy, 2010). Recombinant amphotropic retroviral stock generation and retroviral

infection were performed as described in Bundy and Sealy, 2003. The hemagglutinin (HA)-tagged SUMO-2 expression vector was a kind gift of Dr. Ron Hay (University of St Andrews, St Andrews, UK).

Transient transfections

Cos-7 cells were plated 18-24 hours prior to transfection so that the cells were 80-90% confluent at the time of transfection. Serum-free DMEM replaced complete media on cells 1 hour before transfection. 8ug of total DNA was transfected into cells via 24uL GenJet (SignaGen Laboratories, Gaithersburg, MD, USA) in serum-free media. After 5 hours, the media was changed to complete media. The cells were harvested two days post-transfection.

Preparation of immunoprecipitations, cell lysates and immunoblot analysis

Confluent plates of cells were treated with 50uM MG132 for 8 hours and 5mM N-ethylmaleimide for 30 minutes for the immunoprecipitations. Immunoprecipitations were performed as described in Chapter 3. Cell lysates were prepared from 100 mm dishes of 90% confluent cells as described in Chapter 2. Relative protein concentrations were determined using the Protein Assay Reagent (BioRad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Equal amounts of protein were loaded onto 10% SDS-PAGE and separated by electrophoresis. The proteins were transferred to an Immobilon P or Immobilon FL filter and the blots were processed as described previously (Eaton *et al.*, 2001). After the nonspecific binding sites were blocked, the blots were inclubated with primary antibody (C-terminal C/EBPbeta at a 1:5 000 dilution; T7 at 1:10 000, N-terminal C/EBPbeta at 1:2 000) in TBS-T (100mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 0.5% nonfat dried milk (NFDM) for 1 hour at room temperature. The blots were washed with three successive changes of TBS-T

containing 0.5% NFDM at room temperature for 20 minutes and incubated with a HRP-conjugated goat anti-rabbit (1:5 000 or 1:2 000 dilution) or a HRP-conjugated goat anti-mouse antibody (1:10 000 dilution) as described above for an additional hour. The blots were then washed with three successive changes of TBS-T solution for 15 minutes and the signal was detected by chemiluminescence using SuperSignal West Pico reagent (Pierce, Rockford, IL, USA) and autoradiography with Kodak X-OMAT film (Rochester, NY, USA). Alternatively, the LI-COR ODYSSEY infrared imaging system (Lincoln, Nebraska) was used for immunoblot analysis. Quantitation was performed as per manufacturer's instruction.

Cross-linking of C/EBPbeta1-specific antibody to protein A beads

The serum from the rabbit polyclonal C/EBPbeta1-specific antibody raised to a 16 amino acid peptide corresponding to the first 16 amino acids in human C/EBPbeta1 described in Eaton *et al.*, 2001, was used in the cross-linking. We began by ammonium sulfate precipitating the antibody out of the serum. Serum containing approximately 4mg antibody was first clarified. An equal volume of saturated ammonium sulfate pH 7.5-8.0 was slowly added dropwise into the clarified serum at 4 degrees Celsius. The serum/ammonium sulfate solution was mixed frequently during the addition of ammonium sulfate. The serum/ammonium sulfate solution was rotated at 4 degrees Celsius overnight. The next day the serum/ammonium sulfate solution was spun in an HB-4 swinging bucket rotor at 3000xg for 30 minutes at 4 degrees Celsius. The supernatant was removed and the pellet containing the antibody was resuspended in 1x PBS. The PBS/antibody solution was then transferred to dialysis tubing and dialyzed in 1x PBS overnight at 4 degrees Celsius. The solution in the dialysis tubing was clarified the following day. To cross-link the antibody to the protein A agarose, 2mL of protein A agarose slurry (Invitrogen) was washed in 1x PBS and collected. 4mg of the ammonium

precipitated antibody was mixed with the beads for 1 hour at room temperature. The beads were then washed with sodium borate and the antibody was cross-linked to the beads with 20uM dimethylpimelimidate in sodium borate rocking for 30 minutes at room temperature. The cross-linking was quenched by rocking the beads in 0.2M ethanolamine pH 8.0 for 2 hours at room temperature. Finally, the beads were washed in 1x PBS and stored at 4 degrees Celsius.

In vitro phosphorylation and sumoylation

5ug of purified rat C/EBPbeta1 (Lap1) protein was incubated with 100uM ATP, 10mM magnesium chloride, and protease and phosphotase inhibitors (10uM sodium vanadate,10mM sodium molybdate, 10mM beta-glycerophosphate, 1ug/mL aprotinin, 1ug/mL leupeptin, 1ug/mL pepstatin, and 1mM phenylmethylsulfonyl fluoride). One half of this original sample was incubated with 0.5ug active, purified Erk-2 (Upstate/Millipore) for 1 hour at 30 degrees Celsius. The other half of the sample was not incubated with Erk-2. Half of the sample incubated with Erk-2 (so one quarter of the original) and half of the sample not incubated with Erk-2 were then incubated with the sumoylation machinery for 1 hour at 37 degrees Celsius. The purified sumoylation machinery included 0.05ug/uL human E2 conjugating enzyme (Ubc9), 0.05ug/uL human SUMO-3 peptide, and 0.0075ug/uL E1 activating enzyme (SAE I/II) (LAE Biotechnology Co.). Sumoylation was performed in the presence of 0.1mM ATP, the protease and phoshotase inhibitors (10uM sodium vanadate,10mM sodium molybdate, 10mM beta-glycerophosphate, 1ug/mL aprotinin, 1ug/mL leupeptin, 1ug/mL pepstatin, and 1mM phenylmethylsulfonyl fluoride), and 1x SUMO Buffer (LAE Biotechnology Co.). After sumoylation, 2x SDS sample buffer was added to the four different samples. The samples were boiled for 5 minutes and stored at -70 degrees Celsius until subjected to SDS-PAGE.

Results

Sumoylation of C/EBPbeta1 in breast cancer cell lines

p52-C/EBPbeta1 is not observed via immunoblot analysis of breast cancer cell lines (Eaton *et al.*, 2001, Figure 36), however p52-C/EBPbeta1 is expressed in the MCF10A immortalized but non-transformed mammary epithelial cell line (Figure 36, lane 1 and Atwood and Sealy, 2010). Figure 36 utilizes an antibody raised to the first 23 amino acids present only in the first isoform of C/EBPbeta. Using this C/EBPbeta1-specific antibody, immunoblot analysis of a panel of breast cancer cells results in higher molecular weight bands. These higher molecular weight bands are likely post-translationally modified C/EBPbeta1, because C/EBPbeta1 can be modified by a variety of post-translational modifications known to increase the apparent molecular weight of the protein via SDS-PAGE. It is likely that these bands are not non-specific, as antibodies raised to other regions of C/EBPbeta also recognize these higher molecular weight bands (Figure 38 and data not shown).

Knowing that C/EBPbeta1 can be sumoylated in Cos-7 cells and after observing higher molecular weight bands on the immunoblots of breast cancer cells using the C/EBPbeta1-specific antibody, we wanted to know if C/EBPbeta1 could be sumoylated in breast cancer cell lines. We expressed T7-tagged C/EBPbeta1 (T7-C/EBPbeta1) by infecting breast cancer cells with a retroviral vector expressing T7-C/EBPbeta1-IRES-eGFP. Infected cells were then sorted by fluoresecence activated cell sorting (FACS) using green fluorescent protein (GFP) as a marker resulting in a homogenous population of cells expressing T7-C/EBPbeta1 and GFP. Immunoprecipitations were performed with T7 antibody beads and resulting immunoblot analysis reveals that C/EBPbeta1 is sumoylated when exogenously expressed in the MDA231 breast cancer cell line (Figure

