# CIGARETTE SMOKE CONDENSATE-INDUCED TRANSCRIPTIONAL REGULATION OF BCL-XL IN SPONTANEOUSLY IMMORTALIZED HUMAN BREAST EPITHELIAL CELLS

By

SHAHNJAYLA KHRISHIDA CONNORS

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2008

To my parents, who never doubted that I could do it—for your continuous love and support, I dedicated this dissertation to you.

ACKNOWLEDGMENTS

I thank God for blessing me and surrounding me with all those who have made it possible for me to finish this dissertation. I thank my parents, extended family, church family, and friends for their prayers, spiritual, and emotional support. I thank Dr. Satya Narayan, my supervisory chair for the opportunity to complete my project in his laboratory, the members of my supervisory committee, and Dr. Aruna Jaiswal for all his technical assistance and support. I thank those whose work was the foundation for my project and those who provided special reagents and supplies. Lastly, I thank all those who were involved in me successfully completing this process including the Interdisciplinary Program in Biomedical Sciences (IDP), the Office of Graduate Minority Programs (OGMP), Department of Anatomy and Cell Biology, and all the faculty and staff, too numerous to name, who offered academic, administrative, and clerical support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

# CIGARETTE SMOKE CONDENSATE-INDUCED TRANSCRIPTIONAL REGULATION OF BCL-XL IN SPONTANEOUSLY IMMORTALIZED HUMAN BREAST EPITHELIAL CELLS

By

Shahnjayla Khrishida Connors

August 2008

Chair: Satya Narayan
Major: Medical Sciences-Molecular Cell Biology

Breast cancer is the second leading cause of cancer deaths in women. It is unclear whether there is a link between cigarette smoking and increased breast cancer risk. Cigarette smoke contains over 4,000 compounds, over 80 of which have been identified as carcinogens. There is evidence to support the fact that smokers metabolize mammary carcinogens and human studies show that tobacco constituents can reach breast tissue where they produce their harmful effects. In previous studies, it has been demonstrated that cigarette smoke condensate (CSC), which has a similar chemical composition as cigarette smoke, is capable of transforming the spontaneously immortalized human breast epithelial cell line, MCF10A, possibly through the upregulation of the anti-apoptotic gene, *bcl-xl*. Upregulation of this gene impedes the apoptotic pathway and allows the accumulation of DNA damage that can lead to cell transformation and carcinogenesis. In the present study, the mechanism of CSC-mediated transcriptional upregulation of *bcl-xl* gene expression in MCF10A cells has been determined. The human *bcl-xl* promoter (pBcl-xLP) was cloned and putative transcription factor binding sites were identified. Deletion constructs that removed the putative *cis*-elements were transfected into MCF10A cells to determine which element or elements were responsive to CSC treatment. The promoter

activity was significantly decreased in constructs lacking C/EBP-binding sites. Site-directed mutagenesis of C/EBP-binding sites on the pBcl-xLP attenuated the CSC-induced increase in promoter activity. Western blot, gel-shift, and super-shift analysis confirmed that C/EBPβ bound to a C/EBP-binding site on the pBcl-xLP. Additionally, overexpression of C/EBPβ isoforms, particularly, LAP2, stimulated pBcl-xLP activity and Bcl-xL protein levels in the absence of CSC treatment. Site-directed mutagenesis of the C/EBP sites on the pBcl-xLP also altered the promoter response to the C/EBPβ overexpression constructs. These results indicate that C/EBPβ-LAP2 regulates *bcl-xl* gene expression in response to CSC treatment. Understanding the mechanism of transcriptional regulation of *bcl-xl* can be used to identify chemotherapeutic targets for the prevention and treatment of breast carcinogenesis, especially that induced by cigarette smoke carcinogens.

# CHAPTER 1
# INTRODUCTION

## Breast Cancer

Breast cancer is the most common cancer and is second only to lung cancer as the leading cause of cancer death in women. The American Cancer Society estimates that in 2008, 67,770 new cases of carcinoma *in situ*, the noninvasive, earliest form of breast cancer, will be diagnosed. In addition, 182,460 new cases of invasive breast cancer will also be diagnosed in the United States (American Cancer Society, 2008). Although breast cancer is 100-times more common in females, 1,990 men will be diagnosed with the disease this year (American Cancer Society, 2008). In 2008, 40,480 women and 450 men will succumb to this disease (American Cancer Society, 2008). Breast cancer death rates have decreased since 1990. This decrease is believed to be the result of early detection, increased awareness, and improved treatment. While breast cancer survival rates have improved about 14% since the 1970, this progress has not impacted all populations equally. When controlled for age and stage at diagnosis, mortality rates vary among racial and ethnic groups (National Cancer Institute, 2007). While minorities have generally have lower incidence rates, they have higher mortality and develop more aggressive forms of breast cancer (American Cancer Society, 2008).

Ordered mammary epithelial architecture is critical to maintaining a differentiated state and control of cell proliferation (Bissell et al., 2003). Disruptions of this ordered architecture can lead to breast carcinogenesis. While the progression of colon cancer has been extensively described in a linear model (Fearon and Vogelstein, 1990; Polyak et al., 1996; Vogelstein et al., 1988), the progression of breast carcinogenesis is less understood. Breast cancer is considered a heterogeneous disease that develops along a continuum; the multi-step process begins at ductal or lobular atypical hyperplasia and progresses to invasive carcinoma and metastasis (Beckmann

et al., 1997; Russo and Russo, 2001). Accumulation of genetic errors in growth control and DNA repair genes occur at each step (Beckmann et al., 1997). Two classes of genes are affected during this progression: oncogenes and tumor suppressors. Oncogenes and tumor suppressor genes regulate epidermal growth factor receptors and genes involved in cell cycle progression, proliferation, and apoptosis. Oncogenes act to increase cell replication and decrease differentiation. The activation of oncogenes, such as *ras* and *c-myc*, by mutation, amplification, or rearrangements are associated with tumorigenesis. Alternatively, tumor suppressor genes such as *TP53* and retinoblastoma (*Rb*) are associated with cell cycle regulation, differentiation, and apoptosis. By definition, these genes act to prevent tumorigenesis. The loss of tumor suppressor function makes cells more susceptible to tumorigenesis and results from a mechanism known as the Knudson's "two-hit" hypothesis. In this model, the loss of function results from two occurrences: the first "hit" is a germline mutation in one copy of the gene and the second "hit", a somatic mutation or deletion in the second copy of the gene, results in the loss of gene function (Knudson, 1971).

## Cigarette Smoke Carcinogens

Epidemiological evidence has shown that not only cigarette smoke, but also unburned tobacco is carcinogenic to man (Hoffmann and Wynder, 1968). Cigarette smoke condensate (CSC), because of its similar composition, is used as a surrogate for cigarette smoke in experimental studies. Studies have been aimed at identifying and classifying the carcinogenic constituents in CSC (Table 1-1). Animal bioassays and advances in analytical chemistry techniques have brought the number of proven carcinogens in cigarette smoke to approximately 80 (Hecht, 2002; Hoffmann et al., 2001; Smith et al., 2003). The International Agency for Research on Cancer (IARC) and the Registry of Toxic effect of Chemical Substances (RTEC) have classified the components of cigarette smoke by potential carcinogenicity and bioactivity,

respectively. The IARC classifies mainstream cigarette smoke as a Group 1 (known human) carcinogen (International Agency for Research on Cancer, 1985). Other categories include: Group 2A, probably carcinogenic to humans, Group 2B, possibly carcinogenic to humans, and Group 3, not classifiable as to their carcinogenicity to humans (International Agency for Research on Cancer 1972-2000). Studies have reviewed the IARC carcinogen groups found in cigarette smoke from Group 1 (Smith et al., 1997), Group 2A (Smith et al., 2000), and Group 2B (Smith et al., 2000a; Smith et al., 2001). These compounds have also been ranked by potential toxicity using IARC and RTECS data. The purpose of this study was to use concentration, metabolism, bioactivity, and lipophilicity to develop "effective toxicities" as a means to compare compounds and to identify the most toxic for further study (Smith and Hansch, 2000). Effective toxicity was used to group the cigarette smoke components into six categories, I: rodent carcinogens and reproductive effectors, II: rodent carcinogens, III: reproductive effectors, IV: benign tumorigens, V: *in vitro* mutagens, and VI: compounds that have insufficient evidence of biological activity (Smith and Hansch, 2000).

Polycyclic aromatic hydrocarbons (PAHs) were the first pure compounds shown experimentally to be carcinogenic and are complete (Hoffmann and Wynder, 1971; Whitehead and Rothwell, 1969; Wynder and Wright, 1957). PAHs are ubiquitous environmental pollutants produced by the incomplete combustion of fossil fuels (Trombino et al., 2000) and during the burning of tobacco (Hoffmann and Wynder, 1968; Wynder, 1967). Benzo[α]pyrene (B[α]P), which was first isolated from coal tar in the 1930s, is one PAH found in cigarette smoke. B[α]P is a mammary carcinogen (el-Bayoumy et al., 1995) and was classified in the most bioactive category I by Smith and Hansch because it is a rodent carcinogen that causes reproductive effects (2000). The PAH, 7, 12-dimethylbenzanthracene (DMBA), is another well-known mammary

carcinogen present in cigarette smoke (Kumar et al., 1990). The strongest PAH carcinogen, dibenzo[alpha,l]pyrene (DB[α,l]P), is a very active mammary carcinogen that has a greater potency than DMBA (Cavalieri et al., 1991). Other carcinogenic compounds in cigarette smoke include *N*-nitrosamines such as tobacco-specific, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN). Both of these compounds are rodent carcinogens and classified in category II (Smith and Hansch, 2000). Aromatic amine and metals are also present in CSC (Hoffmann et al., 2001). Strong carcinogens such as PAHs, nitrosamines, and aromatic amines occur in smaller amounts (1-200 ng per cigarette). Weaker carcinogens, such as acetaldehyde, are present at larger concentrations (1 mg cigarette). The total amount of carcinogens in cigarette smoke is about 1-3 mg per cigarette, and is similar to the amount of nicotine (Hecht, 2003).

**Smoking and Breast Cancer Risk**

One of the most prevalent negative effects cigarette smoking has on human health is cancer (American Cancer Society, 2008). Currently, smoking accounts for approximately 30% of all cancer cases in developed countries (Doll, 1981; Peto et al., 1996; U.S. Department of Human and Health Services, 1989). Smoking causes about 90% of lung cancer cases worldwide. Therefore it the overwhelming cause of lung cancer, which is the leading cause of cancer death worldwide (International Agency for Research on Cancer, 2004). Tobacco is the most extreme example of a systemic carcinogen (DeMarini, 2004) and causes cancer in more organ sites than any other human carcinogen identified thus far. In addition to causing cancers of the lung, mouth, and esophagus, cigarette smoke has been linked to some leukemias and cancers of distant organs such as the pancreas, cervix, kidney, and stomach (U.S. Department of Human and Health Services, 2004). Smoking is also proposed to be an initiator of colorectal carcinogenesis

(Giovannucci et al., 1994a; Giovannucci and Martinez, 1996; Giovannucci et al., 1994b; Services, 1994).

**Epidemiological Studies**

Environmental carcinogens have long been suspected to contribute to human breast cancer. However, no specific agents have been fully implicated except radiation (John and Kelsey, 1993). One such environmental factor is cigarette smoking. Although lung cancer has been concretely linked to cigarette smoking, its relationship to other cancers such as those of the breast is more difficult to establish.

Epidemiological studies reflect conflicting associations between cigarette smoking and increased breast cancer risk. Most studies indicate that cigarette smoking has no effect on breast cancer risk (MacMahon, 1990; Palmer and Rosenberg, 1993). A large population based study found no increased risk even with heavy smokers and those who started to smoke at an early age (Baron et al., 1996). Other studies recorded that cigarette smoking has little or no independent affect on breast cancer risk (Hamajima et al., 2002) and there was no association found with active smoking (Lash and Aschengrau, 2002). Another study suggested that there is no increased risk of breast cancer in women who smoked during pregnancy (Fink and Lash, 2003).

Conversely, studies have also concluded that cigarette smoke is an etiologic factor for breast cancer (Bennett et al., 1999; Wells, 2000). Early exposure to cigarette smoke and increased years since smoking commencement was found to play a role in increased breast cancer risk (Egan et al., 2002; Johnson et al., 2000; Terry et al., 2002). Smoking prior to a first full-term pregnancy may also have a role in breast cancer development (Band et al., 2002; Johnson et al., 2000). In a large California Teachers Study Cohort breast cancer risk was associated with active cigarette smoking (Reynolds et al., 2004). Smoking has also been linked to increased breast cancer risk in women with mutations in carcinogen metabolizing genes.

Women with N-Acetyltransferase 2 (NAT2) slow acetylation phenotypes have increased risk for breast cancer (Ambrosone et al., 1996; Ambrosone et al., 2008). NAT2 is involved in the metabolism of aromatic amines, a major class of cigarette smoke carcinogens. Variants slow the clearance of aromatic amines. Other polymorphic metabolism genes include CYP1A1 and glutathione S-transferase M1 (GSTM1). Polymorphisms in these genes affect the amount of DNA adducts in women with breast cancer, especially in smokers (Firozi et al., 2002). In individuals that favor the metabolism of tobacco carcinogens (due to polymorphisms or mutations) smoking as a cause of breast cancer becomes more plausible (Hecht, 2002).

The link between passive cigarette smoking (second hand smoke) and breast cancer risk has also been considered. Passive smoking has been identified as a breast cancer risk factor in case-controlled studies (Johnson et al., 2000; Morabia et al., 1996). A prospective study from the Nurse's Health Study and others reported that passive smoking is unrelated to breast cancer (Egan et al., 2002; Lash and Aschengrau, 2002). A report from the US Surgeon General concluded that the evidence linking secondhand smoke and breast cancer is suggestive, but not sufficient to infer a causal relationship (U.S. Department of Health and Human Services, 2006). However, the American Society recommends that women should be aware of the possible link and limit their exposure to active as well as passive cigarette smoke (American Cancer Society, 2008).

Studies have probed the reason for conflicting epidemiological results. The effects of smoking on breast cancer risk may differ by menopausal status (Band et al., 2002; Johnson et al., 2000). Additionally, tobacco may also have anti-estrogenic effects that reduce breast cancer risk (Baron et al., 1990; Bremnes et al., 2007; Tanko and Christiansen, 2004). In some studies cigarette smoking was found to have an inverse relationship to breast cancer (Baron et al., 1990)

and to protect rats from mammary tumor formation (Davis et al., 1975). This opposing effect may explain why epidemiological studies reflect inconsistent results on the association between breast cancer risk and cigarette smoking (Bremnes et al., 2007). Additionally, study methods can be skewed by biases in control selection, chance variation, type of stratification, or small sample size (Baron et al., 1996). Other possibilities include the association with risk is too small to detect or that for some women there is increased risk, while others are afforded protection from cigarette smoking (Phillips and Garte, 2008). It is plausible that smoking can cause breast cancer in humans, but this relationship is difficult to establish because of low carcinogen doses (Hecht, 2002).