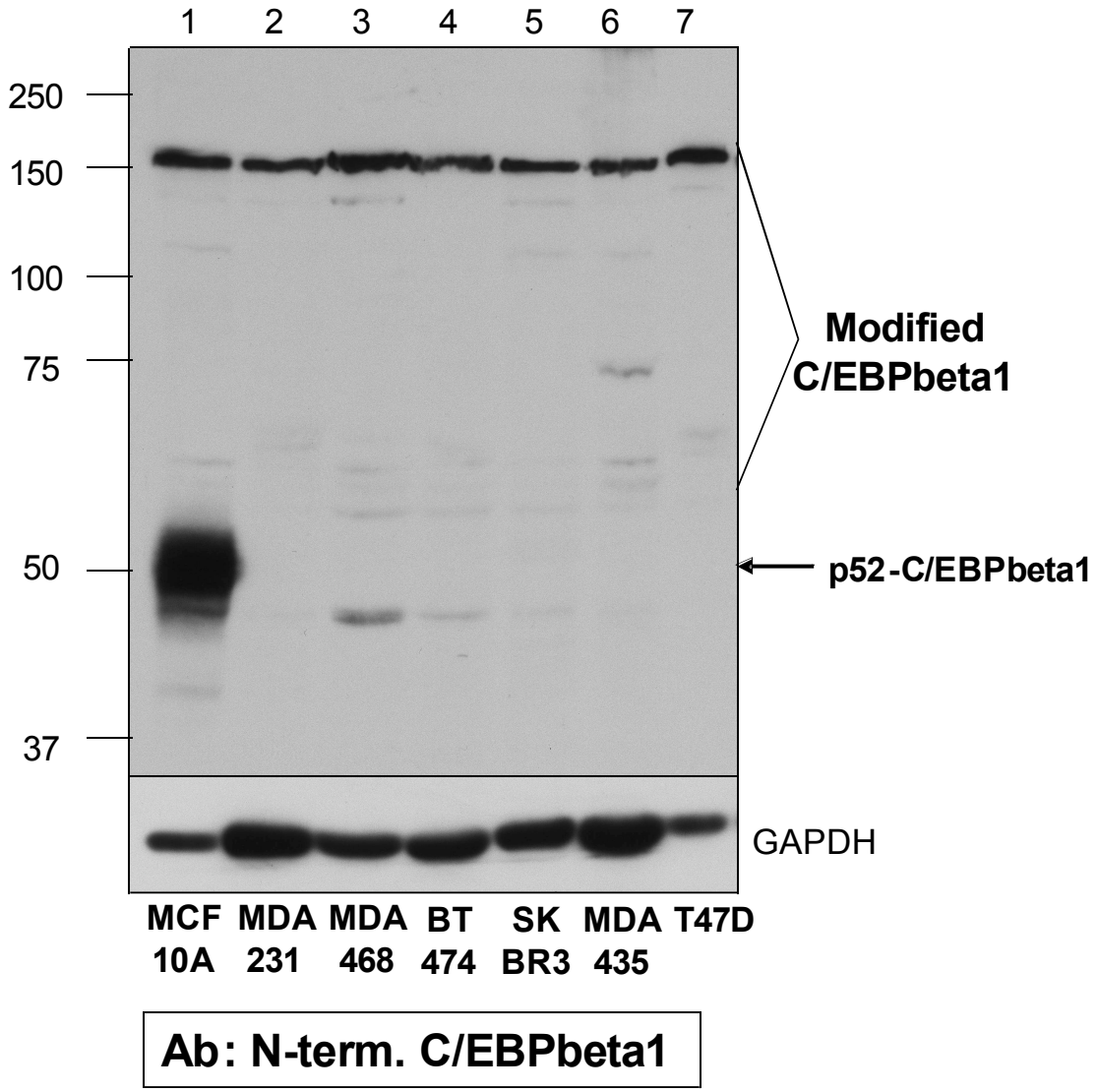

Figure 36. Higher molecular weight bands are present in anti-C/EBPbeta1 immunoblots of breast cancer cell lines. a. Cell lysates were prepared and were run on an 8% SDS-PAGE in the following order: lane 1 MCF10A, lane 2 MDA231, lane 3 MDA468, lane 4 BT-474, lane 5 SK-BR3, lane 6 MDA435 and lane 7 T47D. Immunoblot analysis was performed with a C/EBPbeta1-specific antibody raised to the first 21 amino acids unique to C/EBPbeta1 (Abcam 18F8). The bottom immunoblot was performed as a loading control for GAPDH. Bars indicate the mobility's of standard molecular weight markers, in kilo-Daltons (kDa), in all figures.

37). The immunoblot on the left is with an anti-SUMO-2/3 antibody, and there are distinct higher molecular weight bands in the MDA231-T7-C/EBPbeta1 lane as compared to control MDA231 only (lane 2 compared to lane1). These bands coincide with bands in the MDA231-T7-C/EBPbeta1 lane (lane 4) on the immunoblot on the right in which an anti-C/EBPbeta antibody is used. Similar results were obtained using the SKBR3 and HCC1954 breast cancer cell lines (data not shown), allowing us to conclude that sumoylation of C/EBPbeta1 occurs in breast cancer cells.

Next we wanted to determine if the higher molecular weight bands that are observed in the anti-C/EBPbeta1 immunoblot in Figure 36 are sumoylated C/EBPbeta1. To examine this, we used our C/EBPbeta1-specific antibody described in Eaton *et al.*, 2001, cross-linked to protein A beads. Immunoprecipitations were performed with these beads using cell extracts from MDA468 and MDA231 breast cancer cell lines. The results from the MDA468 immunoprecipitation are shown in Figure 38. The immunoblot on the left uses an anti-SUMO-2/3 specific antibody, the middle immunoblot is with a C-terminal C/EBPbeta antibody, and the immunoblot on the right is with the C/EBPbeta1-specific antibody. Lanes 3, 6, and 9 are the immunoprecipitations with MDA468 extract, whereas the other lanes are negative controls (lanes 1, 4, and 7 are beads only, lanes 2, 5, and 8 are beads not cross-linked to antibody but incubated with extract). Lanes 3, 6, and 9 exhibit higher molecular weight bands that correspond in mobility and are not present in the lanes with control samples. The anti-SUMO-2/3 antibody recognizes these unique higher molecular weight bands (Figure 38 lane 3), which coincide with the bands in the anti-C/EBPbeta immunoblots (Figure 38, lanes 6 and 9), thus demonstrating that endogenous C/EBPbeta1 is sumoylated in breast cancer cell lines.

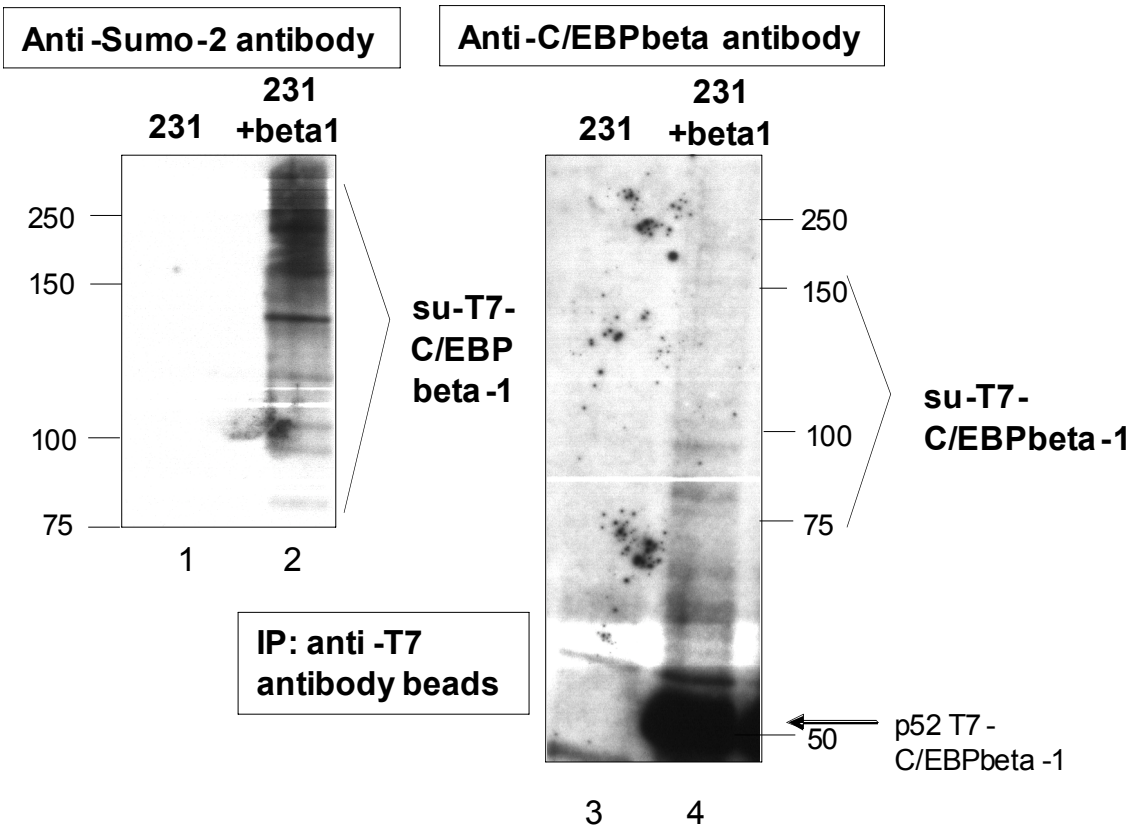

Figure 37. Exogenously expressed T7-C/EBPbeta1 is sumoylated in MDA231 cells. MDA231 breast cancer cells were infected with T7-C/EBPbeta1-IRES-eGFP-LZRS three times and sorted by FACs using GFP as a marker. Immunoprecipitations of confluent 100mm dishes were performed with uninfected MDA231s (lanes 1 and 3) or T7-C/EBPbeta1-MDA231 cells (lanes 2 and 4) using T7 antibody beads. The left is an immunoblot with an anti-SUMO-2/3 antibody and the immunoblot on the right is with an anti-C/EBPbeta antibody. Sumoylated C/EBPbeta1 is indicated and the parent p52 C/EBPbeta1 is indicated by the arrow.

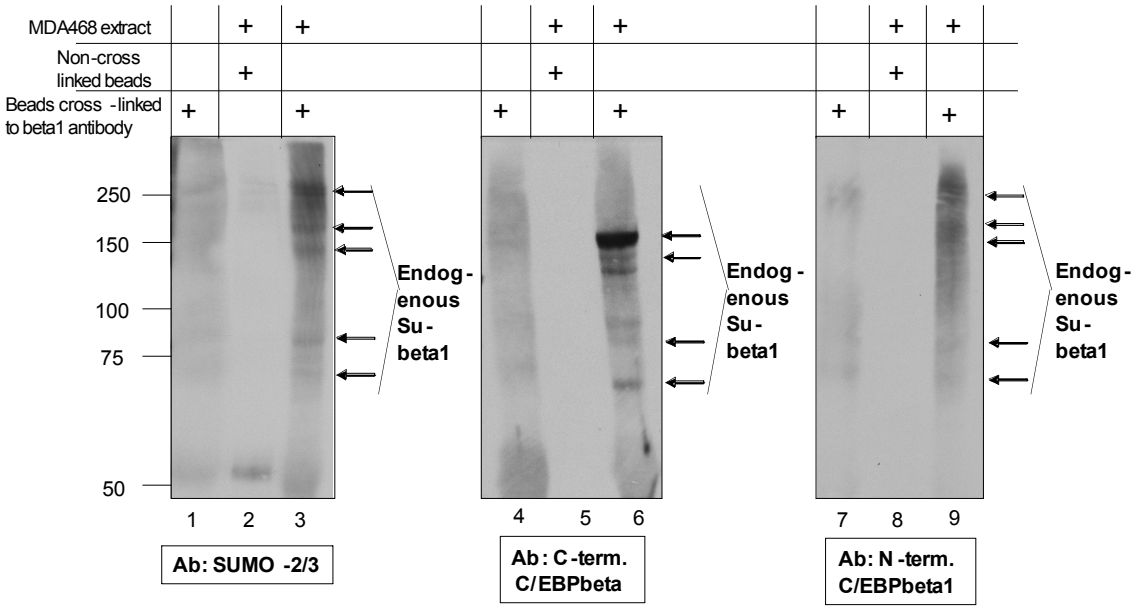

Figure 38. The higher molecular weight bands in anti-C/EBPbeta1 immunoblots of breast cancer cells is sumoylated C/EBPbeta1. Immunoprecipiations of MDA468 cells were performed with protein A agarose beads cross-linked to a C/EBPbeta1-specific antibody (described in Eaton *et al.*, 2001). Lanes 3, 6, and 9 are the immunoprecipitations whereas lanes 1, 4, and 7 are negative control beads only and lanes 2, 5 and 8 are negative control non-crosslinked beads incubated with MDA468 extract. The left immunoblot is performed with an anti-SUMO-2/3 antibody, the middle immunoblot with a C-terminal C/EBPbeta antibody (Abcam 47A1) and the right hand immunoblot with a C/EBPbeta1-specifc antibody (Abcam 18F8). Arrows indicate sumoylated C/EBPbeta1. (beta1 = C/EBPbeta1, su = sumoylated)

C/EBPbeta1 is phosphorylated on Thr235 by Erk2 and this phosphorylation enhances sumoylation of C/EBPbeta1 *in vitro*

It is well-known that C/EBPbeta is phosphorylated on Thr235 by Erk, however few studies have examined which particular isoform of C/EBPbeta is phosphorylated by Erk-2. One study has determined that C/EBPbeta2 is phosphorylated by Erk-2 on Thr235 (Hanlon *et al.*, 2001), but no one has determined whether C/EBPbeta1 can be phosphorylated on this residue by Erk-2. To examine this, we took purified rat C/EBPbeta1 protein and incubated it with purified, active Erk-2. Figure 39 is an immunoblot with an anti-pThr235 C/EBPbeta-specific antibody illustrating that C/EBPbeta1 is phosphorylated on Thr235 after incubation with Erk-2 (compare lane 4 with lane 3), *in vitro.* Figure 39 is the same immunoblot only with the anti-T7 tag antibody demonstrating approximately equal amounts of protein are present in both lanes (compare lanes 1 and 2).

After determining that Erk-2 phosphorylates C/EBPbeta1 on Thr235 *in vitro*, we wanted to determine if this phosphorylation could enhance sumoylation of C/EBPbeta1, as this has been found to be true for several other transcription factors. To do this we incubated purified C/EBPbeta1 protein with Erk-2 and followed by the addition of purified SUMO-2/3 peptide, the SUMO activating (E1) enzyme, and the SUMO conjugating (E2) enzyme. Figure 40 is an immunoblot with the anti-T7 tag antibody indicates that incubation with Erk-2 prior to incubation with SUMO-2/3 enhances sumoylation of C/EBPbeta1 (compare lanes 2 and 4).

Next we wanted to determine if sumoylated C/EBPbeta1 is phosphorylated on Thr235. T7-C/EBPbeta1 and HA-SUMO-2/3 were transiently transfected into Cos-7 cells and immunoprecipitations were performed with T7 antibody beads. The anti-

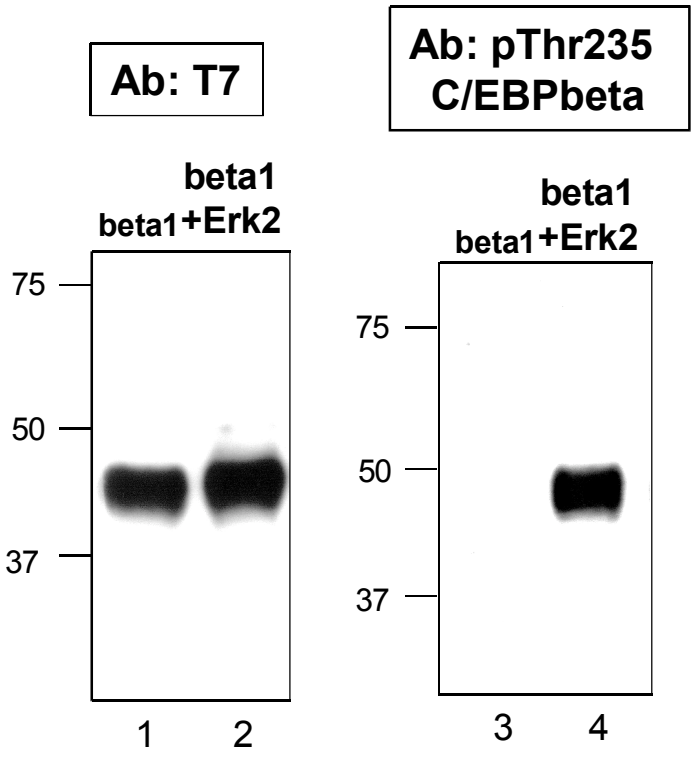

Figure 39. C/EBPbeta1 is phosphorylated on Thr235 by Erk-2 *in vitro.* Immunoblot analysis of purified rat C/EBPbeta1 (Lap1) (lanes 1 and 3) and C/EBPbeta1 incubated with purified, active Erk-2 (lanes 2 and 4). The immunoblot on the left is with the anti-T7 tag antibody and on the right is with the anti-phosphoThr235 C/EBPbeta antibody. Rat C/EBPbeta1 migrates faster via SDS-PAGE because it is smaller in size than human C/EBPbeta1.

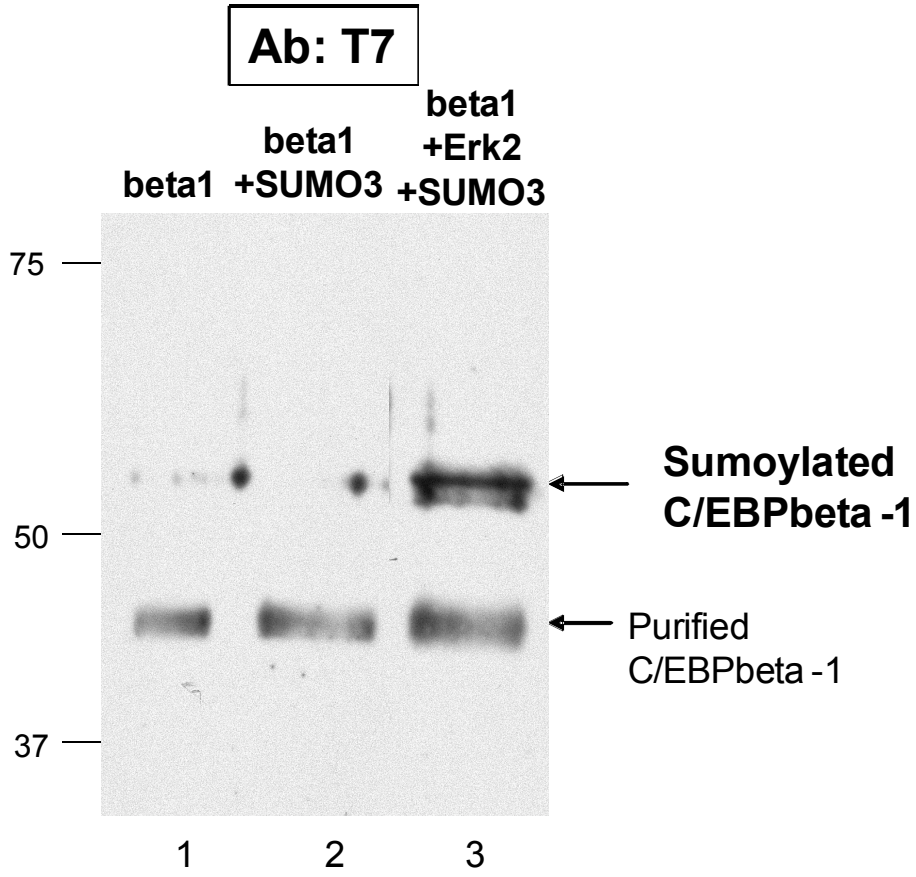

Figure 40. Phosphorylation of C/EBPbeta1Thr235 by Erk-2 enhances sumoylation of C/EBPbeta1 *in vitro.* Immunoblot analysis with the anti-T7 tag antibody. Lane 1 is purified rat C/EBPbeta1, lane 2 is C/EBPbeta1 incubated with purified E1 SUMO activating enzyme, purified E2 SUMO conjugating enzyme, and purified SUMO-3 peptide, and lane 3 is C/EBPbeta1 with Erk-2, E1, E2 and SUMO-3. Arrows indicate C/EBPbeta1 and sumoylated C/EBPbeta1.

phosphoThr235 C/EBPbeta antibody was used on the immunoblot in Figure 41. Figure 41 lane 2 demonstrates that sumoylated C/EBPbeta1 is phosphorylated on Thr235. The middle immunoblot is with the anti-HA tag antibody confirming that the higher molecular weight band is sumoylated C/EBPbeta1 and the immunoblot on the right is with the anti-C/EBPbeta C-terminal antibody.

Mutation of Thr235 to alanine decreases sumoylation of C/EBPbeta1

After observing that phosphorylation of C/EBPbeta1 by Erk-2 enhances sumoylation *in vitro*, we mutated the Thr235 phosphoryation site to an alanine to determine if preventing phosphorylation of this residue would lead to a decrease in sumoylation. T7-C/EBPbeta1 or T7-C/EBPbeta1T235A with HA-SUMO-2/3 were transiently transfected into Cos-7 cells and immunoprecipitations were performed with T7 antibody beads. The middle immunoblot in Figure 42 is with the anti-HA tag antibody and demonstrates that mutant C/EBPbeta1T235A that cannot be phosphorylated at Thr235 is sumoylated to a lesser extent than C/EBPbeta1 (compare lanes 5 and 6). This is confirmed in lanes 8 and 9, which are the same samples only with the anti-C/EBPbeta antibody. The anti-phosphoThr235 C/EBPbeta antibody was used on the immunoblot on the left and lane three demonstrates that T7-C/EBPbeta1T235A does not with the anti-phosphoT235 antibody because this residue has been mutated.