**Biological Studies**

Despite conflicting epidemiological results, biological studies support the hypothesis that cigarette smoke can play a role in breast carcinogenesis. The anatomy of the breast makes it a susceptible target for chemical carcinogens. Carcinogens in tobacco smoke can pass though alveolar membranes in the lung, enter the blood stream, and be transported to the breast tissue by plasma lipoproteins (Shu and Bymun, 1983), and can be readily stored and concentrated in the breast adipose tissue (Obana et al., 1981). Since many of these compounds are lipophilic in nature, their concentration in breast adipose tissue increases exposure to adjacent epithelial cells (Perera et al., 1995). Human mammary epithelial cells have a high capacity to metabolize carcinogens into DNA-binding substances and are therefore the ultimate targets for carcinogenesis (MacNicoll et al., 1980; Pruess-Schwartz et al., 1986; Stampfer et al., 1981).

Cigarette smoke components have been found in the breast milk (Catz and Giacoia, 1972; O'Brien, 1974) and the presence of smoking products in nipple aspirates resulted in positive Ames Salmonella mutagenesis tests (Ames et al., 1975). The concentration of compounds in breast ducts may provide a means by which cancer-initiating and promoting substances reach the

breast epithelium (Petrakis, 1977a, b). Additionally, evidence suggests smokers metabolize cigarette constituents in their breast tissue. Nicotine and its metabolite, cotinine, have been found in the breast secretions of non-lactating, women smokers (Petrakis et al., 1978). These studies support the hypothesis that mutagenic substances reach the breast epithelia and may have implications in the pathogenesis of benign breast disease and cancer (Petrakis et al., 1980).

**The Mechanism of CSC-induced Breast Carcinogenesis**

Tobacco is a significant human mutagen. DNA damage is the primary effect from exposure to cigarette smoke carcinogens. Studies indicate that CSC can induce DNA strand breaks (DSBs) in rodents, mammalian cells in culture, and DNA *in vitro* (DeMarini, 2004). CSC also causes DSBs in human cells *in vitro* (Luo et al., 2004; Nakayama et al., 1985). In animal models the potency of carcinogens is strongly correlated with the ability to form covalent adducts with DNA (Bartsch et al., 1983; Pelkonen et al., 1980). Therefore, DNA adducts, the covalent binding products of a carcinogen, its metabolite, or related substances to DNA, are central to the carcinogenic properties of tobacco products, including cigarette smoke (Hecht, 1999). Cigarette smoking has been associated with increased DNA damage in the lungs of smokers (Cuzick et al., 1990; Routledge et al., 1992) and studies suggest that similar damage may occur in the tobacco-induced neoplasms of other tissues (Cuzick et al., 1990). DNA adducts known to be associated with exposure to PAHs and tobacco smoke have been found in breast tissue. DNA adducts related to tobacco exposure were found in the breast tissue of women with breast cancer. All of the positive samples were from smokers as compared to no adducts found in nonsmoker tissue (Perera et al., 1995). Increased levels of aromatic DNA adducts were even found in the adjacent normal tissue of breast cancer patients (Li et al., 1996a). These and other studies indicate that exposure to environmental carcinogens, such as those found in cigarette smoke may be associated with the etiology of human breast cancer (Li et al., 1996a).

CSC also causes cytogenetic damage to cells, including chromosomal deletions, in rat cells and murine models *in vivo* (Dertinger et al., 2001; Rithidech et al., 1989). It also causes anaphase bridges in normal human fibroblast cells (Luo et al., 2004). Anaphase bridges are chromosomal segregation defects first described in maize (McClintock, 1942). These bridges probably originate from DNA DSB repair (Luo et al., 2004; Zhu et al., 2002) and are linked to chromosomal instability (CIN) in cancer cells (Gisselsson et al., 2000; Montgomery et al., 2003) and to tumorigenesis in mice (Artandi et al., 2000). Anaphase bridges break during anaphase, exposing telomerase-free ends that can fuse with other broken strands or sister chromatids resulting in fused chromosomes. These fused chromosomes can repeatedly undergo breakage-fusion-bridge cycles during subsequent mitoses (Gisselsson, 2003). Additionally, CSC transformed MCF10A-CSC3 cells (Narayan et al., 2004), in contrast to parental MCF10A cells, display polyploidy (Jaiswal, 2008).

Hecht offers a model linking cigarette-induced DNA damage to lung carcinogenesis that can also be applied to other tobacco-induced cancers including breast carcinogenesis (Hecht, 1999, 2003, 2006) (Figure 1-1). Nicotine addition causes continual cigarette smoking and chronic exposure to cigarette smoke carcinogens. Most of these carcinogens must be metabolically modified. Glutathione-S-transferases and UDP-glucuronosyl transferases convert carcinogen metabolites into less harmful forms (Armstrong, 1997; Burchell, 1997) and the detoxified components are excreted out of the body. Conversely, cytochrome P450 enzymes (P450s) convert the carcinogens to electrophilic compounds that can bind DNA and form adducts (Guengerich, 2001; Jalas et al., 2005). P450 enzymes, are part of mammalian system that responds to foreign matter in the body (Guengerich, 2001). P450s, CYP1A1 and CYP1B1, are inducible by the aryl hydrocarbon receptor which is important in the activation of PAHs

(Nebert et al., 2004). The balance between activation and detoxification enzymes varies among individuals and affects cancer susceptibility (Vineis et al., 2003). Cellular repair systems can remove DNA adducts and return the DNA structure to its original state (Goode et al., 2002). If the adducts are not repaired (overwhelming of repair system or polymorphisms in repair enzymes) and persist during DNA replication, miscoding and permanent mutations can occur in the DNA. DNA adducts lead to genotoxic damage including CIN, DNA strand breaks, chromosomal/gene mutations, and cytogenetic changes (DeMarini, 2004). Damaged cells may be removed by apoptosis and the balance between mechanisms leading to and opposing apoptosis has a significant effect on tumor formation (Bode and Dong, 2005). Mutations that cause loss of function in pro-apoptotic genes or the upregulation of anti-apoptotic genes allows DNA damage to persist and may result in abnormal gene expression. The chronic DNA damage from cigarette smoke exposure is consistent with the genetic changes that occur as normal tissues progress from hyperplasia to invasive cancer (Osada and Takahashi, 2002; Park et al., 1999; Wistuba et al., 2002). Mutations that occur in oncogenes or tumor suppressors can also contribute to the loss of normal cell growth control (Hecht, 1999) resulting in cell transformation and eventually tumorigenesis.

## Chemical Transformation of Human Breast Epithelial Cells

Chemicals contribute to carcinogenesis by inducing cellular transformation: the conversation of normal cells into cells with cancerous properties (Rudin, 1997). Transformation primarily results from carcinogen-induced DNA damage. The most significant characteristic of chemical transformation is increased proliferation. Proliferating cells can readily metabolize carcinogens and harbor the resulting genetic mutations into subsequent generations (Russo and Russo, 1980, 1987; Russo et al., 1982). Other characteristics of transformation are clonal growth (McCormick and Maher, 1989) and anchorage-independent growth, which is a relatively late

marker and can be correlated with tumorigenecity (DiPaolo, 1983; Shin et al., 1975). Neoplastic cells display invasiveness (Ochieng et al., 1991) and locomotion (Albini et al., 1987; Repesh, 1989) and malignant transformation is manifested by the ability to form tumors in mice (Change, 1966; DiPaolo, 1983; McCormick and Maher, 1989). These characteristics contribute to the six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, that are acquired by a cell as it becomes cancerous (Hanahan and Weinberg, 2000).

The transformation of human breast epithelial cells with cigarette smoke carcinogens has been observed repeatedly. Spontaneously immortalized human breast epithelial cells, MCF10F (Soule et al., 1990; Tait et al., 1990), displayed transformed characteristics after treatment with B[α]P and DMBA. The cells had increased proliferation, anchorage-independent growth, and altered patterns when grown in collagen matrix when compared to control cells, but were not tumorigenic *in vivo*. These cells also displayed greater chemoinvasive and chemotactic abilities when compared to control cells (Calaf and Russo, 1993). Being chemoinvasive and chemotactic are characteristics enhanced in transformed cells that correlate with malignant characteristics *in vivo* (Bonfil et al., 1989; Liotta, 1984; MacCarthy, 1988; Mensing et al., 1984; Ochieng et al., 1991; Zimmermann and Keller, 1987). MCF10A, the counterpart of MCF10F cells that grow attached *in vitro* (Soule et al., 1990; Tait et al., 1990), can be transformed with a single treatment of CSC. These cells displayed increased growth and anchorage-independent growth that were stable in re-established cell lines (Narayan et al., 2004).(Chen et al., 1997; Martin and Leder, 2001) NNK transformed MCF10A cells in a study that utilized low doses over a period of time to mimic long-term exposure to the carcinogen (Mei et al., 2003). The transformed cells exhibited increased anchorage-independent growth, cell motility, and tumorigenecity in nude

mice (Mei et al., 2003), meaning the cells had become malignant. These studies provide evidence that cigarette smoke components play a role in the multi-step oncogenesis of the breast.

## Apoptosis

Programmed cell death (PCD), also known as apoptosis, was first described in 1972 (Kerr et al., 1972). It is an evolutionary conserved process that regulates cell proliferation and turnover and maintains genomic integrity by selectively removing highly mutated cells from a population (Cherbonnel-Lasserre et al., 1996). In healthy cells, apoptosis is tightly regulated; too much cell death can lead to degenerative conditions, while too little can lead to autoimmune disorders and cancers (Thompson, 1995).

Apoptosis is a process of death in which the cell takes an active role in its own demise. Characteristics of apoptosis include cell shrinkage, chromatin condensation, and disintegration of the cell, before it is removed by phagocytosis (Kerr et al., 1972). Other forms of apoptosis include anoikis and amorphosis. The survival of epithelial cells requires continual attachment to the extracellular matrix (ECM) (Streuli and Gilmore, 1999). Anoikis occurs upon the detachment of epithelial cells from the extracellular matrix (Frisch and Francis, 1994). The maintenance of cellular morphology is also necessary for the survival of epithelial cells (Chen et al., 1997; Martin and Leder, 2001). Amorphosis is triggered by the alteration of cell shape (Martin and Vuori, 2004). Classical apoptosis can occur through two major pathways.

### Intrinsic Pathway

The intrinsic pathway eliminates cells in response to ionizing radiation, chemotherapy, mitochondrial damage, and certain developmental cues (Kuribayashi et al., 2006). The mitochondrion is the central response unit to this pathway. Mitochondrial swelling and outer mitochondrial membrane rupture results from a wide variety of apoptotic stimuli (Vander Heiden et al., 1997). DNA damage or cell stress causes stabilization of p53 and subsequent activation of

Bcl-2 pro-apoptotic proteins such as Bax and Bak that induce the mitochondrial release of cytochrome c. Bax mediates cell death (Chittenden et al., 1995) by homodimerizing to itself (Zha et al., 1996) and promoting the release of cytochrome c from the mitochondria (Reed, 1997). In the presence of liberated cytochrome c and ATP, the adaptor protein, Apaf-1, recruits pro-caspase-9. It is believed that the presence of cytochrome c changes the conformation of the Apaf-1 negative regulatory domain of WD40 repeats, and allows for its association with pro-caspase-9 (Li et al., 1997). Apaf-1, cytochrome c, and procaspase-9 form the apoptosome complex that activates procaspase-9 (Li et al., 1997; O'Connor and Strasser, 1999). Activated caspase-9 cleaves and activates downstream effector caspases such as caspase-3, -7, which execute apoptosis (Li et al., 1997). Smac/DIABLO is also released from the mitochondria. These compounds inhibit inhibitor of apoptosis proteins (IAPs) and further promoting the activation of caspases (Du et al., 2000; Verhagen et al., 2000).

**Extrinsic Pathway**

The extrinsic pathway eliminates unwanted cells during development, immune system maturation, and during the immunosurveillance removal of tumor cells (Kuribayashi et al., 2006). This pathway bypasses the steps that are regulated by Bcl-2 family members. It is triggered by receptors of the tumor necrosis factor (TNF) receptor type I family, TRAIL receptors, or Fas (CD-95/APO-1) receptors and their ligands. The Fas-induced death pathway is the major pathway that occurs in the lymphoid system (Newton et al., 1998; Strasser et al., 1995) and has become the paradigm for the extrinsic pathway (Kuribayashi et al., 2006). Ligand binding results in receptor trimerization and formation of the death-inducing signaling complex (DISC). The adaptor molecule, Fas-associated protein with death domain (FADD), is then recruited to the receptor's cytosolic tail by its death domain (Chinnaiyan et al., 1995; Green and Kroemer, 2004). Procaspase-8 or -10 are recruited to FADD by an interaction of the N-terminal

death effector domain (DED) of both proteins (Chittenden et al., 1995). The DISC allows for the auto-activation and maturation of caspase-8, -10 (Boatright et al., 2003; Donepudi et al., 2003). The activation of these caspases initiates the death signaling cascade by cleaving and activating the downstream effector caspase-3, -7. The intrinsic and extrinsic apoptotic pathways are interconnected. Activated caspase-8 cleaves the BH3-only protein, tBID, which in turn facilitates the release of cytochrome c from the mitochondria (Li et al., 1998).

**Apoptosis and Cancer**

Studies support the hypothesis that apoptosis selectively removes the most damaged cells from the population (Cherbonnel-Lasserre et al., 1996). Apoptosis is a critical defense against radiation-induced mutations, malignant transformation, and neoplastic progression. Damaged cells that escape this pathway are more likely to have increased levels of mutations due to heavily damaged DNA. DNA damage-induced mutations that occur can contribute to a proliferative advantage that might drive the cell towards malignancy (Cherbonnel-Lasserre et al., 1996). From this and other studies, the concept emerged that an increased threshold for apoptosis represents a central step in tumorigenesis. The surviving damaged cells are the most likely to develop into neoplastic clones (Adams and Cory, 1998; Cherbonnel-Lasserre et al., 1996). Anti- and pro-apoptotic proteins therefore play opposing roles in the prevention or progression of tumorigenesis, respectively. Since many chemotherapeutic drugs kill cancer cells by triggering apoptosis, the modulation of cell apoptosis threshold is of critical therapeutic potential (Chinnaiyan, 1999).

## The B cell leukemia-2 (Bcl-2) Protein Family

The B cell leukemia-2 (Bcl-2) protein family is involved in the regulation of apoptosis. The founding member, Bcl-2, was identified as a translocation found in human follicular lymphoma cells (Tsujimoto et al., 1984) and has anti-apoptotic activity (Vaux et al., 1988). At

least twenty other Bcl-2 members have been identified in mammalian cells (Adams and Cory, 1998; Cory et al., 2003; Gross et al., 1999). All members contain at least one of the four Bcl-2 homology (BH) domains which influence the dimerization required for the function of some members (Kelekar and Thompson, 1998; Yin et al., 1994). The anti-apoptotic members: Bcl-2 (Tsujimoto et al., 1984), Bcl-xL (Boise et al., 1993), Bcl-w (Gibson et al., 1996), Mcl-1 (Kozopas et al., 1993), and A1 (Lin et al., 1996) contain all four BH domains. Anti-apoptotic proteins function by directly or indirectly binding and inhibiting the activity of pro-apoptotic proteins that activate effector caspases (Cory and Adams, 2002; Opferman and Korsmeyer, 2003). Pro-apoptotic members fall into two categories. Bax is the founding member of the first category (Hsu et al., 1997; Hsu and Youle, 1998). Bax and the remaining proteins in this group, Bak and Bok, have domains BH1, BH2, and BH3 and directly induce the release of cytochrome c from the mitochondria. BH3 only proteins (Bad, Bim, Bid), as the name implies, possess only the the BH3 domain (Chittenden et al., 1995; Kelekar and Thompson, 1998). These proteins bind anti-apoptotic proteins and prevent them from sequestering the first group of pro-apoptotic proteins (Letai et al., 2002). BH3-only proteins function upstream of, and are dependent on Bax and Bak and can not kill cells that lack the two proteins (Zong et al., 2001). The dimerization of Bcl-2 proteins can titrate each others functions, suggesting that relative concentrations and ratios of Bcl-2 family proteins act as a rheostat controlling the apoptosis program and cell survival (Farrow and Brown, 1996; Lohmann et al., 2000; Oltvai et al., 1993).

### ***Bcl-x*** **Gene and Promoter Structure**

The human *bcl-x* gene was identified by the cross-hybridization of gene libraries with a *bcl-2* probe (Boise et al., 1993). The gene structure is similar to that of *bcl-2* (Seto et al., 1988). *Bcl-x* is composed of three exons (Figure 1-2A); the first exon is untranslated, while exons II and III code for *bcl-x* mRNAs. Exon II contains translation initiation codons, while exon III contains

the translation termination codons. The exons are separated by a 283 bp intron between exons I and II and a large 9 kb intron between exons II and III. The 5' untranslated region (UTR) spans from exon I to the beginning of exon II (Grillot et al., 1997).

The initial promoter studies occurred in mice. Two *bcl-x* murine promoters were cloned and described (Grillot et al., 1997). The first promoter was 57 bp upstream of the second exon and most active in FL5.12 and K542 cell lines by primer extension. A major transcriptional initiation site was mapped to this region. This promoter lacked a TATA box and instead contained a consensus initiator (Inr) element (YYANT/AYY) at -149 to -142 (Grillot et al., 1997). Inr sites are involved in transcription initiation at TATA-less promoters and the transcription start site usually overlaps the Inr consensus sequence (Smale and Baltimore, 1989). However, the Inr here is probably not involved in transcription initiation because the major start site mapped outside the Inr sequence (Grillot et al., 1997). The second promoter was further 5', upstream of exon I. This promoter was utilized mostly in the brain and thymus. This GC-rich region had Sp protein binding motifs and two major transcription start sites: in the brain the position was -727 and in the thymus the site was mapped to -655 before the initiation codon in exon II (Grillot et al., 1997). Later, studies indicated that the mouse *bcl-x* promoter was active in many tissues and three additional tissue specific murine *bcl-x* promoters were been identified (Pecci et al., 2001).

Human and murine *bcl-x* open reading frames have 93% nucleotide identity (Gonzalez-Garcia et al., 1994). *Bcl-x* mRNAs are transcribed from the human *bcl-x* gene as the result of alternative mRNA splicing, each coding for a single protein isoform (Figure 1-2B). Three *bcl-x* mRNAs and proteins have been reported in humans: Bcl-xLong (Bcl-xL) (Boise et al., 1993), Bcl-xShort (Bcl-xS) (Boise et al., 1993), and Bcl-xBeta (Bcl-xβ) (Ban et al., 1998). *Bcl-xl*

results from the splicing together of the two coding exons (II and III). *Bcl-xs* results from the use of an alternative 5' splice site in exon II and lacks the 3' terminal 63 amino acids that comprise BH1 and BH2 which are needed to inhibit apoptosis. It codes for a 178 amino acid protein (Boise et al., 1993) that is approximately 18 kDa (Gonzalez-Garcia et al., 1994) and functions as a pro-apoptotic protein by antagonizing Bcl-2 and Bcl-xL to promote apoptosis. Bcl-xS is expressed in cells with high turnover rates (Boise et al., 1993). *Bcl-xβ* results from the unspliced *bcl-x* transcript of Exon II that introduces a new stop codon before the third exon. Therefore, it lacks the carboxy-terminal hydrophobic 19 amino acid domain and has a unique stretch of 21 amino acids at the carboxy terminus (Gonzalez-Garcia et al., 1995). *In vitro* studies show that Bcl-xβ interacts with the pro-apoptotic protein Bax (Ban et al., 1998). Whether the protein has anti- or pro-apoptotic effects remains unclear.

**Bcl-xL Protein**

*Bcl-xl* is the major, most abundant *bcl-x* mRNA and protein expressed in murine and human tissues (Boise et al., 1993; Gonzalez-Garcia et al., 1995; Gonzalez-Garcia et al., 1994; Rouayrenc et al., 1995). The human *bcl-xl* promoter is upstream (5') to exon I and codes for the majority of *bcl-x* transcripts in humans. *Bcl-xl* mRNA originates from the 5' untranslated region (UTR) of the promoter (Grillot et al., 1997; Sevilla et al., 1999). A novel *bcl-x* promoter and exon located upstream of exon I has been identified in human lymphoma cells (MacCarthy-Morrogh et al., 2000).

Bcl-xL is a 241 amino acid protein (Boise et al., 1993) of 29-30 kDa (Yin et al., 1994). The structure of human Bcl-xL has been crystallized and characterized (Muchmore et al., 1996). The protein is composed of a total of seven α-helices. The two central anti-parallel hydrophobic helices, α5 and α6, are flanked by helices α3 and α4 on one side and α1, α2, and α7 on the other side. The α-helices 5 and 6 form a hairpin that shares homology with the hairpin structure found

in the translocation domain of diphtheria toxin (Muchmore et al., 1996) and the carboxy-terminal end contains a hydrophobic segment (Huang et al., 1998; Yin et al., 1994). The α5-α6 hairpin and the carboxy-terminal end of Bcl-xL are involved in anchoring the protein to mitochondrial membranes.

A large non-conserved flexible loop connects α1 and α2 (Muchmore et al., 1996) and has been shown to negatively regulate the activity of the protein (Chang et al., 1997). This loop domain comprises about one quarter of the protein and contains all the phosphorylation sites of Bcl-xL between amino acids 32 and 83 (Chang et al., 1997). Similar to Bcl-2, the phosphorylation of Bcl-xL decreases its anti-apoptotic function (Biswas et al., 2001; Poruchynsky et al., 1998). Bcl-xL lacking the flexible loop renders the protein unable to be phosphorylated thus causing the protein to block apoptosis more efficiently than wild-type Bcl-xL (Muchmore et al., 1996). Bcl-xL is also deaminated on this the flexible loop. Deamination is a modification in which an asparagine is converted into an aspartate (Takehara and Takahashi, 2003). Bcl-xL is deaminated at two asparagines in response to anti-neoplastic agents. Deamination negatively modulates the pro-survival activity of Bcl-xL and the inhibition of this modification increases the cells' resistance to these agents (Deverman et al., 2002). Proteins with such long regions of random coil do not normally have long half-lives because the region is vulnerable to cellular proteases (Ciechanover, 1994). It is likely that the loop region of Bcl-xL and other similar proteins are protected by associations with other proteins. A similar loop has been found on Bcl-2 (Chang et al., 1997). Bcl-xL binds to itself with the weakest affinity, indicating that is monomeric in nature (Muchmore et al., 1996) and is localized to the nuclear envelope, extra-nuclear membranes, the mitochondria, and is also present in the cytosol (Gonzalez-Garcia et al., 1994; Hsu et al., 1997).

**Bcl-xL functions as an anti-apoptotic protein**

Bcl-xL is an anti-apoptotic member of the Bcl-2 protein family and is most closely related to Bcl-2 (Boise et al., 1993; Grillot et al., 1997). The two proteins display 43% amino acid identity (Muchmore et al., 1996; Petros et al., 2001). Bcl-xL and Bcl-2 are in the group of oncogenes that function as repressors of apoptosis and do not affect proliferation rates (Korsmeyer, 1992; Miyashita et al., 1994). The role of Bcl-xL in apoptosis is evident; disruption of *bcl-x* gene leads to death in E12-E13 mouse embryos due to massive apoptosis of neuronal and hematopoietic progenitors (Motoyama et al., 1995). It is therefore essential for neurogenesis (Gonzalez-Garcia et al., 1994; Motoyama et al., 1995) and is a key protein during cytokine-regulated myelopoiesis (Packham et al., 1998). Bcl-xL inhibits staurosporine-induced cell death, caspase-3 and caspase-7 activation, and PARP cleavage (Chinnaiyan and Dixit, 1996) and is capable of suppressing apoptosis of IL-3 dependent cells upon growth factor withdrawal (Boise et al., 1993; Gonzalez-Garcia et al., 1994). It also inhibits anoikis in breast cancer cells (Fernandez et al., 2002).

**Dimerization with pro-apoptotic proteins**. Bcl-xL prevents the intrinsic apoptosis pathway (Chinnaiyan and Dixit, 1996; Gonzalez-Garcia et al., 1994) by localizing to mitochondrial membranes, inhibiting the release of cytochrome c, and preventing the downstream activation of apoptotic signal transduction cascades (Boise et al., 1993; Fang et al., 1994; Gonzalez-Garcia et al., 1994). Bcl-xL does so by heterodimerizing with pro-apoptotic proteins (Boise et al., 1993; Oltvai et al., 1993; Yin et al., 1994). Bax monomers must oligomerize to permeate membranes and lead to apoptosis (Annis et al., 2005). The overexpression of Bcl-xL prevents the oligermization of Bax (Finucane et al., 1999; He et al., 2003). Bcl-xL can also bind to and inhibit the BH3-only protein Bad. Normally Bad is phosphorylated and sequestered by the scaffold protein, 14-3-3. Apoptotic signaling results in

the dephosphorylation of Bad, that then binds to Bcl-xL and counteracts its pro-survival activity (Zha et al., 1996). The overexpression of Bcl-xL can sequester Bad to the mitochondria (Cheng et al., 2001; Jeong et al., 2004), leaving excess Bcl-xL to continue its pro-survival functions. The BH1 and BH2 domains have been shown to be important for Bcl-xL to antagonize Bax (Minn et al., 1999; Yin et al., 1994). Later studies determined that BH1-BH4 and the carboxy-terminal domains are required for the sequestering of Bax. Alternatively, BH1, BH3, and the carboxy-terminal tail are necessary for Bcl-xL to sequester Bad to the mitochondria (Zhou et al., 2005).

**Mitochondrial stability**. Studies indicate that Bcl-xL acts in dimerization-independent mechanisms to inhibit apoptosis (Chang et al., 1997; Cheng et al., 1996; Fiebig et al., 2006) in the absence of Bax and Bak. (Chang et al., 1997). Bcl-xL physically inhibits the release of mitochondrial contents such as cytochrome c. It can prevent apoptosis by maintaining mitochondrial membrane potential and volume homeostasis (Boise and Thompson, 1997; Vander Heiden et al., 1997). The loss of $F_1F_0$-ATPase activity, that occurs through the permeability transition pore complex (Zoratti and Szabo, 1995), terminates mitochondrial respiration and triggers the release of cytochrome c (Cai et al., 1998). The three-dimensional structure of the Bcl-xL pore-forming domain (Muchmore et al., 1996) has been implicated in the regulation of membrane permeability (Cramer et al., 1995; London, 1992). Bcl-xL has been shown to function like bacterial toxins that have similar pore domains. It can insert into synthetic lipid vesicles or planar lipid bilayers and form ion-conducting channels (Minn et al., 1997). It is possible that the channels formed by Bcl-xL serve to prevent the release of proteins, such as cytochrome c. Pores formed by Bcl-xL may also serve to stabilize mitochondrial volume potential. The ability of Bcl-xL to prevent apoptosis, however, is probably not solely dependent

on it pore-forming capability (Minn et al., 1997). Bcl-2 and Bax can also form ion channels in synthetic membranes (Antonsson et al., 1997; Schendel et al., 1997).

**Interactions with the apoptosome**. Bcl-xL has been found to form a ternary complex with the apoptotic effector caspase, pro-caspase-9, and Apaf-1. The first indication of this characteristic was discovered when the nematode, *Caenorhabditis elegans* protein, CED-9, and its mammalian homologue, Bcl-xL, bound to and inhibited the function of CED-4, the mammalian counterpart to Apaf-1. This interaction suggested that Bcl-xL may block cell death by a similar mechanism in mammalian cells (Chinnaiyan and Dixit, 1997). Subsequent studies found that in 293 human embryonic kidney cells, caspase-9 and Bcl-xL bound distinct regions of Apaf-1 and formed a ternary complex (Pan et al., 1998). This interaction inhibited the activation of caspase-9 in human embryonic kidney cells with SV40 large T antigen, 293T (Hu et al., 1998). These studies offer an alternative model to the anti-apoptotic mechanism of Bcl-xL in which the protein directly prevents the release of cytochrome c, and also inhibits the activation of pro-caspase-9 through direct interaction with Apaf-1. The mechanism by which Bcl-xL inhibits apoptosis while bound to Apaf-1 may be cell-type dependent. In prostate epithelial cells, Bcl-xL interacts with Apaf-1 but it inhibits apoptosis by preventing the release of cytochrome c from the mitochondria (Chipuk et al., 2001). Caspase-9 and -3 are still activated because the addition of cytochrome c results in their cleavage. It is possible that the interaction between Bcl-xL and Apaf-1 may also depend on experimental conditions or an unidentified protein (Chipuk et al., 2001).

**Bcl-xL and the extrinsic apoptotic pathway**. Bcl-xL also inhibits extrinsic apoptotic pathways. Tumor necrosis factor (TNF)-induced apoptosis was inhibited in the human myeloid leukemia cell line , HL-60, by overexpressing Bcl-xL which was thought to block an early step

in TNF signaling. Bcl-xL may have blocked TNF-induced apoptosis in these cells by reducing the expression of a downstream target of TNF, AP-1 and JNK and MAPK kinases, which regulate AP-1 (Manna et al., 2000). Bcl-xL also inhibited TNF-induced apoptosis in MCF-7 breast carcinoma cells (Jaattela et al., 1995; Srinivasan et al., 1998).

Bcl-xL inhibits Fas-induced cell death by several mechanisms. It protected primary B cells from Fas-mediated apoptosis (Schneider et al., 1997). Bcl-xL inhibited Fas-induced apoptosis in Bcl-xL-tranfected Jurkat cells treated with Fas antibodies not by blocking caspase activation, but by inhibiting the subsequent loss of $\Delta\Psi_m$ (Boise and Thompson, 1997). In Jurkat T lymphocytes and breast carcinoma cells, Bcl-xL inhibited apoptosis induced by microtubule damaging drugs such as paclitaxel and vincristine. In these cells, overexpression of Bcl-xL inhibited the Fas pathway by binding calcineurin and interfering with the nuclear translocation of NFAT proteins that transcriptionally activate the Fas ligand (Biswas et al., 2001).