Figure 43 quantitates the difference in sumoylation between C/EBPbeta1 and C/EBPbeta1T235A. T7-C/EBPbeta1 and the T7-C/EBPbeta1T235A mutant were transiently transfected into Cos-7 cells along with HA-SUMO-2/3. The immunoblot with the anti-T7 tag antibody shown in Figure 43 demonstrates that the T7-C/EBPbeta1T235A mutant is sumoylated to a lesser extent than wild type T7-C/EBPbeta1 (compare lanes 3 and 2). Using the Odyssey system, we quantified the

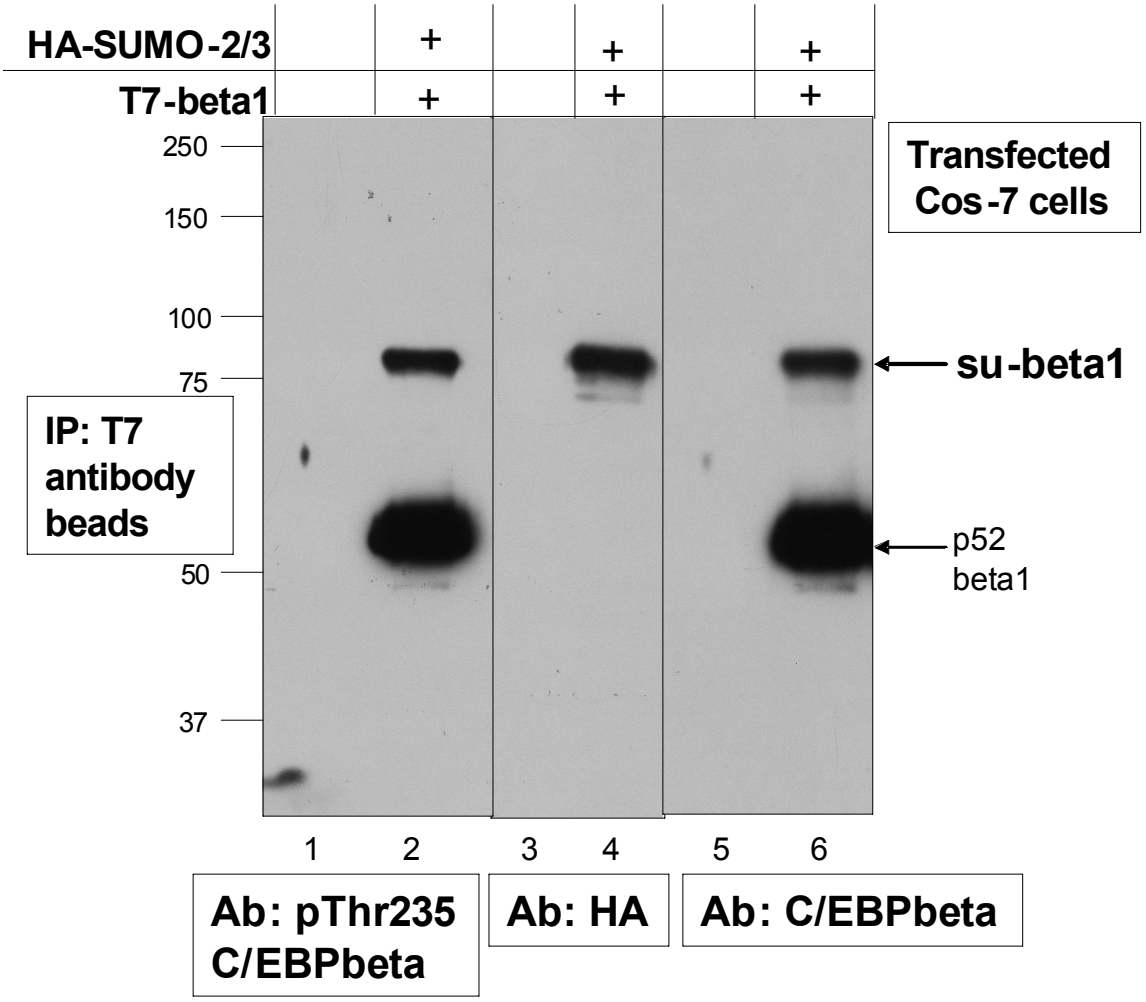

Figure 41. Sumoylated C/EBPbeta1 is phosphorylated on Thr235. Cos-7 cells were untransfected (lanes 1, 3, and 5) or transfected with T7-C/EBPbeta1-pcDNA3.1 and HA-SUMO-2-pcDNA3 (lanes 2, 4 and 6). All samples were immunoprecipiated with T7 antibody beads. Immunoblot analysis was performed with the anti-phosphoThr235 C/EBPbeta antibody (left), anti-HA tag (middle), and anti-C/EBPbeta antibody (right). Arrows indicate sumoylated T7-C/EBPbeta1 and p52-T7-C/EBPbeta1. (beta1 = C/EBPbeta1)

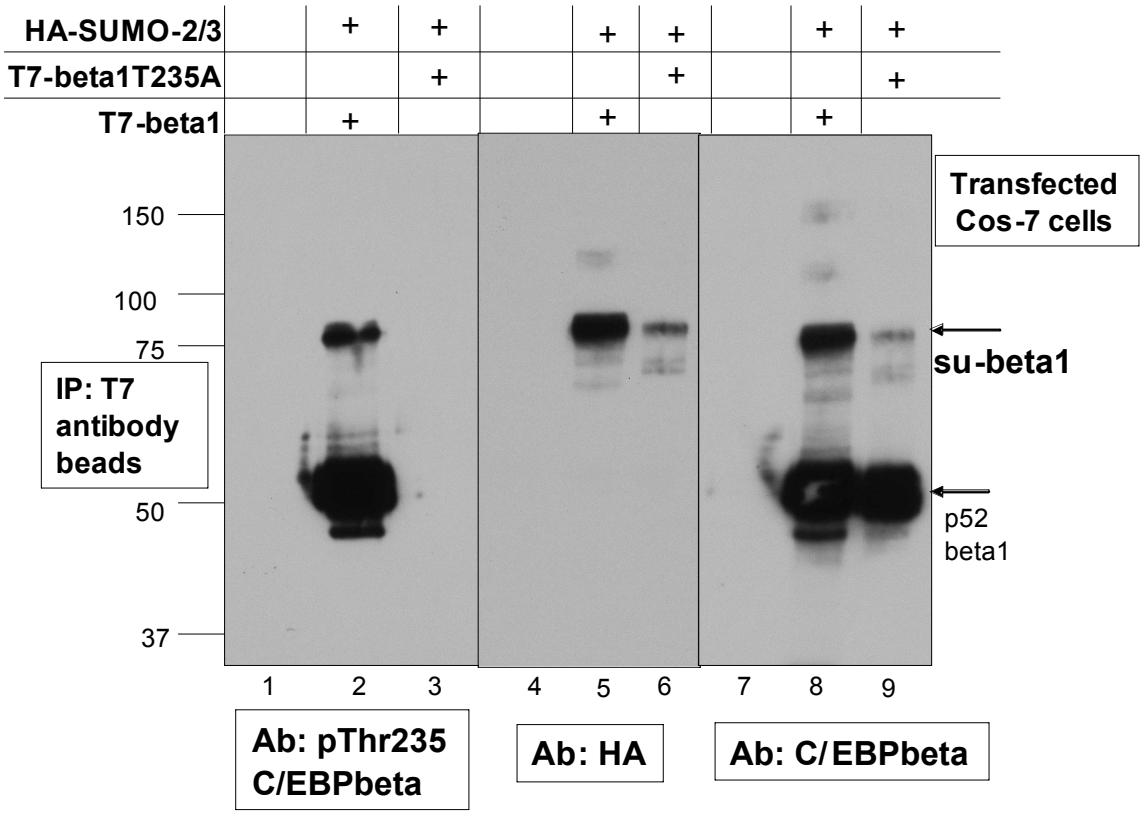

Figure 42. Mutation of Thr235 to alanine decreases sumoylation of C/EBPbeta1. Cos-7 cells were untransfected (lanes 1, 4, and 7), transfected with T7-C/EBPbeta1-pcDNA3.1 and HA-SUMO-2-pcDNA3 (lanes 2, 5 and 8), or transfected with T7-C/EBPbeta1T235A-pcDNA3.1 and HA-SUMO-2-pcDNA3 (lanes 3, 6, and 9). All samples were immunoprecipitated with T7 antibody beads. Immunoblot analysis was performed with the anti-phosphoThr235 C/EBPbeta antibody (left), anti-HA tag (middle), and anti-C/EBPbeta antibody (right). Arrows indicate sumoylated T7-C/EBPbeta1 and p52-T7-C/EBPbeta1.