**Mechanism by which Bcl-xL inhibits caspase-8-dependent apoptosis**. It was discovered in early studies that Bcl-xL and Bcl-2 could block caspase-8 activation (Chinnaiyan and Dixit, 1996). It was hypothesized that Bcl-xL might act upstream of the CED-3 homologue, caspase-8 (Chinnaiyan and Dixit, 1997), by inhibiting its interaction with the DISC or that the protein acted downstream of caspase-8, by preventing its action on target proteins. In peripheral human T cells resistance to CD95-induced apoptosis is characterized by lack of caspase-8 recruitment to the DISC and increased Bcl-xL levels (Peter et al., 1997). However, most studies support the latter theory. The overexpression of Bcl-xL did not affect caspase-8 activation in MCF-7 cells expressing high levels of CD95 (MCF-7-Fas), but the cells still become resistant to CD-95-induced apoptosis (Jaattela et al., 1995). In MCF-7-Fas cells there were no associations found between Bcl-xL and pro-caspase-8 or active caspase-8 subunits, however, PARP cleavage

was completely blocked. Therefore, Bcl-xL seemed to inhibit Fas-induced apoptosis downstream of caspase-8, but upstream of PARP cleavage. Bcl-xL probably inhibited the activity of another caspase-3-like protein that cleaved PARP in these cells but the mechanism remained unclear (Medema et al., 1998). In the next issue of the same publication, it was reported that Bcl-xL inhibited not only Fas-induced apoptosis, but also TNF receptor induced apoptosis in MCF-7 cells transfected with Fas and Bcl-xL cDNAs (MCF-7/FB) (Srinivasan et al., 1998). Bcl-xL was capable of inhibiting apoptosis, despite the full activation of caspase-8. This inhibition manifested as changes in cytochrome c localization and cell morphology. Bcl-xL could even inhibit apoptosis when the cell was microinjected with active caspase-8. However, the activity of caspase-7, a downstream target of caspase-8, was attenuated after treatment with Fas antibody or TNF, and was totally blocked in cells treated with UV. The inhibition of apoptosis by Bcl-xL was therefore, also found to occur downstream of caspase-8 but upstream of one of the caspase-8 targets. Bcl-xL may have inhibited caspase-8 activity by translocating the protein from the plasma membrane, sequestering caspase-8 targets, or by regulating the availability of cofactors necessary for caspase-8 to cleave its targets (Srinivasan et al., 1998). The property of Bcl-xL to inhibit apoptosis downstream of initiator caspases but upstream of their targets (possibly effector caspases) have been previously observed (Boise and Thompson, 1997; Medema et al., 1998), however the mechanism by which Bcl-xL inhibits extrinsic pathway of apoptosis seems to be cell-type dependent.

**Bcl-xL and breast cancer**

*Bcl-x* proteins are involved in normal mammary involution and development. In humans, the expression of Bcl-2 and related proteins such as Bcl-xL is not well studied in the mammary gland. Studies focused on the expression of Bcl-2 family members in rodents have been extrapolated for the analysis of human samples. *Bcl-x* isoform expression changes in alveolar

cells during involution, a period of mammary cell apoptosis and remodeling, compared to lactation (Heermeier et al., 1996). *Bcl-xl* and *bcl-xs* expression were analyzed with RT-PCR and differential hybridization. In virgin mice, during lactation, and pregnancy, *bcl-xl* mRNA was ten-fold higher than *bcl-xs* expression. During involution, *bcl-xs* levels increased up to six-fold compared to *bcl-xl* levels and *bax* is also upregulated during this time (Heermeier et al., 1996; Li et al., 1996b, c). Transfection experiments showed that cells expressing Bcl-xL had higher cell viability (27% died) after DNA damage. Co-expression of Bcl-xS and Bcl-xL proteins resulted in the inhibition of the Bcl-xL protective effect (80% cells died). This study further supports the theory that the ratio of pro-apoptotic and anti-apoptotic species in a single cell can determine cell fate.

Bcl-xL is up-regulated in some cancers (Packham et al., 1998) and is implicated in having a role in colorectal carcinogenesis (Krajewska et al., 1996; Maurer et al., 1998). In many cases, Bcl-xL expression occurs at the adenoma to carcinoma transition, continuing through metastasis (Krajewska et al., 1996; Liu and Stein, 1997). Reduction of apoptosis is associated with the development to fibrocystic changes in the breast and increased cancer risk (Allan et al., 1992). However, Bcl-xL has not been fully implicated in human breast tumorigenesis. The effects Bcl-xL has on breast carcinogenesis primarily hinge on the protein's ability to prevent apoptosis and promote survival. The role of Bcl-xL in breast carcinogenesis is evident from biological studies. Bcl-xL has roles in breast carcinogenesis on the levels of primary tumor growth, metastasis, and chemotherapy resistance.

**Bcl-xL and primary tumor growth.** Bcl-xL is overexpressed in some primary human breast carcinomas and the breast cancer cell line, T47D (Olopade et al., 1997; Schott et al., 1995) and is a marker for increased tumor grade and nodal metastasis (Olopade et al., 1997). It is also

increased cancerous, but not normal breast epithelium and may serve as an indicator or patient prognosis (Krajewski et al., 1999). Although Bcl-xL overexpression in mouse tumors, it does not increase the number of mitotic figures (Liu et al., 1999) is therefore does not affect cell proliferation or cell cycle progression.

**Bcl-xL and metastasis.** Bcl-xL has a more important role in metastasis than in primary tumor development. The overexpression of Bcl-xL does not induce primary tumor formation but enhances MEK-induced tumorigenesis in the mammary gland environment (Martin et al., 2004). Metastasis is the primary cause of treatment failure in cancer patients (Chambers et al., 2001). Overexpression of Bcl-xL in MDA-MB-435 breast cancer cells increased cell metastatic activity. Resistance to cytokine-induced apoptosis, increased cell survival in circulation, and increased anchorage-independent growth were all characteristics of these cells (Fernandez et al., 2002). MDA-MB-435 cells transfected with Bcl-xL metastasized to the lung, lymph nodes, and bone when inoculated into the mammary fat pads of nude mice (Rubio et al., 2001). Bcl-xL increased tumor cell survival in the bloodstream (Fernandez et al., 2000) and the metastatic properties of breast cancer cells that had already lost extracellular matrix dependence by improving cell survival under conditions with no cellular adhesion, enhancing anchorage-independent growth. Surprisingly, Bcl-xL did not increase metastatic activity in cells that had not escaped the extracellular matrix. MDA-MB-435 cells transfected with the *bcl-xl* gene and inoculated into nude/SCID mice resulted in increased lymph node metastasis (Fernandez et al., 2002).

The mechanisms by which Bcl-xL increases metastasis have been investigated. It has been suggested that the key event in breast cancer metastatic progression is the deregulation of cell death (Fernandez et al., 2002). Therefore, apoptosis resistance has a role in metastasis (Fernandez et al., 2000; McConkey et al., 1996). Additionally, Bcl-xL overexpression could

functionally associate with genes that control the events that result in the acquisition of metastatic phenotypes and shorten the dormancy of metastatic cells in several organs (Mendez et al., 2006). Tumor dormancy is the prolonged quiescent period in which the metastatic progression is not clinically detected (Yefenof et al., 1993). Studies suggested that Bcl-xL shortens the dormancy of metastatic cells. Experiments with mice injected with cells overexpressing Bcl-xL indicated that Bcl-xL has role in dormancy by promoting the survival of cells in metastatic foci (Rubio et al., 2001). Pro-survival proteins, such as Bcl-xL, displace the offset the balance between death and proliferation, shortening the period between dissemination and the appearance of clinical metastasis (Karrison et al., 1999). Bcl-xL does not appear to affect the actual movement of metastatic cells to foci because, breast cancer cells overexpressing Bcl-xL reach target organs in similar numbers as the vector controls. Since the Bcl-xL tumors developed more metastases than control cells, Bcl-xL may promote the survival of and harbor metastatic cells at metastatic foci (Rubio et al., 2001) allowing the metastatic cells to adapt to changes in their cellular environment (Fernandez et al., 2002; Li et al., 2002).

The loss of apoptosis is also instrumental in accumulating genomic damage. The extended lifespan of cells overexpressing Bcl-xL allows for more genetic mutations. This is evident in that the loss of apoptosis in breast carcinoma is more frequent in tumors with microsatellite instability (MSI) (Mendez et al., 2001) and leads to the appearance of variants with malignant potential such as survival at metastatic foci (Zhivotovsky and Kroemer, 2004). It has been proposed that genetic instability correlates with anti-apoptotic proteins, such as Bcl-xL, that are involved in the selection of highly metastatic cells during tumorigenesis. Therefore the accumulation of genetic alternations caused by the deregulation of Bcl-xL in breast cancer are essential to metastasis (Mendez et al., 2005). These studies suggests that the primary role of

Bcl-xL in the breast cancer metastasis is allowing for the accumulation of genetic mutations and alterations, decreasing tumor cell dormancy, and providing a mechanism for which metastatic cells can adapt to new microenvironments (Fernandez et al., 2002).

**Bcl-xL and chemotherapy resistance**. The molecular mechanisms responsible for chemoresistance are unclear. One mechanism involves altering the expression of anti-apoptotic proteins such as Bcl-xL because many chemotherapy drugs kill tumor cells by inducing apoptosis (Barry et al., 1990; Kaufmann, 1989). Cells expressing Bcl-xL are more likely to be chemo-and radiotherapy-resistant (Cherbonnel-Lasserre et al., 1996; Simonian et al., 1997). The role of Bcl-xL in chemotherapy resistance overlaps it role in metastatsis and is primarily the survival and therefore subsequent adaptation of cancer cells to their new environments (Gu et al., 2004). It has been suggested that chemotherapy treatment selects for tumor clones that overexpress Bcl-xL. This is evident because the staining intensity of such proteins increased after chemotherapy of primary tumors (Campos et al., 1993; Castle et al., 1993; Maung et al., 1994; Weller et al., 1995). Cancer cells overexpressing Bcl-xL are more easily selected for resistance after drug treatment because of their lack of apoptosis (Fernandez et al., 2000). Additionally, Bcl-xL increases genetic instability in cells that can result in phenotypes that are more adaptive than others (Gu et al., 2004). Resistant to chemotherapy in SCC 25 squamous carcinoma cells *in vitro* is associated with Bcl-xL expression (Datta et al., 1995). Animal studies indicated that Bcl-xL promoted chemotherapy resistance in mouse models. The tumors caused by SCK mammary cells transfected with Bcl-xL are resistant to apoptosis induced by chemotherapeutic agents, methotrexate and 5-fluorouracil. The protein may function in a similar fashion in human cells the overexpress Bcl-xL (Liu et al., 1999). The role of Bcl-xL in organ

specificity and overcoming dormancy (Rubio et al., 2001) indicates that it may be a hallmark of metastasis and contributes to therapy resistance in doing so (Gu et al., 2004).

## The CCAAT/Enhancer Binding Protein (C/EBP) Family

The CCAAT/enhancer-binding protein (C/EBP) family is a group of leucine zipper transcription factors that plays roles in the differentiation of adipocytes, myeloid, and other cells, metabolism, inflammation, proliferation, and other cellular functions (Ramji and Foka, 2002). Each family member is composed of divergent (<20% homology) amino-terminal region, and a conserved carboxy-terminal domain. This carboxy-terminal region consists of a basic DNA-binding domain followed by α-helical leucine zipper region which is involved in dimerization (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002). The specificity of DNA-binding is dictated by the amino acids in the basic region (Johnson, 1993) and dimerization is required for DNA-binding (Landschulz et al., 1989). Dimers bind DNA at the sequence A/G TTGCG C/T AA C/T (Johnson, 1993; Osada et al., 1996; Vinson et al., 1989), as an inverted Y, each arm a single α-helix that binds one half of the palindromic sequence in the DNA major groove like a pair of scissors (Tahirov et al., 2001; Tahirov et al., 2002).

Three C/EBP proteins are expressed in mammary tissue: C/EBPα, C/EBPβ, and C/EBPδ (Gigliotti and DeWille, 1998; Sabatakos et al., 1998) and have been studied extensively (Osada et al., 1996). C/EBPα is expressed in but is not required for the normal development of the mammary gland (Seagroves et al., 1998). The expression of C/EBPα causes growth $G_0$-$G_1$ cell cycle arrest and inhibits mammary cell proliferation (Gery et al., 2005). In fact, the protein is downregulated in and has been considered a potential tumor suppressor gene for breast cancer (Gery et al., 2005). C/EBPδ functions in the maintenance of mammary epithelial cells (Gigliotti et al., 2003). The protein functions in cell cycle exit/$G_0$ entry and it inhibits mammary cell growth *in vitro* (Gigliotti et al., 2003; O'Rourke et al., 1997; O'Rourke et al., 1999; Sivko and

DeWille, 2004). C/EBPδ is tightly regulated during $G_0$ growth arrest of human mammary epithelial cells which allows cells to quickly re-enter cell cycle and proliferate upon growth factor stimulation (Sivko and DeWille, 2004). C/EBPδ is also down-regulated in breast cancer; with the progression from normal mammary epithelium to breast carcinoma (Porter et al., 2003). Both C/EBPα and C/EBPδ are correlated with cell-cycle inhibitory proteins, Rb, p27, and p16 (Milde-Langosch et al., 2003).

**C/EBPβ protein**

Human C/EBPβ (NF-IL6) was identified as a protein with high DNA-binding homology to rat C/EBP[α] (Landschulz et al., 1988a; Landschulz et al., 1988b) that mediated IL-6 signaling by binding to IL-6 responsive elements on the tumor necrosis factor (TNF), interleukin-8 (IL-8), and granulocyte colony-stimulating factor (G-CSF) promoters (Akira et al., 1990; Poli et al., 1990). Homologues have been found in other species including: IL6-DBP (Poli et al., 1990), LAP (Descombes et al., 1990), AGP/EBP (Chang et al., 1990), CRP2 (Williams et al., 1991), and NF-M (Kowenz-Leutz et al., 1994). Later, a Greek letter notation was coined for each C/EBP protein (α, β, γ, δ, ε, ζ) (Cao et al., 1991).

**C/EBPβ protein function**

C/EBPβ differs from other C/EBP members in that it promotes the proliferation and represses the differentiation of many cell types (Lekstrom-Himes and Xanthopoulos, 1998). Knockout mice have been used to determine the biological functions of the protein. The primary defects occur in several different categories: the immune system (Screpanti et al., 1995; Tanaka et al., 1995), adipocyte differentiation (Tanaka et al., 1997), liver function (Croniger et al., 1997; Greenbaum et al., 1998), and female fertility (Sterneck et al., 1997). C/EBPβ knockout mice also displayed defects in mammary development (Milde-Langosch et al., 2003). Glandular development was impaired in virgin, pregnant, and lactating C/EBP-deficient mice (Robinson et

al., 1998). Functional markers of murine mammary gland differentiation, where low or absent in these mice and they displayed dysfunctional differentiation of secretory epithelium, even in response to lactation specific hormones (Seagroves et al., 1998). Impaired mammary glands had delayed growth, enlarged ducts, and decreased branching. The defects seen in these mice were intrinsic to the epithelial cells because the lack of C/EBPβ in the stroma did not affect ductal elongation and branching during puberty or alveolar development during pregnancy (Grimm and Rosen, 2003). The protein acts as the mediator of mammary cell fate by influencing hormonal receptors such as progesterone receptor (PR) (Seagroves et al., 2000). Additionally, C/EBPβ is required for ductal morphogenesis, lobuloalveolar development, and functional differentiation of murine mammary epithelial cells and for the proper proliferation and morphogenic responses during mammary gland maturation and for differentiation of milk-producing secretory cells during pregnancy (Robinson et al., 1998). These studies emerge C/EBPβ as a critical component in the control of mammary epithelial cell proliferation and differentiation and the in hormonal signaling cascades responsible for the healthy, fully developed, and lactating mammary gland (Robinson et al., 1998).