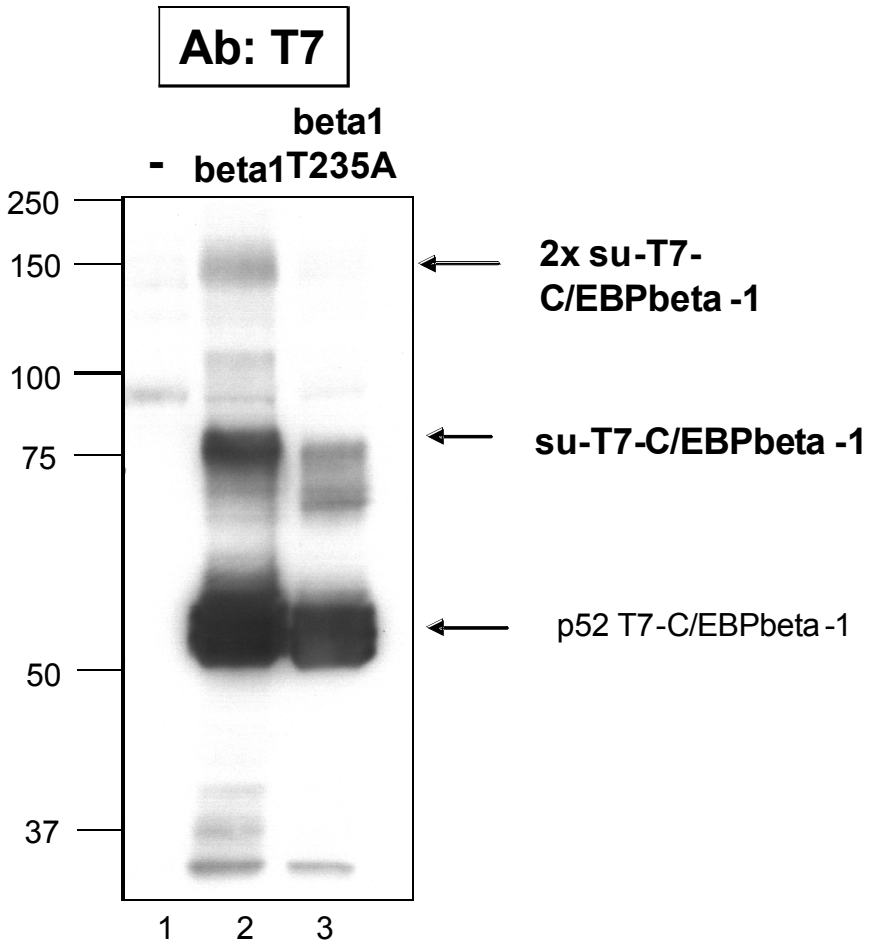

Figure 43. C/EBPbeta1T235A is sumoylated to a lesser extent than wild type C/EBPbeta1. Immunoblot analysis using the anti-T7 tag antibody of cell lysates from Cos-7 (lane 1), Cos-7 cells transfected with T7-C/EBPbeta1-pcDNA3.1 and HA-SUMO-2-pcDNA3 (lane 2), and Cos-7 transfected with T7-C/EBPbeta1T235A-pcDNA3.1 and HA-SUMO-2-pcDNA3 (lane 3). Arrows indicate p52-T7-C/EBPbeta1 and sumoylated T7-C/EBPbeta1. The relative amount of protein in the parent T7-C/EBPbeta1 band and the 75kDa sumoylated T7-C/EBPbeta1 band was measured using the LI-COR Odyssey system. It was determined that there is 3.25 times more sumoylated 75kDa T7-C/EBPbeta1 as there is 75kDa T7-C/EBPbeta1T235A. This was calculated relative to the p52-T7-C/EBPbeta1 and p52-T7-C/EBPbeta1T235A bands. This was repeated three times with a standard deviation of +/-0.26. (beta1 = C/EBPbeta1, su-beta1 = sumoylated C/EBPbeta1)

percentage of sumoylated T7-C/EBPbeta1 in the 75kDa band compared to the non-sumoylated parent 52kDa band and determined that there is 3.25 +/- 0.26-fold less sumoylated T7-C/EBPbetaT235A compared to wild type. This supports our findings in Figure 41 that phosphorylation of C/EBPbeta1 on Thr235 enhances sumoylation.

Discussion

p52-C/EBPbeta1 is not observed via immunoblot of breast cancer cell lines with C/EBPbeta antibodies (Eaton *et al.*, 2001, Figure 36), however breast cancer cell lines exhibit higher molecular weight bands that react with C/EBPbeta antibodies, including C/EBPbeta1-specific antibodies (Figure 36). C/EBPbeta1 is known to be modified by post-translational modifications that can affect the apparent molecular weight of the protein via SDS-PAGE, including the post-translational modification sumoylation. C/EBPbeta1 is modified by SUMO-2/3 when transfected into Cos-7 cells, and C/EBPbeta1 is the only isoform of C/EBPbeta known to be modified by sumoylation (Eaton and Sealy, 2003). Modification by sumoylation adds 10-20kDa to the apparent molecular weight of the target protein. Additionally, SUMO-2/3 itself contains a SUMO consensus site, so that SUMO-2/3 can be further sumoylated to form SUMO-2/3 chains. This can result in a ladder of higher molecular weight bands. We demonstrate that when T7-C/EBPbeta1 is expressed in breast cancer cells such as MDA231s, a ladder of higher molecular weight bands are observed with both a C/EBPbeta antibody and a SUMO-2/3 antibody, indicating sumoylation of C/EBPbeta1 (Figure 37). Furthermore, we demonstrate that the higher molecular weight bands observed in the anti-C/EBPbeta1 immunoblot of breast cancer cells in Figure 36 are sumoylated C/EBPbeta1 (Figure 38).

Phosphorylation oftentimes enhances sumoylation, and it is well known that signaling that activates phosphorylation of proteins is commonly activated in breast cancer cells. One example of this is the frequent activation of the Ras pathway in breast cancer cells. Ras pathway activation leads to the activation of numerous kinases known to phosphorylate C/EBPbeta on Threonine 235 (Thr235) including Erk-2 (Hanlon *et al.*, 2001), cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007), and p38 (Engelman *et al.*, 1998, Horie *et al.*, 2007). Therefore we wanted to determine whether phosphorylation of Thr235 in C/EBPbeta1 was enhancing sumoylation of C/EBPbeta1. Figure 39 demonstrates that full length C/EBPbeta1 is phosphorylated on Thr235 by Erk-2 *in vitro*, and Figure 40 reveals that this phosphorylation by Erk-2 on Thr235 enhances sumoylation of C/EBPbeta1, *in vitro*. Moreover, Figure 41 demonstrates that sumoylated C/EBPbeta1 in Cos-7 cells is phosphorylated on Thr235. To further examine the effect phosphorylation of C/EBPbeta1Thr235 has on sumoylation of C/EBPbeta1, we mutated C/EBPbeta1Thr235 to an alanine so that this residue could no longer be phosphorylated. Figures 42 and 43 demonstrate that this mutant is sumoylated to a lesser extent than wild type C/EBPbeta1, confirming that phosphorylation of Thr235 of C/EBPbeta1 enhances sumoylation of this protein.

We show here that breast cancer cells express sumoylated C/EBPbeta1. Non-transformed cells may also express sumoylated C/EBPbeta1, however normal cells express high levels of p52-C/EBPbeta1 (Figure 36 and Eaton *et al.*, 2001). With this loss of p52-C/EBPbeta1 in transformed cells through degradation (Chapter 3) and/or enhancement of sumoylation, the proportion of C/EBPbeta1 that is sumoylated in these cells is very high. This is in contrast to normal cells where p52-C/EBPbeta1 appears to be the predominant form of C/EBPbeta1 expressed (Figure 36, lane 1). Therefore the functional affect of sumoylation of C/EBPbeta1 would be much greater in the

transformed cells than in nontransformed cells. Sumoylation of transcription factors oftentimes leads to transcriptional repression. It would be advantageous for breast cancer cells to repress the transcriptional abilities of C/EBPbeta1 leading to repression of IL6 and thus evasion of senescence, a tumor suppressive mechanism. Sumoylation of proteins can lead to an alteration in binding partners of the target protein. Repression of transcriptional ability by sumoylated transcription factors is due to the sumoylated protein binding to transcriptional co-repressors such as HDACs, Daxx, members of the NURD co-repressor complex, and Polycomb group proteins (Gill, 2005). C/EBPbeta has been shown to interact with transcriptional co-repressors such as HDAC1 (DePoi *et al.*, 2005, Xu *et al.* 2003) and Daxx (Wethkamp and Klempnauer, 2009). Therefore sumoylated C/EBPbeta1 may be interacting with these transcriptional co-repressors in breast cancer cells thus repressing C/EBPbeta1-mediated transcription of IL6 leading to the evasion of senescence. It will be important to determine if sumoylation of C/EBPbeta1 is leading to transcriptional repression of this protein in breast cancer cells and the mechanism by which this repression is occurring.

This study is important because the original study that examined expression of the C/EBPbeta isoforms in breast cancer cells indicated that C/EBPbeta1 is not expressed in these transformed cells (Eaton *et al.*, 2001). Although it is true that the parent p52-C/EBPbeta1 is not observed via immunoblot of breast cancer cell lines (Eaton *et al.*, 2001 and Figure 36), we demonstrate here that sumoylated C/EBPbeta1 is present in anti-C/EBPbeta immunoblots of breast cancer cells. It is important to clarify that C/EBPbeta1 is expressed in breast cancer cells, it is just not observed in anti-C/EBPbeta immunoblots at its non-modified apparent molecular weight of 52kDa. Sumoylated C/EBPbeta1 is observed in anti-C/EBPbeta immunoblots at higher molecular weights ranging from 75kDa to 150kDa (Figure 38). It has been suggested

that C/EBPbeta1 may be a good biomarker to distinguish normal mammary epithelia from tumor using immunohistochemical methods, because C/EBPbeta1 is not expressed in breast cancer cells. However we illustrate here that C/EBPbeta1 continues to be expressed in breast cancer cells, albeit in a modified form. Moreover, these studies demonstrate that it is important to consider post-translational modifications of proteins when identifying biomarkers.

Taken together, these studies show that C/EBPbeta1 is expressed in breast cancer cells in a post-translationally modified, sumoylated form, which results in higher molecular weight bands in immunoblots. Additionally, this sumoylation is enhanced by phosphorylation of C/EBPbeta1Thr235. Further studies are necessary to determine the functional significance of sumoylated C/EBPbeta1. Current studies in our laboratory are investigating the functional significance of sumoylated C/EBPbeta1. A C/EBPbeta1K173R non-sumoylatable mutant has been constructed along with a C/EBPbeta1-SUMO2 fusion construct. These constructs, along with C/EBPbeta1T235A that demonstrates a decrease in sumoylation, are being introduced into WI-38 normal human fibroblasts to determine their ability to induce IL6 and senescence in these cells. If our hypothesis is correct and sumoylation of C/EBPbeta1 leads to transcriptional repression, then the non-sumoylatable C/EBPbeta1K173R protein and the C/EBPbeta1T235A mutant that demonstrates less sumoylation will be able to induce IL6 and senescence to a greater extent than wild type C/EBPbeta1, and the C/EBPbeta1-SUMO2 fusion protein will not be able to induce IL6 and senescence to the extent of C/EBPbeta1. Therefore, these studies will give insight into the functional role of sumoylation of C/EBPbeta1.

CHAPTER V

SUMMARY AND DISCUSSION

C/EBPbeta1 and oncogene-induced senescence

Leading up to the current studies, the transcription factor C/EBPbeta had been implicated in oncogene-induced senescence (OIS), a tumor suppressive mechanism. OIS is considered a tumor suppressive mechanism because when activated oncogenes such as activated Ras or Raf are introduced into normal cells these cells undergo senescence instead of transformation. As the small but quickly growing field of OIS was gaining support, Sebastian *et al.*, 2005 reported that C/EBPbeta is critical for activated Ras-induced senescence. They showed that C/EBPbeta-/- mouse embryonic fibroblasts (MEFs) forced to express Ras(V12) continued to proliferate and lacked morphological features of senescence, whereas control wild type MEFs senesced when forced to express activated Ras(V12). In 2008 Kuilman *et al.* demonstrated that C/EBPbeta is critical for activated Raf-induced senescence. Kuilman and colleagues went on to show that this was due to the ability of C/EBPbeta to induce the cytokine IL6. It is well known that C/EBPbeta induces IL6, however Kuilman *et al.* were the first to show that OIS is dependent on the induction of an inflammatory network. Since there is increasing evidence for functional differences between the three different isoforms of C/EBPbeta, we wanted to determine which isoform of C/EBPbeta was responsible for OIS. We had previously shown that overexpression of C/EBPbeta2 in MCF10A immortalized but non-transformed human mammary epithelial cells transformed this cell line (Bundy and Sealy, 2003). In addition, overexpression of C/EBPbeta3 in mammary epithelial cells

and breast cancer leads to autophagic cell death (Bundy *et al.*, 2005, Abreu and Sealy, 2010). However, the function of C/EBPbeta1 at that point in time was not clear. We knew that p52-C/EBPbeta1 was expressed in normal mammary epithelial cells but not in breast cancer cell lines (Eaton *et al.*, 2001). Moreover, studies from Chapter 3 indicated that introduction of activated Ras into MCF10A immortalized mammary epithelial cells transformed this cell line and led to the degradation of C/EBPbeta1. Because transformation of cells by Ras negatively regulated C/EBPbeta1, we hypothesized that C/EBPbeta1 was not degraded during Ras-induced senescence and was therefore the isoform responsible for OIS.

In Chapter 2 we demonstrate that C/EBPbeta1 is expressed in the normal human fibroblast WI-38 cells commonly used to study OIS. Importantly, C/EBPbeta1 is expressed in these cells when activated Ras is introduced and these cells undergo senescence. We go on to show that C/EBPbeta1 inhibits growth of these cells. Figure 12 demonstrates that C/EBPbeta1 induces IL6 expression over 6-fold whereas the other transactivator isoform of C/EBPbeta, C/EBPbeta2, only induces IL6 expression 2-fold. Furthermore, in the senescence-associated beta-galactosidase assay, C/EBPbeta1 induced senescence in over 50% of the cells whereas C/EBPbeta2 induced senescence in just over 35% of the cells. This data allowed us to conclude that C/EBPbeta1 is the primary isoform responsible for the induction of IL6 and subsequent senescence. Because approximately 35% of WI-38-C/EBPbeta2 cells senesce despite only a small (2.3 fold) induction in IL6 expression, C/EBPbeta2 is likely upregulating other cytokines involved in OIS. In fact Kuilman *et al.* found that other cytokines were induced during OIS. Alternatively, C/EBPbeta2 may also be heterodimerizing with endogenous C/EBPbeta1 to induce IL6 and thus senescence to lower levels.

This study is important because it describes a functional role for C/EBPbeta1 in normal cells. Many groups in the C/EBPbeta field frequently treat the two transactivator isoforms of C/EBPbeta as functionally equal. However our lab and others continue to demonstrate functional differences between these two isoforms. The functional differences of the isoforms of C/EBPbeta likely account, at least in part, for the numerous studies on C/EBPbeta demonstrating that this protein is important for a variety of seemingly conflicting functions including proliferation, senescence, transformation, cell death, and cell survival. C/EBPbeta1 is expressed in normal cells and is the primary transactivator isoform responsible for OIS. In contrast, C/EBPbeta2 is expressed at high levels in breast cancer cells and overexpression of this second isoform transforms MCF10A mammary epithelial cells (Eaton *et al.*, 2001, Bundy and Sealy, 2003). Importantly, activated Ras leads to OIS in normal cells yet leads to transformation of many immortalized cell lines. These two very different functional outcomes likely occur because of differences in signaling, the details of which remain unclear at this point. One possible explanation for this could be that Ras is signaling through C/EBPbeta1 in normal cells when senescence is induced, whereas during transformation Ras may be signaling through C/EBPbeta2. Since C/EBPbeta1 is the predominant isoform of C/EBPbeta expressed in normal mammary epithelial cells (Eaton *et al.*, 2001) and C/EBPbeta1 is the primary transactivator isoform able to induce IL6 and senescence in normal cells, Ras is likely signaling through this full length isoform of C/EBPbeta1 to induce senescence. How Ras signals to C/EBPbeta1 during OIS is currently unknown. Activation of Ras in normal cells may be leading to phosphorylation of C/EBPbeta1 at a different site by a different kinase or may be leading to a different post-translational modification of C/EBPbeta1. C/EBPbeta is known to be phosphorylated by other kinases in the Ras pathway and be modified by other post-translational modifications, such as methylation or acetylation (Engleman *et al*., 1998, Horie *et al.*, 2007, Hanlon *et*

al., 2001, Xu *et al.*, 2003, Cesena *et al.*, 2007, Kowenz-Leutz *et al.*, 2010). Conversely, C/EBPbeta2 is the predominant C/EBPbeta isoform expressed in breast cancer cells, transformed cells in which the Ras pathway is activated (Eaton *et al.*, 2001). Additionally, we show in Chapter 3 and 4 that p52-C/EBPbeta1 is negatively regulated by activated Ras in transformed mammary epithelial cells. Therefore Ras is likely signaling through this second isoform of C/EBPbeta during transformation. This signaling is likely through phosphorylation and subsequent activation of C/EBPbeta2, allowing C/EBPbeta2 to transcriptionally activate genes involved in proliferation and epithelial to mesenchymal transition (Nakajima *et al.*, 1993, Hanlon and Sealy, 1999, Hanlon *et al.*, 2000, Hanlon *et al.*, 2001, Bundy and Sealy, 2003, Bundy *et al.*, 2005, Russell *et al.*, 2010).

The goal of many current cancer therapies is to target specific proteins. If a particular protein is oncogenic and is overexpressed in a tumor, the goal of the targeted drug will likely be to inhibit the protein. On the contrary, if a protein suppresses tumorigenesis and is downregulated in a particular cancer, the therapy may be to re-express or overexpress this protein. In the case of C/EBPbeta, the different isoforms of this protein have functional differences. Therefore if C/EBPbeta is the desired target, it would be important to target a specific isoform of this transcription factor. Since C/EBPbeta2 expression is acquired in breast cancers and promotes tumorigenic characteristics, it would be beneficial to inhibit this isoform in breast cancer. However, C/EBPbeta1 and C/EBPbeta3 are involved in tumor suppressive mechanisms, senescence and cell death, respectively, so it would not be advantageous to inhibit these isoforms. Unfortunately targeting specific isoforms of C/EBPbeta is difficult, because the isoforms consist of shared sequence with the only difference being their size. The study of the functional differences of the isoforms of C/EBPbeta sheds light on

the fact that it is important to consider different isoforms of proteins when therapeutically targeting specific factors no matter what the size difference, because desired results may not occur if isoforms with different functions are also targeted.

Degradation of C/EBPbeta1 by activated Ras in transformed cells

Our lab has previously shown that p52-C/EBPbeta1 is expressed in normal mammary epithelial cells but not breast cancer cell lines (Eaton *et al.*, 2001). This observation led us to ask, what happens to p52-C/EBPbeta1 in breast cancer cell lines? Answering this question was an overriding goal of my thesis work. It is important to determine how proteins involved in tumor suppressive mechanisms such as OIS are negatively regulated during tumorigenesis. Understanding this negative regulation may shed light on how to re-express or re-activate proteins and pathways that would suppress tumorigenesis. To examine what happens to p52-C/EBPbeta1 in transformed mammary epithelial cells, the MCF10A/MCF10A-Ras cell system was utilized. When activated Ras(V12) is introduced into MCF10A cells, an immortalized but nontransformed mammary epithelial cell line, these cells become transformed (Figures 17 and 18). This system allowed us to study a “normal” mammary epithelial cell line compared to a transformed line simply by introducing Ras(V12). First we examined p52-C/EBPbeta1 expression in these cell lines. p52-C/EBPbeta1 is expressed in MCF10A cells whereas there is a striking loss of p52-C/EBPbeta1 expression in the transformed MCF10A-Ras cells (Figure 19). We went on to examine the mechanism of this loss of p52-C/EBPbeta1 during Ras transformation, and determined that p52-C/EBPbeta1 is being degraded by the proteasome, likely in a ubiquitin-dependent manner. To extend these studies to breast cancer cell lines, T7-C/EBPbeta1 was expressed in the MDA231 and MDA435 breast cancer cell lines. After several weeks in culture, expression of p52 T7-C/EBPbeta1 decreases and treatment of these cells with the proteasomal inhibitor

MG132 stabilizes expression of p52 T7-C/EBPbeta1, indicating that p52 T7-C/EBPbeta1 is being degraded by the proteasome in breast cancer cells also. Therefore, proteasomal degradation of p52-C/EBPbeta1 is one mechanism by which breast cancer cells negatively regulate C/EBPbeta1.

Next we wanted to determine what was triggering degradation of C/EBPbeta1 in transformed mammary epithelial cells. The Ras pathway is activated in most breast cancer cells, whether it be through activation of upstream receptors, mutation in Ras itself, or activation of downstream effectors. Activation of the Ras pathway activates several phosphorylation cascades known to phosphorylate C/EBPbeta. The most well-characterized phosphorylation site in C/EBPbeta is Thr235. Kinases including Erk-2 (Hanlon *et al.*, 2001), cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007), and p38 (Engelman *et al.*, 1998, Horie *et al.*, 2007) are known to phosphorylate C/EBPbeta at this Thr235 residue. We hypothesized that phosphorylation of C/EBPbeta1 by a specific kinase activated during Ras transformation was leading to proteasomal degradation of C/EBPbeta1.