**C/EBPβ protein isoforms**

The *cebpb* gene consists of single exon gene with no introns and the transcription of the gene results in a single 1.4 kb mRNA (Zahnow, 2002). Three C/EBPβ protein isoforms are generated post-transcriptitionally: LAP1, LAP2, and LIP (Figure 1-3) through a leaky ribosome scanning mechanism that uses alternative translation initiation start sites (Descombes and Schibler, 1991; Ossipow et al., 1993). LIP has also been hypothesized to result from the proteolytic cleavage of other C/EBPβ isoforms (Baer and Johnson, 2000; Dearth et al., 2001; Welm et al., 1999)

LAP1 (liver-enriched activation protein 1), also called LAP*, is the full-length isoform. The protein is 38 kDa in mice (Calkhoven et al., 2000; Williams et al., 1995) and 45 kDa in humans (Eaton et al., 2001). Human LAP1 represses the cyclin D1 promoter and is proposed to regulate transcription of genes in non-proliferating or differentiating cells (Eaton et al., 2001). LAP1 is detected in the normal mammary glands of mice (Dearth et al., 2001; Eaton et al., 2001). In humans, the protein is detectable in normal, mostly non-dividing human breast tissue and in secretory mammary epithelial cells exfoliated in human breast milk (Eaton et al., 2001; Milde-Langosch et al., 2003).

Human LAP2 (liver-enriched activation protein 2), also called LAP, differs from human LAP1 by 23 amino acids in humans, and 21 amino acids in mouse, rat, and chicken (Kowenz-Leutz et al., 1994; Williams et al., 1995). The protein is 32 kDa-35 kDa in rodents (Calkhoven et al., 2000; Descombes et al., 1990) and 42 kDa in humans (Eaton et al., 2001). It is the most transcriptionally active form of C/EBP (Williams et al., 1995) and promotes cell proliferation, motility, and invasion (Bundy and Sealy, 2003). The growth-promoting functions of C/EBPβ are carried out in large by LAP2. Human LAP2 activates cyclin D1 promoter and has been proposed to promote epithelial cell growth (Eaton et al., 2001). LAP2 is expressed throughout rodent mammary development; two–three fold during pregnancy, decreases at parturition, but is still readily detectable through lactation and involution and modestly decreased at lactation (Raught et al., 1995; Seagroves et al., 1998). In humans, LAP2 is expressed in normal and malignant breast tissue (Eaton et al., 2001; Milde-Langosch et al., 2003).

LIP (liver-enriched inhibitory protein) lacks a 49 amino acid portion of its amino-terminal transactivation domain but retains the dimerization and DNA-binding domains. Therefore it can antagonize the transcriptional activation of the LAP isoforms, C/EBP proteins,

and other leucine zipper proteins. It does so by forming heterodimers with target proteins, resulting in C/EBP protein dimers unable to transactivate target promoters, or it binds C/EBP sites on target promoters with a greater affinity, competing with functional C/EBP dimers (Descombes and Schibler, 1991). LIP is 20 kDa in rodents (Calkhoven et al., 2000; Descombes and Schibler, 1991) and humans (Eaton et al., 2001). Its expression is associated with rapid mammary epithelial cell proliferation and it inhibits cell differentiation (Raught et al., 1995). LIP isn't detectable in virgin rat mammary gland (Seagroves et al., 1998) but it increases 100 times during pregnancy which coincides with increased alveolar cell proliferation during this time. LIP expression is nearly undetectable at parturition and remains low throughout lactation and involution (Dearth et al., 2001; Raught et al., 1995; Seagroves et al., 1998).

The ratios of LAP/LIP are an important determinant of C/EBPβ function (Seagroves et al., 1998) and critical in mediating the expression of C/EBPβ target genes (Descombes and Schibler, 1991). These ratios rather than the absolute amounts of each isoform are an important indication of transcriptionally activity of C/EBPβ (Zahnow et al., 2001) and have a dramatic effect on mammary gland development. Several lines of evidence indicate that C/EBPβ-expressing cells exhibit unique LAP/LIP ratios, depending on cell type and that C/EBP does not always function in a positive manner when the expression of LIP exceeds negligible levels (Shimizu et al., 2007).

Several mechanisms have been described for the differential expression of C/EBPβ isoforms. It is hypothesized that the LAP1 and LAP2 translation start sites and a small uORF are embedded within a stem loop structure on the C/EBPβ mRNA and that both play an important roles in the regulation of AUG recognition and isoform translation (Raught et al., 1996; Xiong et al., 2001). The mRNA binding protein, CUG repeat binding protein (CUG-BP1) (Baldwin et al.,

2004; Timchenko et al., 1999), calreticulin (Timchenko et al., 2002), and eukaryotic translation initiation factors, eIF-2α and eIF-4E (Calkhoven et al., 2000) bind *cebpb* mRNA and direct isoform translation. eIF2 plays a role in translation start site recognition (Donahue et al., 1988) and catalyzes the binding of Met-tRNA to the 40S ribosomal subunit (Schreier and Staehelin, 1973) while eIF4E recognizes the 5' mRNA cap as the first step in ribosomal scanning (Pause et al., 1994).

**C/EBPβ and Breast Cancer**

C/EBPβ mRNA is present in murine virgin mammary glands. It increases during pregnancy, declines at mid-lactation, and increases again within 48 hours of involution (Gigliotti and DeWille, 1998). *In situ* localization studies in the mouse mammary gland have identified the localization of C/EBPβ mRNA *in vivo*. In humans, C/EBPβ mRNA is present in low levels in virgin mammary gland, increases during pregnancy, declines slightly during lactation, and is induced 24-28 hours after the onset of involution (Gigliotti and DeWille, 1998; Robinson et al., 1998; Sabatakos et al., 1998).

C/EBPβ plays a role in rodent breast carcinogenesis (Zahnow, 2002). Mice overexpressing the gene in the mammary gland develop hyperplasia and carcinoma (Wang et al., 1994). C/EBPβ probably contributes to tumorigenesis by increases in mRNA and protein levels rather than somatic mutations (Grimm and Rosen, 2003). Studies indicate that C/EBP proteins may also be involved in the etiology or progression of human mammary carcinomas, however, sparse information has been acquired so far (Milde-Langosch et al., 2003; Zahnow, 2002). Studies have indicated that the protein has a role in human breast carcinogenesis (Raught et al., 1996; Zahnow et al., 1997). C/EBPβ mRNA was two-five fold higher in MMTV/c-neu mammary tumors than the levels normally expressed during lactation or involution (Dearth et al., 2001).

Since all C/EBPβ isoforms originate from a single mRNA, protein levels of each isoform provide a more accurate depiction of the role of C/EBPβ in breast carcinogenesis. Changes in the ratios of C/EBPβ isoforms LAP/LIP have been observed in breast cancer (Eaton et al., 2001; Zahnow et al., 1997). Each C/EBPβ isoform can contribute to breast carcinogenesis separately. LAP1 is expressed in normal breast epithelial cells and tissue from rodents and humans (Dearth et al., 2001; Eaton et al., 2001), so it plays few if any roles in breast carcinogenesis. LAP2 is expressed in infiltrating ductal carcinoma extracts (Zahnow et al., 1997), is acquired in primary human breast tumors, and is present in cultured breast cancer cell lines (Eaton et al., 2001). It was expressed at high levels of invasive primary breast tumor samples and was the only transactivator isoform expressed in breast cancer cell lines (Eaton et al., 2001). LAP2 was also associated with advanced stages and increased proliferation in human breast tumors (Milde-Langosch et al., 2003). LAP1 and LAP2 functions differ and the altering of the ratio between the two isoforms may contribute to the transformation of human breast epithelial cells (Bundy and Sealy, 2003).

The expression of LIP is tightly regulated during mouse mammary gland development and breast cancer progression (Raught et al., 1996; Zahnow et al., 1997). The LIP isoform was detected in 10 different rat tumor lines and its expression was restricted to mammary tumors and not detectable in pre-neoplastic lesions or other primary tumors (Raught et al., 1996; Sundfeldt et al., 1999). Generally, LIP is increased in more proliferative tumors or developmental time points and is highly expressed in the most aggressive, poorly differentiated human cancers (Raught et al., 1996; Zahnow et al., 1997). Overexpression of LIP in mouse mammary epithelial cells increased proliferation, foci formation, and loss of contact inhibition. It has been suggested that LIP overexpression stimulates a growth cascade that makes cells susceptible to additional

oncogenic hits, resulting in tumorigenesis (Zahnow et al., 2001). LIP was correlated with ER-negative phenotypes and increased proliferation (Milde-Langosch et al., 2003). In fact, one study suggested that LIP expression should be evaluated further as a prognostic marker for human breast cancer (Zahnow et al., 1997).

The role of LIP in breast carcinogenesis is controversial. There was no significant level of LIP detected in high grade infiltrating mammary carcinomas (Eaton et al., 2001) and LIP overexpression in the non-transformed mouse mammary epithelial cell line, HC11, did not significantly affect cell proliferation or cell cycle progression (Dearth et al., 2001).
The overexpression of LIP in NIH3T3 cells lead to cell death (Eaton et al., 2001) and strongly inhibited growth in MCF10A cells (Bundy et al., 2005). Its role may also be concentration dependent. Moderate LIP expression in mouse mammary epithelial cells (SCp2) promoted luminal morphogenesis, while increased LIP expression induced apoptosis (Hirai et al., 2001). The fact that LIP may result from cleavage in addition to *de novo* translation (Baer and Johnson, 2000; Dearth et al., 2001; Welm et al., 1999), also makes it difficult to determine its role in carcinogenesis (Welm et al., 1999). Together, these studies indicate that more research is needed to determine the role of the LIP isoform in breast tumorigenesis.

Table 1-1. Carcinogens Present in Cigarette Smoke. A partial list of these carcinogens is below. The IARC group reflects the likelihood of human carcinogenicity: (1) human carcinogen; (2A) probably carcinogenic to humans; (2B) possibly carcinogenic to humans; (3) not classifiable as to their carcinogenicity to humans. Classifications reflect data up to 2004 (International Agency for Research on Cancer, 2004). Table is adapted with permission from Macmillian Publishers for Hecht, 2003. (*) Adapted with permission from The American Chemical Society for Hoffman et al., 2001. (**) Adapted with permission from Springer Science and Business Media for Hecht, 2006.

| Chemical class | Examples* | IARC group** |
|---|---|---|
| Aldehydes | Formaldehyde | 1 |
| | Acetadehyde | 2B |
| Aromatic amines | 4-Aminobiphenyl | 1 |
| | 2-Naphthylamine | 1 |
| Inorganic compounds | Arsenic | 1 |
| | Lead | 2B |
| Polycyclic hydrocarbons (PAHs) | Benzo(α)pyrene (B[α]P) | 1 |
| | Dibenzo(a,h)anthracene | 2A |
| Phenols | Caffeic acid | 2B |
| | Catechol | 2B |
| Nitroamines | 4(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) | 1 |
| | N-nitrosonornicotine (NNN) | 1 |
| Volatile hydrocarbons | Benzene | 1 |
| | Styrene | 2B |

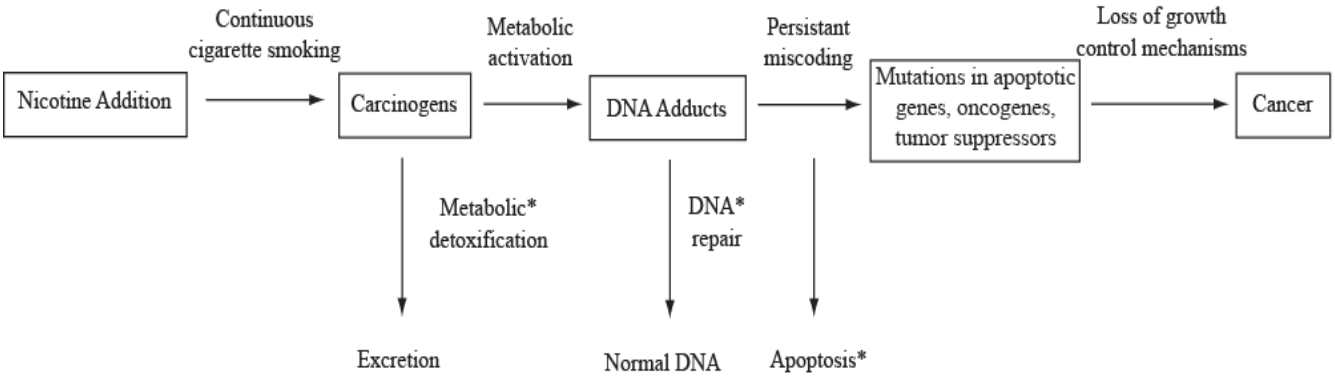


Figure 1-1. Mechanism of cigarette smoke-induced cancer. Nicotine addition causes continual cigarette smoking and chronic exposure to cigarette related carcinogens. Most of these carcinogens are either metabolically detoxified and excreted out of the body or activated. The carcinogens that are metabolically activated form intermediates that bind to DNA and cause adducts. If the adducts are not repaired and persist during DNA replication, miscoding and permanent mutations can occur in the DNA. Damaged cells may be removed by apoptosis. However, if a mutation occurs in an oncogene or tumor suppressor, there could be a loss of normal cell growth control. Inactivation of apoptosis genes or upregulation of anti-apoptotic genes allows the DNA damage to persist and may result in abnormal gene expression. Loss of cell cycle control, cell transformation, and eventually tumorigenesis can result. Asterisks (*) represent the body's endogenous defense systems. Adapted with permission from Oxford University Press for Hecht, 1999.