To examine this, MCF10A-Ras cells expressing T7-C/EBPbeta1 were treated with a panel of kinase inhibitors that inhibit kinases known to phosphorylate C/EBPbeta. Figure 28 demonstrates that treatment with the cdk inhibitor roscovitine stabilizes T7-C/EBPbeta1 expression. cdk2 phosphorylates C/EBPbeta on Thr235 and this phosphorylation can be inhibited by treatment with roscovitine (Shuman *et al.*, 2004, Li *et al.*, 2007). Furthermore, knockdown of cdk2 leads to a decrease in phosphorylation of C/EBPbetaThr235. Figure 29 shows that cdk2 can phosphorylate C/EBPbeta1 specifically, as the previous studies did not examine or indicate the isoform(s) of C/EBPbeta being studied. In addition, cdk2 is activated in MCF10A-Ras and MDA231 cells when compared to MCF10A cells, consistent with activated cdk2 phosphorylating

C/EBPbeta1 on Thr235 and promoting degradation of C/EBPbeta1 in the transformed cells lines. To confirm phosphorylation of C/EBPbeta1 Thr235 was responsible for degradation of C/EBPbeta1, a non-phosphorylatable C/EBPbeta1T235A mutant was constructed and expressed in MCF10A-Ras cells. This mutant is stabilized in the MCF10A-Ras cells when compared to wild type C/EBPbeta1, indicating that phosphorylation of this Thr235 residue in C/EBPbeta1 is responsible for degradation of C/EBPbeta1 in transformed mammary epithelial cells.

To expand these studies to a different cell system, the WI-38 normal human fibroblasts from Chapter 2 were utilized. It has been well-characterized that introduction of a small set of oncogenes into these cells allows for transformation of these cells (Hahn *et al.*, 1999). Introduction of hTERT and SV40 large and small T antigen immortalizes WI-38 cells, whereas introduction of hTERT, SV40T/t, and Ras(V12) transforms these cells (Hahn *et al.*, 1999). An advantage of this cell system is that we have the ability to examine normal cells that are capable of senescing (WI-38), immortalized cells (WI-38-hTERT-SV40T/t), and transformed cells (WI-38-hTERT-SV40T/t-Ras(V12), wheras in the MCF10A/MCF10A-Ras system we only have immortalized and transformed cells available. Utilization of the WI-38 cell system also allows for the examination of p52-C/EBPbeta1 expression in a different cell type, fibroblasts, transformed by activated Ras. Examination of p52-C/EBPbeta1 expression in this cell system demonstrates that p52-C/EBPbeta1 is expressed in the WI-38, WI-38-hTERT, and immortalized WI-38-hTERT-SV40T/t, but is not expressed in the transformed WI-38-hTERT-SV40T/t-Ras(V12) cells (Figure 33). This is consistent with the results in the MCF10A/MCF10A-Ras where p52-C/EBPbeta1 is expressed in the MCF10A cells but not the transformed MCF10A-Ras cells.

To further extend these studies in the WI-38 cell system, cdk2 activity was examined in the WI-38 cells since activated cdk2 leads to phosphorylation of C/EBPbeta1Thr235 and subsequent degradation of C/EBPbeta1 in transformed MCF10A-Ras cells. Figure 34 demonstrates that cdk2 is not activated in the WI-38 and WI-38-hTERT cells, but cdk2 is activated in the WI-38-hTERT-SV40T/t and WI-38-hTERT-SV40T/t-Ras(V12) cells. These results allow us to conclude that phopshorylation of C/EBPbeta1 by cdk2 is necessary but not sufficient for degradation of C/EBPbeta1. Other post-translational modifications of C/EBPbeta1 may be necessary for degradation of C/EBPbeta1. Post-translational modifications are tightly regulated, can affect other modifications through cross-talk and can alter the function of proteins. C/EBPbeta is modified by other post-translational modifications, such as acetylation or methylation, which may be required for or may prevent degradation of C/EBPbeta1 (Xu *et al.*, 2003, Cesena *et al.*, 2007, Kowenz-Leutz *et al.*, 2010). In addition, ubiquitination of proteins oftentimes requires a specific ubiquitin E3 ligase to assist in the conjugation of ubiquitin to the target protein. Expression or proper localization of a specific ubiquitin E3 ligase may be necessary for ubiquitination and subsequent degradation of C/EBPbeta1.

Sumoylation of C/EBPbeta1 in breast cancer cells

p52-C/EBPbeta1 is not expressed in breast cancer cell lines likely because C/EBPbeta1 is critical for OIS, a tumor suppressive mechanism (Chapter 2). We demonstrate in Chapter 3 that p52-C/EBPbeta1 is not expressed in transformed cells because it is degraded by the proteasome. However we show in Chapter 4 that breast cancer cells modify C/EBPbeta1 through sumoylation so that C/EBPbeta1 migrates at a higher molecular weight. The process of sumoylation is deregulated in cancer (Sarge and Park-Sarge, 2009). Conjugation of SUMO increases the apparent molecular weight

of the target protein and can alter protein function. Our lab frequently observes higher molecular weight bands on anti-C/EBPbeta immunoblots of breast cancer cells (Figure 35). Knowing that C/EBPbeta1 can be modified by sumoylation, we hypothesized that these higher molecular weight bands are sumoylated C/EBPbeta1 (Eaton and Sealy, 2003). Figure 36 demonstrates that exogenously expressed T7-C/EBPbeta1 is sumoylated in breast cancer cells. Moreover, the higher molecular weight bands observed in anti-C/EBPbeta immunoblots of breast cancer cells is sumoylated C/EBPbeta1 (Figure 37).

Phosphorylation oftentimes enhances sumoylation and phosphorylation cascades are frequently activated in cancer. The Ras pathway is commonly upregulated in breast cancers, and activation of this pathway activates various downstream kinases known to phosphorylate C/EBPbeta. Therefore we examined whether phosphorylation of C/EBPbeta1 enhanced sumoylation. Figure 38 demonstrates that phosphorylation of C/EBPbeta1 on Thr235 by Erk-2 enhances sumoylation of C/EBPbeta1, *in vitro*. Additionally, sumoylated C/EBPbeta1 is phosphorylated on Thr235 (Figure 39). To verify that phosphorylation of C/EBPbeta1Thr235 enhances sumoylation, C/EBPbeta1Thr235 was mutated to an alanine so that this residue could no longer be phosphorylated. Figure 39 shows that the non-phosphorylatable C/EBPbeta1T235A mutant is sumoylated to a lesser extent than wild type C/EBPbeta1, confirming that phosphorylation of C/EBPbeta1 on Thr235 enhances sumoylation of C/EBPbeta1.

Sumoylation of transcription factors frequently leads to transcriptional repression by these target proteins. Although the functional significance of sumoylated C/EBPbeta1 has not yet been examined, it would be beneficial for breast cancer cells to repress the transcriptional abilities of C/EBPbeta1. C/EBPbeta1 induces IL6 which promotes OIS, a tumor suppressive mechanism. In order to escape this barrier to tumorigenesis,

sumoylated (i.e. repressed) C/EBPbeta1 would not be able to activate IL6 and therefore the cells would avoid undergoing senescence. This repression in transcriptional ability is oftentimes due to the ability of sumoylated proteins to recruit and interact with transcriptional co-repressors. C/EBPbeta is already known to interact with two transcriptional co-repressors, HDAC1 (DePoi *et al.*, 2005, Xu *et al.* 2003) and Daxx (Wethkamp and Klempnauer, 2009). Therefore sumoylated C/EBPbeta1 may be interacting with these transcriptional co-repressors in breast cancer cells thus repressing C/EBPbeta1-mediated transcription of IL6. This would in turn lead to the evasion of senescence. Understanding the functional significance of sumoylated C/EBPbeta1 will give insight into why this modified form of C/EBPbeta1 is present in breast cancer cells.

Future Directions

The work in this thesis demonstrates that in normal cells C/EBPbeta1 is involved in OIS, a tumor suppressive mechanism, and that this transcription factor is negatively regulated in breast cancer cells either through proteasomal degradation or sumoylation. There are some remaining questions, especially regarding regulation of the degradation and sumoylation of C/EBPbeta1. Chapter 3 demonstrates that activation of cdk2 and subsequent phosphorylation of C/EBPbeta1 on Thr235 by activated cdk2 is necessary for the degradation of C/EBPbeta1 in cells transformed by Ras. In the WI-38 cells however, this activation of cdk2 and phosphorylation of C/EBPbeta1 on Thr235 is not sufficient for degradation of C/EBPbeta1. This is demonstrated in Figure 34 where WI-38-hTERT-SV40T/t still express C/EBPbeta1 even though cdk2 is activated. As discussed above, the addition or removal of other post-tranlational modifications of C/EBPbeta1 may be necessary for degradation of C/EBPbeta1 in the immortalized WI-38-hTERT-SV40T/t. Alternatively, expression or correct localization of a particular ubiquitin E3 ligase may be necessary for ubiquitination and degradation of C/EBPbeta1.

The WI-38 cell system will be a good system to address this question since the introduction of Ras(V12) into the WI-38-hTERT-SV40T/t cells leads to the degradation of C/EBPbeta1. First, it will be important to confirm that C/EBPbeta1 is undergoing proteasomal degradation. This can be done by treating the WI-38-hTERT-SV40T/t-Ras(V12) cells with a proteasomal inhibitor such as MG132, preparing cell lysates, subjecting these samples to SDS-PAGE and performing immunoblot analysis for C/EBPbeta1 expression. After proteasomal degradation of C/EBPbeta1 in the WI-38-hTERT-SV40T/t-Ras(V12) cells is verified, mass spectrometry analysis should be performed on the stabilized C/EBPbeta1 in these cells treated with MG132. C/EBPbeta1 will be immunoprecipitated out of WI-38-hTERT-SV40T/t cells and WI-38-hTERT-SV40T/t-Ras(V12) cells treated with MG132. Post-translational modifications of C/EBPbeta1 as well as binding partners will be identified via co-immunoprecipitation. Post-translation modifications or binding partners unique to the WI-38-hTERT-SV40T/t-Ras(V12) cells treated with MG132 will give insight into necessary modifications or E3 ligases/binding partners required for degradation of C/EBPbeta1 during transformation.