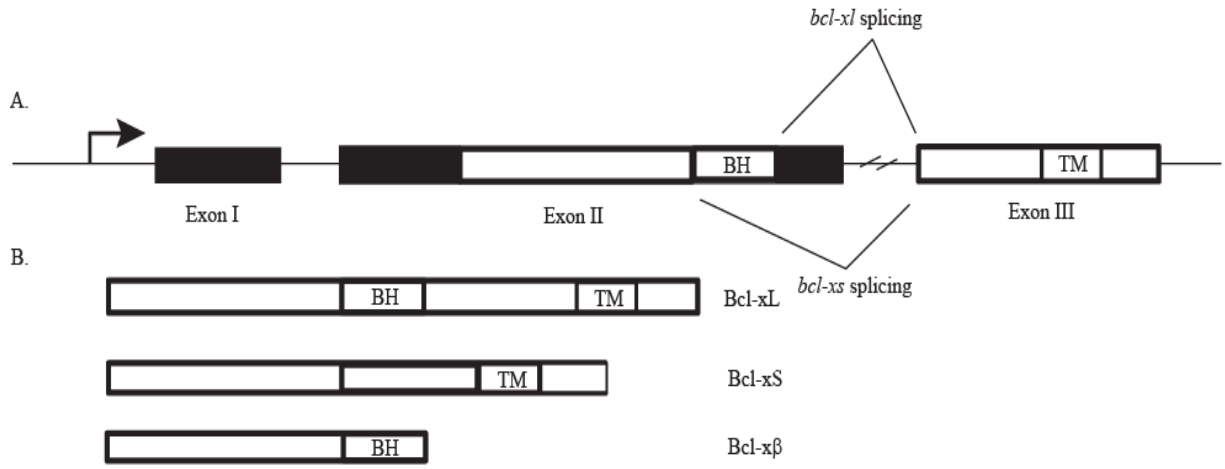


Figure 1-2. Human *bcl-x* gene structure and proteins. (A) *Bcl-x* gene structure is composed of three exons. Exon I is non-coding, while exon II and exon III code for *bcl-x* mRNAs. The *bcl-xl* promoter is located 5' of Exon I. (B) Human *bcl-x* mRNAs. Bcl-x pre-mRNA is alternatively spliced into three different mRNAs, each coding for a single protein. Bcl-xL is anti-apoptotic, while Bcl-xS is pro-apoptotic and lacks the BH domain (BH) due to an alternative splicing site in Exon II. Bcl-xβ results from unspliced mRNA and lacks the transmembrane domain. Its role in apoptosis remains unclear. The isoforms share several domains. The BH Domain (BH) is the 63 amino acid region containing BH1 and BH2 domains, having the most homology to Bcl-2. The transmembrane domain (TM) is responsible for mitochondrial localization. Adapted with permission from the American Society for Biochemistry and Molecular Biology for Pecci et al., 2001.

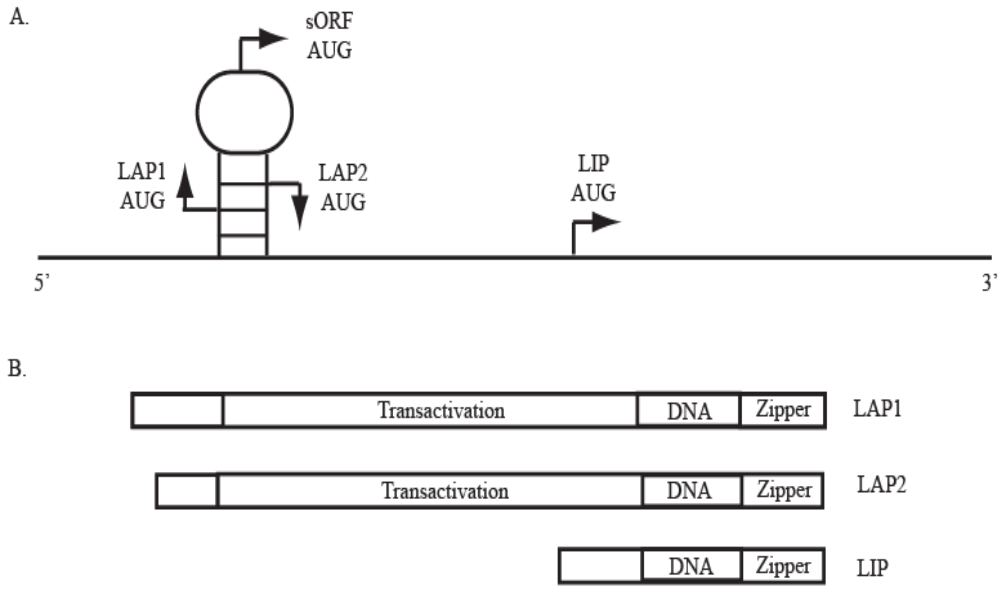


Figure 1-3. Human C/EBPβ mRNA structure and protein isoforms. (A) C/EBPβ mRNA contains three translation initiation sites (AUGs) from which isoforms are translated. The 5' end contains a RNA hairpin region. Between the LAP1 and LAP2 AUGs, there is an AUG associated with a small open reading frame (sORF), which are important for the translational control of C/EBPβ isoforms. (B) The mRNA is alternatively translated into three different isoforms by a leaky ribosome scanning mechanism. LAP1 and LAP2 differ by only 21 amino acids. LIP is considerably shorter and lacks the transactivation domain. It retains the dimerization and leucine zipper domains, therefore it acts as a dominant negative to LAP1 and LAP2 protein function. LIP also results from the proteolytic cleavage of the other LAP isoforms. Adapted from Zahnow, 2002.

# CHAPTER 2
# MATERIALS AND METHODS

## Preparation of CSC

CSC was prepared from the University of Kentucky Reference Cigarette IR4F (Davis, 1984; Sullivan, 1984) which contains 9 mg tar and 0.8 mg nicotine per cigarette and approximates the average full flavor, low-tar cigarette available on the American market (Chepiga et al., 2000). The CSC was prepared by a procedure previously described (Hsu et al., 1991). In short, the particulate phase (tar) was collected on a Cambridge filter pad from cigarettes smoked under standard Federal Trade Commission conditions (35 mL puff volume of a 2 sec duration) on a specialized machine (Griffith and Hancock, 1985). The particulate matter was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 40 mg/mL, aliquoted into vials, and stored at -80°C. For treatment, the stock solutions were diluted to the appropriate concentrations in complete medium.

## Culturing of MCF10A Cells

MCF10A cells were maintained in 1X Dulbecco's modification of Eagle's medium/Ham's F12 (DMEM/F12) 50/50 Mix with L-glutamine and 15 mM Hepes (MediaTech, Inc., Manassas, VA). This medium was supplemented with 5% horse serum, 100U/mL penicillin/streptomycin, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, and 10 ng/mL epidermal growth factor. The cells were incubated in a 5% $CO_2$ incubator at 37°C.

## Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) as per manufacturer instructions. Cells were seeded on 60 mm tissue culture plates and treated at 50-60% confluency. At the appropriate times, the plates were rinsed with cold 1X Dulbecco's

phosphate buffered saline (DPBS) (MediaTech, Inc., Manassas, VA), three milliliters of TRIzol were added directly into each plate and the plates were rotated for 15-20 min until the cells detached. The TRIzol-cell solution was pipetted from each plate into a round bottom Falcon tube (BD Biosciences Pharmingen, Mississauga, ON), 700 µl of chloroform was added, and the tubes were incubated for 15 min, shaking every 2 min. The tubes were centrifuged at 10,000 rpm at 4°C for 20 min. The upper aqueous phase was removed into a fresh tube, 1.8 mL isopropanol was added, and the tubes were incubated at room temperature for 10 min with intermittent mixing. After centrifugation at 10,000 rpm for 20 min at 4°C, the supernatant was carefully removed, leaving the pellet. The pellet was washed with 700 µl of 75% ethanol in diethylpyrocarbonate (DEPC) water; after a final centrifugation, the alcohol was removed, and the pellet was resuspended in 10-20 µl DEPC water. The samples were incubated at 65°C for 10 min, allowed to cool, quantitated, and stored at 80°C until use. All procedures were performed with RNAse free tubes and equipment.

The isolated RNA (0.5 µg) was used to make cDNA with the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA) as per manufacturer instructions. Two microliters of cDNA was then used for PCR with primers specific for Bcl-xL (800 bp) or GAPDH (320 bp). The primers used were Bcl-xL sense: 5'- TTGGACAATGGAC TGGTTGA-3', Bcl-xL antisense: 5'-GTAGAGTGGATGGTCAGTG-3' and GAPDH sense: 5'-GGGAAGCCACTGGCATGGCCTTCC-3', GAPDH antisense: 5'- CATGTGGGCCATGAG GTCCACCAC-3'. The PCR cycles were: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 20 sec, 58°C for 30 sec, 72°C for 1 min; and a 4°C hold.

## Western Blot Analysis

MCF10A cells were plated on 150 mm plates and treated at 50-60% confluency with CSC as described in the figure legends. After treatment the cells were processed into whole cell extract. Cells were scraped into 50 mL conical tubes and pelleted at 1,500 rpm for 5 minutes at 4°C. Pellets were rinsed with 1X DPBS (Mediatech, Herndon, VA) and resuspended in 100-500 µl lysis buffer (20 mM Tris pH 7.4, 100 mM NaCl, 1 mM PMSF, 0.5% deosycholate, 1% NP 40, 1% SDS, 1.2 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM sodium methavandate, 50 mM NaFl, 1 µg each of aproptin, leupeptin, pepstatin). The cells were rotated for 20 min at 4°C and then centrifuged at 13,200 for 10 min at 4°C. The supernatant was removed into a fresh tube and used for Western analysis. Lysates were prepared for electrophoresis with lysis buffer and 6X Western dye to a final concentration of 1X dye, and then boiled for 5 min. After cooling and a brief centrifugation, proteins were separated on a 10% SDS-PAGE gel and electroblotted onto a Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ). The blots were blocked with 5% milk in Tris buffered saline –1% Tween (TBS-T). The blots were then probed with the appropriate antibodies diluted in 2.5% milk in TBS-T. The blots were incubated, rocking, at room temperature for 2 h for primary antibodies and 1 h for secondary antibodies. The antibodies used were: anti-Bcl-xL (sc-1041), and anti-C/EBPβ (sc-150), both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Blots were rinsed between antibodies with three washes of TBS-T, 10 min each. ECL Plus Western Blotting Detection System (Ambersham Biosciences, Piscataway, NJ) and autoradiography were used to detect protein levels. Blots were stripped at 65°C for 30 min to 1 h, shaking every 10-15 min. The stripped blots were rinsed with TBS-T, blocked again, and re-probed with the anti-Actin (sc-1616) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for the loading control

## Cloning of the Human *Bcl-xl* Promoter (pBcl-xLP)

The human *bcl-xl* promoter was previously cloned and sequenced in my lab. Using primers modelled from Sevilla *et al*., (1999), nucleotides 226-915 from the published human *bcl-xl* promoter (Gene Bank Accession No. D30746) were cloned into a PGL-3 Basic Luciferase Vector (Promega Corp., Madison, WI) at *Xho*I and *Hind*III restriction enzyme sites. The promoter *cis*-elements were determined with the TRANSFEC v4.0 Program (TESS: Transcription Element Search System, University of Pennsylvania) and two transcription initiation sites were identified.

## Cloning of pBcl-xLP Deletion Constructs

PCR was used to make nine sequential deletion pBcl-xLP constructs. The full-length pBcl-xLP (-54,+647) was used as template for PCR with specific primers that amplified the appropriate regions resulting in the deletion constructs. The primers were pBcl-xLP (-28,+707) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+707) anti-sense: 5'-CCCAAGCTTGTCCAAAACACCTGCTCA-3'; pBcl-xLP (-28,+542) sense: 5'-CCGCTCG AGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+542) anti-sense: 5'-CCCAAGCTTCC AGAACTGGTTTCTTTGTGG-3'; pBcl-xLP (-28,+462): sense 5'-CCGCTCGAGCCACCTCC GGGAGAGTACTC-3', pBcl-xLP (-28,+462) anti-sense: 5'-CCCAAGCTTCCAGTGGACTC TGAATCTCCC-3'; pBcl-xLP (-28,+375) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGT ACTC-3', pBcl-xLP (-28,+375) anti-sense: 5'-CCCAAGCTTCCCCCGCCCCCACTCCCGCT C-3'; pBcl-xLP (-28,+342): sense 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+342) anti-sense: 5'-CCCAAGCTTTACATTCAAATCCGCCTTAG-3'; pBcl-xLP (-28,+282) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+282) anti-sense: 5'-CCCAAGCTTTCACAGGTCGGAGAGGAGG-3'; pBcl-xLP (-28,+222) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+222)

anti-sense: 5'-CCCAAGCTTGCTGGCAAAAAAACCAGCTC-3'; pBcl-xLP (-28,+132) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+132) anti-sense: 5'-CC AAGCTTAACCAGCCCCCTCGTTGCT-3'; pBcl-xLP (-28,+42): sense: 5'-CCGCTCGAGCC ACCTCCGGGAGAGTACTC-3', pBcl-xLP (-28,+42) anti-sense: 5'-CCCAAGCTTCCCCTCT CTTGCACGCCC-3'. The PCR cycles were: 1 cycle of 94°C for 3 min; 32 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min; 1 cycle of 72°C for 10 min; and 4°C hold. The PCR products were gel extracted with the QIAXEXII Gel Extraction Kit (Qiagen, Inc., Valencia, CA) as per manufacturer instructions. Samples and empty PGL-3 vector were digested with *Xho*I and *Hind*III for 4 h. Digested products were ligated into the digested vector overnight at 16°C using T4 DNA ligase (New England BioLabs, Inc., Ipswich, MA). The ligation products were transformed into Max Efficiency DH5α Chemically Competent cells (Invitrogen Life Technologies, Carlsbad, CA) according to package instructions and spread on Ampicillin-Luria Broth (LB) plates. Colonies were screened for the correct insert with the QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA) as per manufacturer instructions. The isolated DNA was digested with *Xho*I and *Hind*III to confirm the presence of the correct insert. The constructs were sent for sequencing and upon confirmation, were further amplified with the QIAGEN Plasmid Maxi Kit (Qiagen, Inc., Valencia, CA). The resulting DNA was used in transfection assays.

**Promoter Activity Assays**

Approximately 0.5 million cells were seeded on 60 mm tissue culture plates. *Bcl-xl* promoter constructs (pBcl-xLPs) were transfected into cells with FuGENE 6 Transfection Reagent (Roche Applied Bioscience, Indianapolis, IN) as per manufacturer instructions. Nine microliters of FuGENE 6.0, 2 µl of the appropriate promoter construct, 0.5 µg pCMV β-galactosidase plasmid, and 100 µl serum free medium (SFM) were mixed for each plate. The

solution was incubated for 30 min at room temperature. During this time, the plates to be transfected were rinsed with SFM. After incubation, an additional 1.9 mL SFM was added to the DNA solution for each plate. The DNA-lipid solution was mixed well and 2 mL was added to each transfection plate (after SFM rinse was removed). Five hours later, the DNA-lipid solution was removed from the cells and 4 mL of fresh medium was added. Sixteen hours later, the cells were treated and harvested at the appropriate time points.

At the appropriate time points, cells were scraped into 15 mL conical tubes and pelleted at 1,500 rpm for 5 min at 4°C. Pellets were rinsed with 1X DPBS (Mediatech, Herndon, VA) and resuspended in 50-100 µl 1X reporter lysis buffer, depending on the size of the pellet. The 1X reporter lysis buffer was diluted from 5X Reporter Lysis Buffer (Promega Corp., Madison, WI). The samples were lysed with five freeze-thaw cycles of alternating liquid nitrogen and 37°C water bath incubations for 5 min at a time. After each water bath incubation, the samples were vortexed to ensure proper lysing. The samples were then centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant was removed to a fresh tube and used for promoter activity analysis.

The pBcl-xLP promoter activity was measured with luciferase assays. Luciferase assay reagent (20 mM tricine, 1.07 mM magnesium carbonate hydroxide, 2.67 mM magnesium sulfate, 0.1 mM EDTA) was used to prepare luciferase assay buffer (4 mL luciferase assay reagent, 470 µM luciferin, 270 µM coenzyme A, 530 µM ATP, 33.3 mM DTT). All of the reagents for the luciferase assay buffer were purchased from Sigma-Aldrich (St. Louis, MO). The assay was performed with a Monolight 3010 Luminometer (BD Biosciences Pharmingen, Mississauga, ON). Ten milliliters of lysate were put into a Luminometer cuvette (BD Biosciences

Pharmingen, Mississauga, ON) and it was placed into the luminometer. The machine injected 100 µl luciferase assay buffer and recorded the resulting luciferase activity.

β-galactosidase assays were performed in 96-well flat bottom tissue culture plates. In each well, 10 µl of lysate was incubated with 50 µl double distilled water (DDW), 15 µl 5X Reporter Lysis Buffer (Promega Corp., Madison, WI), and 75 µl 2X assay buffer (200 mM sodium phosphate buffer pH 7.2, 2 mM $MgCl_2$, 100 mM β–mercaptoethanol, 1.33 mg/mL σ-Nitrophenyl-B-D-galactopyranoside (ONPG)). Wells were mixed and incubated at room temperature for 5 min to 1 h to allow the yellow color to develop. The reactions were stopped with 75 µl 1M sodium carbonate and the plates were read for absorbance with 490 nm wavelength on a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). These readings were divided into luciferase values to normalize for transfection efficiency.

**Electrophoretic Mobility Shift Assay (EMSA)**

Single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides for EMSA analysis. For annealing, 25 µg of each oligonucleotide and its compliment were combined with 10 µl 1M KCl to a total of 50 µl DDW. The mixture was heated at 90°C for 10 min and then allowed to slowly cool to 25°C. Double-stranded oligonucleotides were diluted to 100 ng/µl. The oligonucleotides used were C/EBP site-I wild-type sense: 5'-AAAAACAAAAACCAACTAAA-3', C/EBP site-I wild-type anti-sense: 5'-TTTAGTTGGTTTTTGTTT TT-3'; C/EBP site-I mutant sense: 5'-AAAA**GGGCCCAA**AAC TAAA-3'; C/EBP site-I mutant anti-sense 5'-TTTTAGTTTGGGCCCTTTTT-3'; C/EBP site-II wild-type sense: 5'-CCTGAGCTTCGCAATTCCTG-3', C/EBP site-II wild-type anti-sense: 5'-CAGGAATTGCGAAGCTCAGG-3'; C/EBP site-II mutant sense: 5'-CCTAGC**CACAGC** ATTCCTG-3', C/EBP site-II mutant anti-sense 5'-CAGGAATGCTGAGGCTCAGG-3'. The

underlined nucleotides are the core of the C/EBP consensus sequence and the bold nucleotides were mutated.

Double-stranded oligonucleotides were end-labelled in reactions of 200 ng of oligonucleotide, 4 µl 10X T4 Polynucleotide Buffer (New England BioLabs, Inc., Ipswich, MA), 1 µl T4 Polynucleotide Kinase (New England BioLabs, Inc., Ipswich, MA), and 5 µl $^{32}$P γ-ATP to a final volume of 40 µl. The mixture was incubated at 37°C for 30 min and purified through a Sephadex G-50 DNA grade Nick Column (Amersham Biosciences, Piscataway, NJ). The amount of radioactivity was measured with a LS 6500 Multipurpose Scintillation Counter (Beckman Coulter, Fullerton, CA).

EMSA and super-shift analysis was performed with nuclear extracts prepared as described in Shapiro et al. (1988). DNA-protein binding reactions were assembled to a final volume of 20 µl with 20 mM Hepes pH 7.9, 1 mM DTT, 5 mM $MgCl_2$, 80 mM KCl, 10% glycerol, 0.025% NP-40 and 0.5 µg poly(dI.dC). After 10 min incubation at room temperature, 2 µg of nuclear extract was added. Three microliters of the appropriate $^{32}$P-labelled double-stranded probe were added and incubated for another 20 min at room temperature. For competition experiments, appropriate amounts of unlabelled probe were added after the addition of poly(dI.dC) and incubated at room temperature for 10 min before the addition of the $^{32}$P-labelled probe. To perform super-shift analysis the master mix consisted of 20 mM Hepes pH 7.9, 1 mM DTT, 5 mM $MgCl_2$, 37.5 M KCl, 7.5% glycerol, and 1 µg poly(dI.dC). Five micrograms of nuclear extract and 4.5 µl of $^{32}$P-labelled probe were added. Antibodies specific to C/EBPα (sc-9314X), β (sc-150X), and δ(sc-636X) (Santa Cruz Biotechnology, Santa Cruz, CA) proteins and formulated for use in EMSA and ChIP analysis were added to the reaction mixture prior to the addition of $^{32}$P-labelled probe and incubated for 30 min. The reactions were

loaded on a nondenaturing 4% polyacrylamide gel Tris-borate-EDTA (TBE) which was pre-run at 100 volt for at least 30 min. The samples were loaded (without dye) and run at 100 volts for the first 15 min and 150 volts for a total of 1.5 hours with 0.5X TBE used as running buffer. After the run was completed, the gel was transferred to filter paper and dried for 1.5 h at 80°C. The DNA-protein complexes were then visualized by autoradiography. Exposure times varied from 2 h to 24 h.

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP analysis was carried out with the ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY) as per manufacturer instructions. One million cells were seeded onto 100 mm tissue culture plates and treated at appropriate times. The cells were fixed by adding 270 µl of 37% formaldehyde per 10 mL of cell media, and incubating for 10 min at 37°C. The medium was removed, the plates were rinsed with ice-cold 1X DPBS (Mediatech, Herndon, VA) containing protease inhibitors, scraped into fresh tubes, and lysed with SDS lysis buffer containing protease inhibitors. The lysate was sonicated on ice for 4 cycles of 30 sec with 20 intervals using a Branson Sonicator 450 (Branson Power Company, Danbury, CT) at a 5% duty cycle, 20% constant maximal power, and with a control output of 5. The lysate was clarified, diluted, pre-cleared, and immunoprecipitated with 2 µg antibody overnight at 4°C. The antibody used was anti-C/EBPβ (sc-150X) (Santa Cruz Biotechnology, Santa Cruz, CA). The resulting complexes were then rinsed, eluted, and heated to reverse the cross-linkages. DNA was isolated by phenol/chloroform extraction and ethanol precipitation.

The isolated DNA was used in PCR reactions using primers specific to C/EBP site-II on the pBcl-xLP. The primers were C/EBP site-II sense: 5'-CGGGTGGCAGGAGGCCGCGGC-3' and C/EBP site-II anti-sense: 5'-AACTCAGCCGGCCTCGCGGTG-3', resulting in a 190 bp product.

## Site-directed Mutagenesis

The QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to mutate two C/EBP sites on the pBcl-xLP construct, via manufacturer instructions. Primers specific for the *cis*-element mutations were used to perform PCR. The primers were C/EBP site-I sense: 5'-TGGTGCTTAAATAGAAAAAAGGGCCCAAAACTAAATCCATACCAGCCAC C T-3', C/EBP site-I anti-sense: 5'-GGTGGCTGGTATGGATTTAGTTTTGGGCCCTTTTTCT TTTTTCTATCTATTTAAGCACCA; C/EBP site-II sense: 5'-AGCAAGCGAGGGGGCTGGT TCCTGAGCCACAGCATTCCTGTGTCGCCTTCT -3', C/EBP site-II anti-sense: 5'-AGAAG GCGACACAGGAATGCTGTGGCTCAGGAACCAGCCCCCTCGCTTGCT-3'. The PCR products were digested with *Dpn*I to degrade template DNA. Digested products were transformed into XL-1 Blue Supercompetent Cells (Strategene, La Jolla, CA) and spread onto Ampicillin-LB agarose plates. The presence of the correct mutation was confirmed and construct amplification was carried out as described earlier in this section.

## Overexpression of C/EBPβ

MCF10A cells were plated at the density of 0.6 million cells per 60 mm plate and allowed to attach overnight. The next day, 2 μg empty pCDNA3.1 vector, or either C/EBPβ overexpression construct was transfected into the cells with FuGENE 6.0 Transfection Reagent (Roche Applied Bioscience, Indianapolis, IN) as previously described in this section. At the scheduled times, plates were harvested for luciferase assays or whole cell extract as described above.

## Statistical Analysis

Experiments were averaged and appropriate statistics were performed. Significance was calculated with T-tests and p-values below 0.05 were considered significant. Statistics were performed with SigmaPlot 10.0 Software (SPSS Inc, Chicago, IL).

# CHAPTER 3
# CSC TREATMENT RESULTS IN THE TRANSCRIPTIONAL UPREGULATION OF BCL-XL IN MCF10A CELLS

## Introduction

CSC is capable of transforming the spontaneously immortalized breast epithelial cell line, MCF10A in culture (Narayan et al., 2004). A single dose of CSC resulted in characteristics such as anchorage-independent growth, colony formation, and increased expression of NRP-1, a marker of neoplastic progression (Stephenson et al., 2002). Additionally, these characteristics remained stable in cell lines established from the treated cells, with no further CSC treatment. These transformed cells were found to have elevated mRNA and protein levels of Bcl-xL versus the control cells. Even though, most treated cells die, the transformed cells escaped cell cycle arrest and survived due to the overexpression of anti-apoptotic genes such as *bcl-xl* (Narayan et al., 2004). The anti-apoptotic role of Bcl-xL and its implication in cancer, lead to the investigation of how the gene was regulated in response to CSC treatment in MCF10A cells. The hypothesis of this study is that the CSC-induced upregulation of Bcl-xL occurs by a transcriptional mechanism. After CSC treatment, a transcription factor(s) binds the *bcl-xl* promoter, induces its transcriptional activity, and results in the increase of Bcl-xL protein levels.

## Results

### CSC Treatment Induces *Bcl-xl* mRNA and Protein Levels in MCF10A Cells

The previous study suggested that CSC-induced Bcl-xL expression in MCF10A cells was mediated by an increase in *bcl-xl* mRNA (Narayan et al., 2004). To confirm this finding, MCF10A cells were treated with increasing amounts of CSC for 24 h and *bcl-xl* mRNA levels were analyzed with RT-PCR (Figure 3-1A). It is interesting to note the initial decrease in *bcl-xl* mRNA levels at 2.5 μg/mL of CSC treatment; then the levels continued to increase in a concentration-dependent manner. At 50 μg/mL of CSC treatment, the *bcl-xl* mRNA level was

higher than the level in the control cells. To confirm the subsequent induction of Bcl-xL protein levels, cells were treated with 25 μg/mL of CSC for a time course and with increasing concentrations of CSC for 24 h for a concentration curve. Western analysis was used to determine the protein levels in these cells. The time course and concentration curve confirmed the upregulation of Bcl-xL protein levels in a time and concentration-dependent manner (Figure 1-3B). These results confirmed that CSC induced *bcl-xl* mRNA and protein levels in treated MCF10A cells and that the mechanism of Bcl-xL upregulation in these cells was on the level of transcription.

**CSC Induces pBcl-xLP Promoter Activity in MCF10A cells**

One of the most important mechanisms of gene regulation is transcriptional (Weis and Reinberg, 1992). To determine the how *bcl-xl* was induced by CSC, the human *bcl-xl* gene promoter (Grillot et al., 1997; Sevilla et al., 1999) was cloned into a pGL-3 Basic Luciferase vector as described in Materials and Methods and was named pBcl-xLP. In the first step, it was determined which transcription factor(s) were responsible for the upregulation of *bcl-xl* in CSC-treated MCF10A cells. Transcription initiation sites were identified at +1 and +78 and putative *cis*-regulatory elements on the pBcl-xLP were identified (Figure 3-2). The pBcl-xLP promoter had consensus sites for several transcription factors that were reported in earlier studies, such as NF-κB, Oct1, Sp proteins, GATA, STAT, and others (Grillot et al., 1997; Sevilla et al., 1999). The first promoter cloned was pBcl-xLP (-54,+647) according to its length, but further studies indicated that *cis*-elements were located upstream of this first construct. At this point, the second promoter construct, pBcl-xLP (-145,+707), was made.

To determine whether *bcl-xl* promoter activity was induced by CSC treatment in MCF10A cells, the two promoter constructs were separately transfected into MCF10A cells and treated with CSC for time course and concentration curve experiments. The pBcl-xLP

(-145,+707) had no significant promoter activity (data not shown). Alternatively, pBcl-xLP (-54,+647) showed a time dependent and concentration dependent increase in activity after CSC treatment (Figure 3-3). Therefore pBcl-xLP (-54,+647) construct was identified as the basal promoter sequence and became our full-length promoter. It will be referred to as pBcl-xLP from this point forward. This experiment indicated the optimum treatment conditions for the induction of pBcl-xLP in these cells. During the concentration curve the promoter activity peaked at 24 h of treatment (Figure 3-3A). The highest level of promoter activity induced in the concentration curve after treatment with 50 μg/mL of CSC (Figure 3-3B). However, at such a high concentration most of the cells have died. A concentration of 25 μg/mL of CSC was used for the rest of the experiments because it represented a more common treatment concentration and resulted in less cell toxicity and death. Other studies found similar results (Nagaraj et al., 2006). These studies confirmed that pBcl-xLP promoter activity was induced in MCF10A cells by CSC treatment in a time and concentration-dependent manner.

**C/EBP-binding Sites on the pBcl-xLP are CSC-responsive Elements**

Next, the *cis*-elements on the pBcl-xLP responsible for this upregulation were identified. Eukaryotic gene expression is regulated in part by transcriptional mechanisms including transcriptional initiation, which involves site specific protein to DNA and protein to protein interactions at the initiation site (Van Dyke et al., 1988). Transcription factors are essential for the recruitment of RNA polymerase II (RNAP II) and other members of the pre-initiation complex (PIC) to the transcription initiation site. Transcription initiation is the rate-limiting step in the gene transcription and the role of general transcription factors is therefore critical to the transcription of genes.

Putative *cis*-elements on the *bcl-xl* promoter represent possible binding sites for transcription factors that can activate or repress the transcription of the gene. To determine

which transcription factor was increasing pBcl-xLP expression in treated cells, PCR was used to clone nine promoter deletion constructs from the pBcl-xLP promoter. These constructs were designed to sequentially delete potential regulatory elements on the pBcl-xLP (Figure 3-4A). The pBcl-xLP and deletion constructs were individually transfected into MCF10A cells, treated with CSC, and promoter activity was measured (Figure 3-4B). As expected, the pBcl-xLP (-145, +707) showed no significant promoter activity or induction. Conversely, pBcl-xLP (-54,+647) activity was significantly induced by CSC as shown in Figure 3-3. Basal promoter activity was reduced with pBcl-xLP (-28,+707), which reflected the loss of the C/EBP-I site, but the CSC response was maintained, suggesting that C/EBP-I was important for the basal *bcl-xl* promoter activity and that it may or may not have been responsive to CSC treatment. The *bcl-xl* promoter activity continued to decrease as other elements were deleted. However, the CSC response was maintained up to pBcl-xLP (-28,+222). The promoter activity decreased at the next construct, pBcl-xLP (-28,+132), which represented the loss of the site C/EBP-II. Loss of this site resulted in an unrecoverable decrease in promoter activity. These results suggested that the C/EBP-II on the pBcl-xLP may have been the primary CSC-responsive site on the pBcl-xLP promoter.