In Chapters 3 and 4 of this work, we show that phosphorylation of C/EBPbeta1 on Thr235 leads to either proteasomal degradation or enhances sumoylation, respectively. It will be important to establish what determines whether a cell degrades C/EBPbeta1 versus sumoylates C/EBPbeta1. In Chapter 3 we show that phosphorylation of C/EBPbeta1Thr235 by activated cdk2 leads to proteasomal degradation of C/EBPbeta1. In Chapter 4 we do not examine whether a particular kinase is responsible for phosphorylating C/EBPbeta1Thr235 leading to an enhancement of sumoylation in breast cancer cells. *In vitro* phosphorylation of C/EBPbeta1Thr235 by Erk-2 enhances sumoylation. However we do not examine whether Erk-2 is the kinase responsible for phosphorylating C/EBPbeta1Thr235 in

breast cancer cells. Phosphorylation of C/EBPbeta1Thr235 by a specific kinase may be necessary to enhance sumoylation. Various kinases have been shown to phosphorylate C/EBPbetaThr235 including Erk-2 (Hanlon *et al.*, 2001), cdk2 (Shuman *et al.*, 2004, Li *et al.*, 2007), p38 (Engelman *et al.*, 1998, Horie *et al.*, 2007) and GSK3beta (Park *et al.*, 2004). Additionally, phosphorylation of other sites of C/EBPbeta1 may enhance degradation and/or sumoylation of C/EBPbeta1. C/EBPbeta is phosphorylated on various other sites by other kinases including phosphorylation of Ser273 and Ser217 by RSK (Lee *et al.*, 2010, Buck *et al.*, 2001), Ser64 by cdk2 (Shuman *et al.*, 2004), and Ser184 and Thr179 by GSK3beta (Tang *et al.*, 2005). Investigation of phosphorylation of these other sites in C/EBPbeta1 may also play a role in the regulation of this protein. Utilization of mutant C/EBPbeta1 constructs that cannot be phosphorylated at these specific sites as well as using inhibitors that inhibit these particular kinases known to phosphorylate C/EBPbeta will be valuable in these studies.

Further investigation is also necessary to examine the functional role of sumoylated C/EBPbeta1. We show that C/EBPbeta1 is sumoylated in breast cancer cells and that this sumoylation is enhanced by phosphorylation, however we do not yet know the functional implications of this sumoylation. Studies are currently underway in the lab to explore the functional effect sumoylation has on C/EBPbeta1. C/EBPbeta1 induces IL6 which leads to OIS, a tumor suppressive mechanism. Sumoylation of transcription factors frequently leads to transcriptional repression. Taken together, it may be advantageous for breast cancer cells to sumoylate C/EBPbeta1 thus repressing the ability of C/EBPbeta1 to transcriptionally activate IL6. This would prevent cells from undergoing OIS. To examine the functional significance of sumoylated C/EBPbeta1, a C/EBPbeta1K173R non-sumoylatable mutant has been created as well as a C/EBPbeta1-SUMO-2 fusion construct. Currently these constructs are being introduced

into WI-38 cells. We are hypothesizing that the non-sumoylatable mutant will induce IL6 and senescence to a higher extent than wild type C/EBPbeta1 whereas the C/EBPbeta1-SUMO-2 fusion will not be able to induce IL6 and senescence to the extent of wild type. Results from these experiments, which are currently in progress, will indicate the functional effect sumoylation has on C/EBPbeta1.

As mentioned earlier, introduction of activated Ras into normal cells leads to senescence whereas introduction of activated Ras into many immortalized cells leads to transformation. This is probably due in large part to differences in signaling in these different cells. The differences in signaling, however, have not yet been elucidated. Ras-induced senescence and Ras transformation both require C/EBPbeta (Sebastian *et al.*, 2005, Zhu *et al.*, 2002). C/EBPbeta1 in expressed in normal cells and is the primary transactivator isoform responsible for OIS. C/EBPbeta2 on the other hand is expressed at high levels in breast cancer cells and promotes transforming characteristics in the MCF10A mammary epithelial cell line. Therefore one possible mechanism by which the introduction of activated Ras into cells leads to two different outcomes in different cells may be the particular isoform of C/EBPbeta that Ras is signaling through. Unfortunately studying the endogenous function of the different isoforms of C/EBPbeta is difficult because siRNA knockdown of any of the isoforms leads to knockdown of all of the isoforms. This is because all three isoforms of C/EBPbeta are translated from the same mRNA.

One way to study this is to utilize a system engineered by Uematsu *et al.*, 2007. This group generated a knock-in mouse in which the second in-frame methionine necessary for translation of C/EBPbeta2 was replaced with an alanine. This allowed for expression of C/EBPbeta1 and C/EBPbeta3, but not C/EBPbeta2. Manipulation of cells in this way to allow for expression of only one or two isoforms of C/EBPbeta will give

further insight into the functional roles of the endogenous isoforms of C/EBPbeta. This can then be used to study the specific isoforms of C/EBPbeta that Ras signals through to induce senescence versus transformation. For example, mice could be generated where the first in-frame methionine necessary to translate C/EBPbeta1 is replaced with an alanine, so that full-length C/EBPbeta1 could not be expressed but the other two isoforms would be expressed. Induction of IL6 and senescence could then be examined in cells from these mice in which activated Ras(V12) was introduced to determine whether C/EBPbeta1 is necessary for Ras-induced senescence.

In conclusion, this work demonstrates a functional role for full length C/EBPbeta1 in the induction of IL6 and senescence in normal cells. In addition, we show two different mechanisms by which p52-C/EBPbeta1 is regulated in transformed mammary epithelial cells in which the Ras pathway is activated. C/EBPbeta1 can be negatively regulated by Ras transformation through phosphorylation on Thr235 by cdk2 and subsequent proteasomal degradation. C/EBbeta-1 can also be phosphorylated on Thr235 in breast cancer cells which enhances sumoylation of this transcription factor. Therefore, degradation or sumoylation of C/EBPbeta1 by Ras activation may represent mechanisms to bypass OIS (Figure 45).

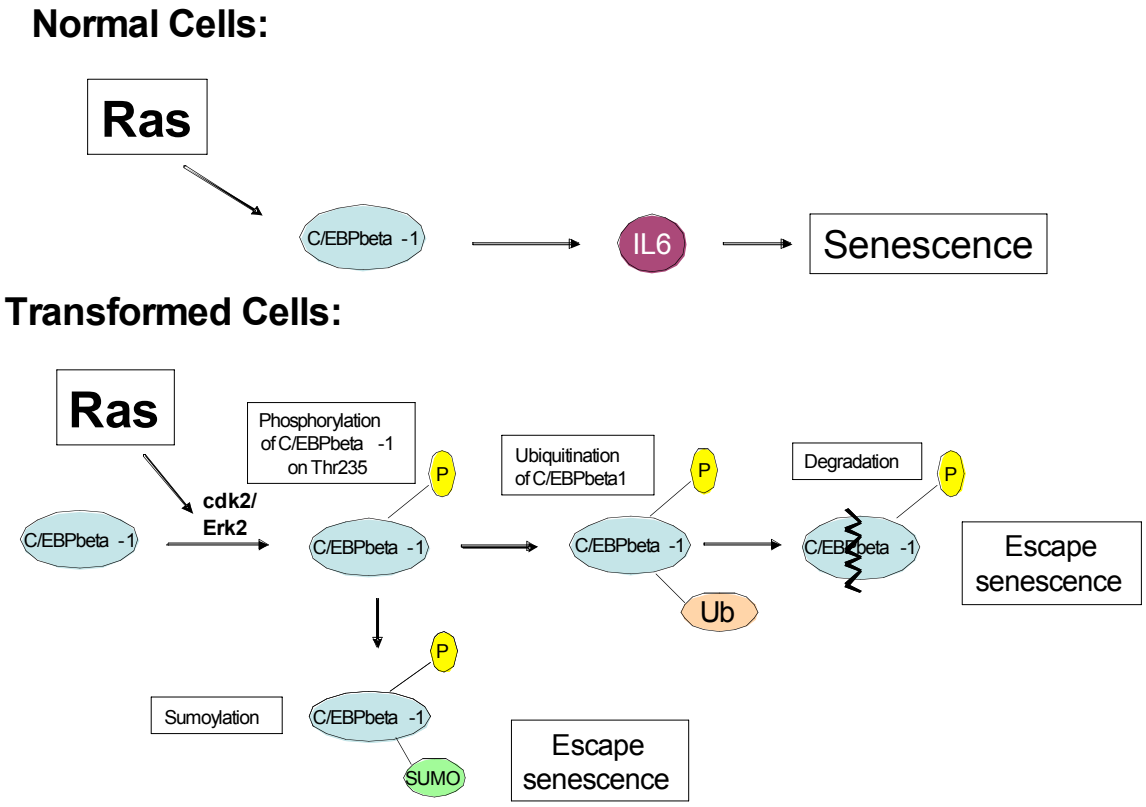

Figure 44. C/EBPbeta1 is involved in OIS in normal cells by inducing IL6, however this transcription factor is negatively regulated by Ras in transformed cells through phosphorylation and subsequent proteasomal degradation and/or sumoylation. Chapter 2 of this work demonstrates that C/EBPbeta1 is the primary C/EBPbeta transactivator isoform that induces IL6 and senescence in normal cells (top). Chapter 3 shows that introduction of activated Ras and transformation leads to phosphorylation of C/EBPbeta1 on Thr235 by cdk2, which promotes ubiquitination of C/EBPbeta1 and consequently degradation (middle). Chapter 4 demonstrates that C/EBPbeta1 is sumoylation in breast cancer cells and that this sumoylation is enhanced by phosphorylation of Thr235 (bottom). Regulation of C/EBPbeta1 via degradation and sumoylation may be mechanisms by which cells escape senescence.

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