Site-directed mutagenesis was used to examine whether C/EBP sites were necessary for CSC-induced promoter activity. Site-directed mutagenesis was performed separately on the C/EBP site-I and site-II of the pBcl-xLP promoter (Figure 3-5). The mutant constructs were then transfected into MCF10A cells that were subsequently treated with CSC. While the wild-type promoter showed a significant induction of activity in response to CSC treatment, neither mutant promoter construct showed a significant induction in response to treatment (Figure 3-6). These

results indicate that C/EBP site-I and site-II elements on the pBcl-xLP respond to CSC treatment in MCF10A cells.

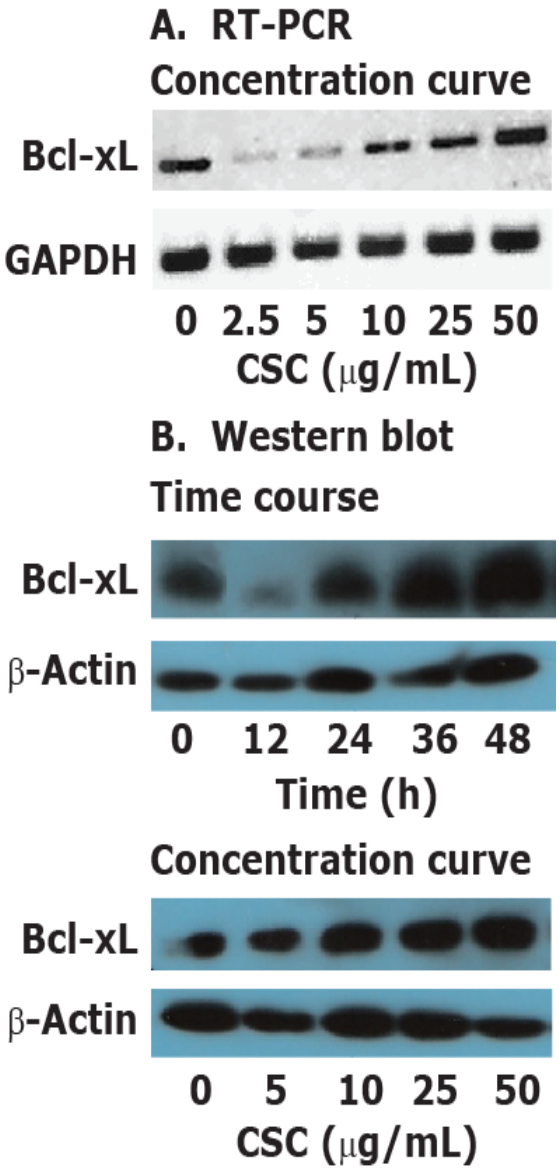


Figure 3-1. *Bcl-xl* mRNA and protein levels are induced in MCF10A cells treated with CSC. (A) RNA was isolated from cells treated with increasing concentrations of CSC for 24 h. RT-PCR was performed with primers specific to the *bcl-xl* cDNA sequence. GAPDH primers were used on the same samples as a loading control. (B) Treated cells were processed for whole cell extract. For the time course, cells were treated with 25 μg/mL of CSC for various time points. For the concentration curve, cells were treated with increasing amounts of CSC for 24 hours. Blots were stripped and probed for β-Actin as a loading control. Data is representative of three separate experiments.

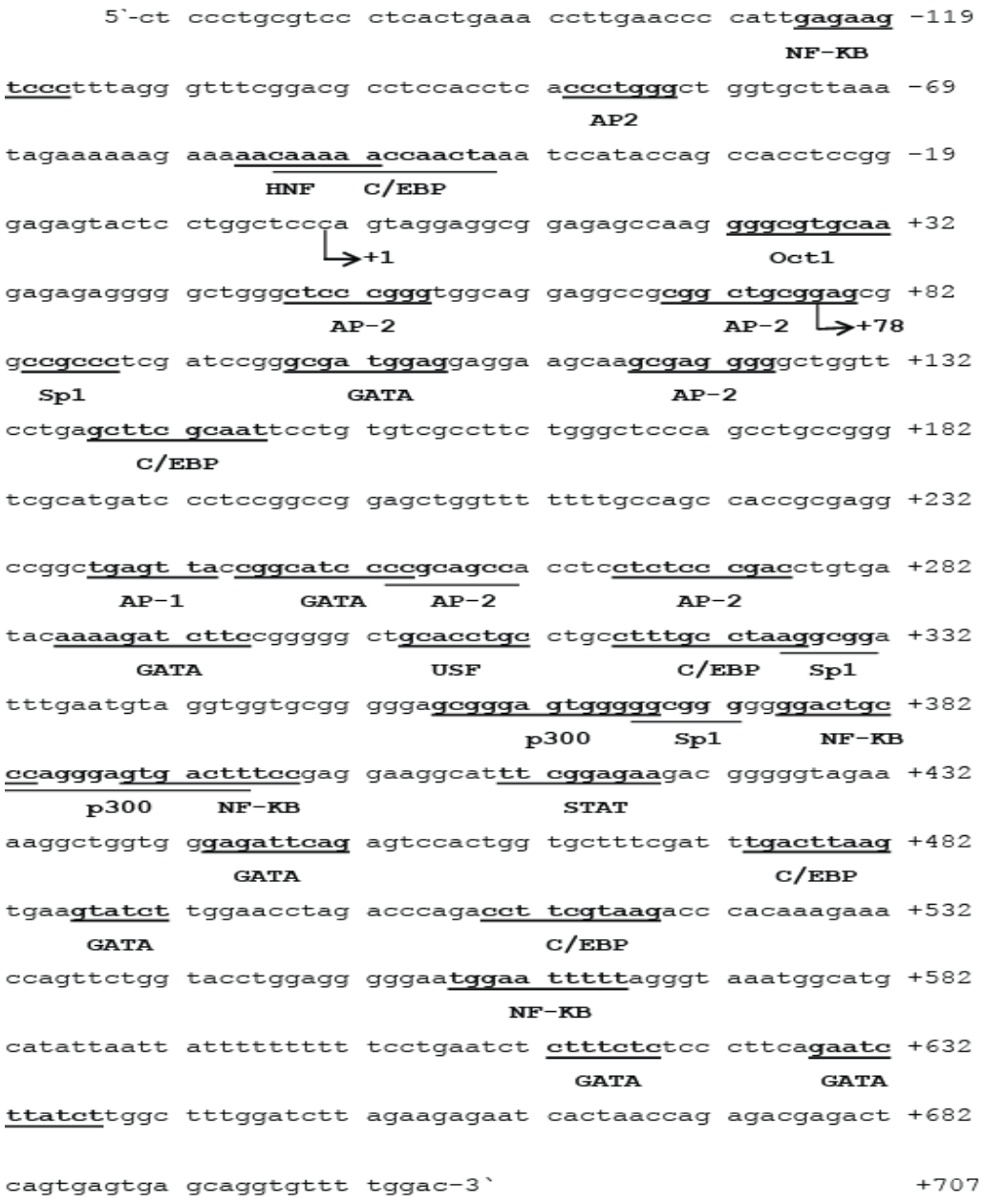


Figure 3-2. Sequence of the cloned human *bcl-xl* promoter, pBcl-xLP. Nucleotides 226 to 915 from the human *bcl-xl* promoter were cloned into a pGL-3 Basic Luciferase Vector and was named pBcl-xLP. The pBcl-xLP contains binding sites for several common transcription factors and the transcription initiation sites are located at +1 and +78.

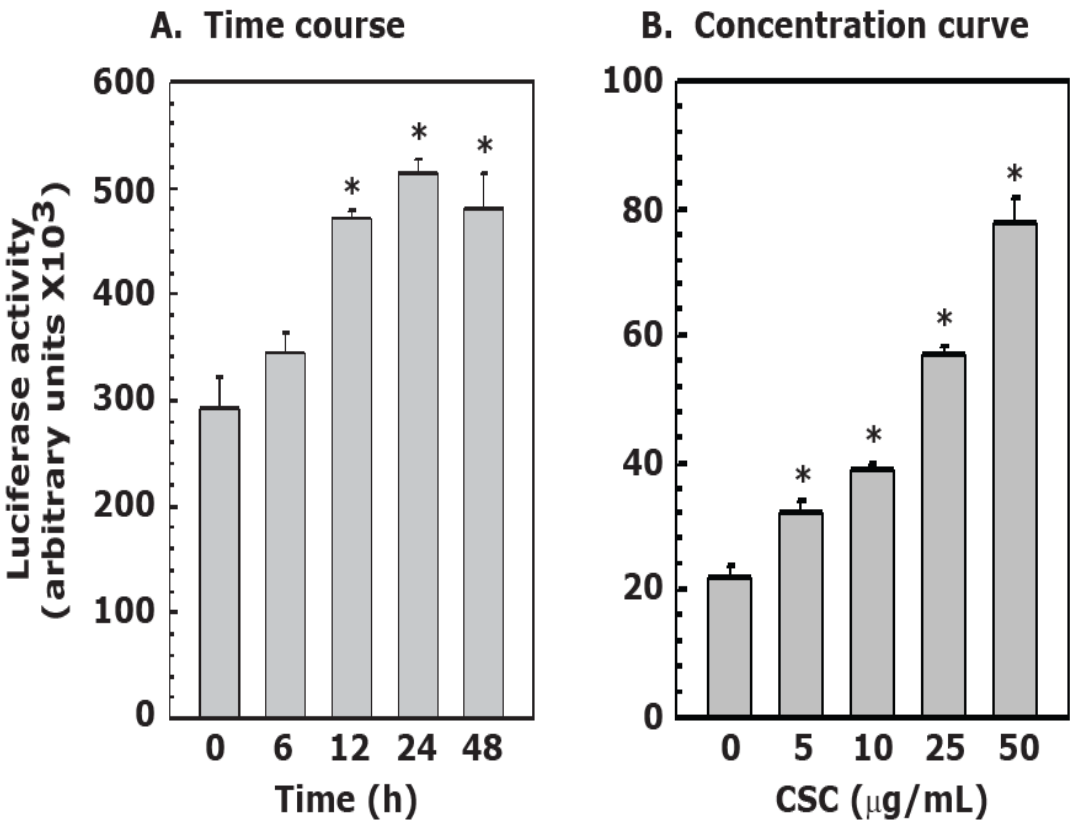


Figure 3-3. CSC treatment induces pBcl-xLP promoter activity in vitro. The pBcl-xLP construct was transfected into MCF10A cells, which were subsequently treated with CSC. Cells were harvested for the (A) time course and (B) concentration curve and promoter activity was measured with luciferase assays normalized with β-galactosidase activity. Data is the average of three replicates + SE and representative of three independent experiments. Asterisks (*) indicate a significant difference compared to the promoter activity of the untreated cells.

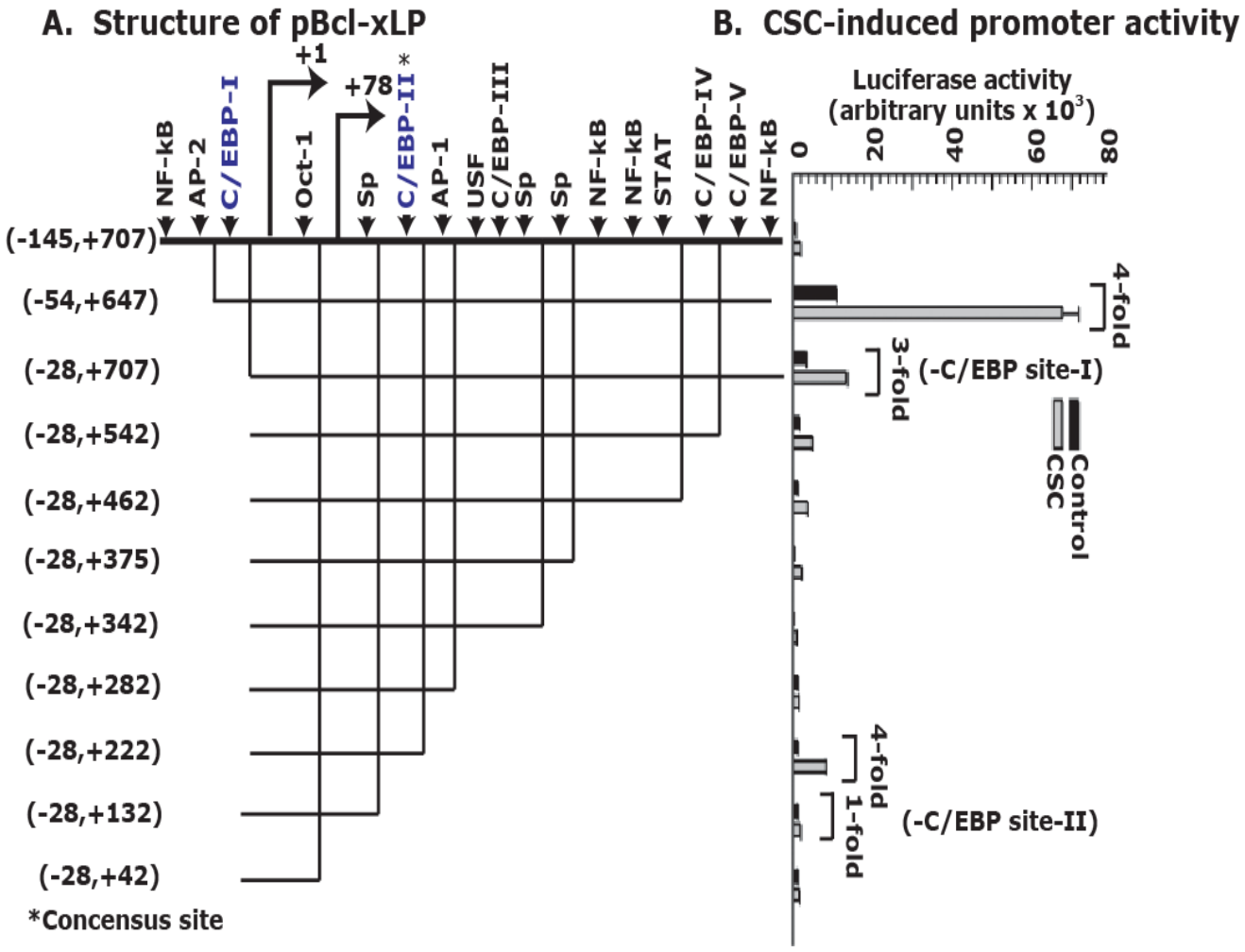


Figure 3-4. The pBcl-xLP promoter contains CSC-responsive *cis*-elements. (A) The basal promoter construct was identified as pBcl-xLP (-54,+647). Nine pBcl-xLP deletion constructs (labelled according to their lengths) were designed to sequentially delete putative *cis*-elements. Arrows indicate the transcription initiation sites. (B) For the determination of the CSC-responsive *cis*-elements on the pBcl-xLP, luciferase assays normalized with β-galactosidase activity were used to measure pBcl-xLP promoter activity in MCF10A cells separately transfected with each construct and then treated with CSC. Data is the average of three replicates $\pm$ SE and representative of three independent experiments.


Figure 3-5. C/EBP mutations introduced on the pBcl-xLP. Site-directed mutagenesis was used to introduce two separate mutations in the pBcl-xLP construct. The resulting constructs were named C/EBP site-I mutant and C/EBP site-II mutant, respectively. The mutations (in red) mirror the mutations used for subsequent EMSA experiments.

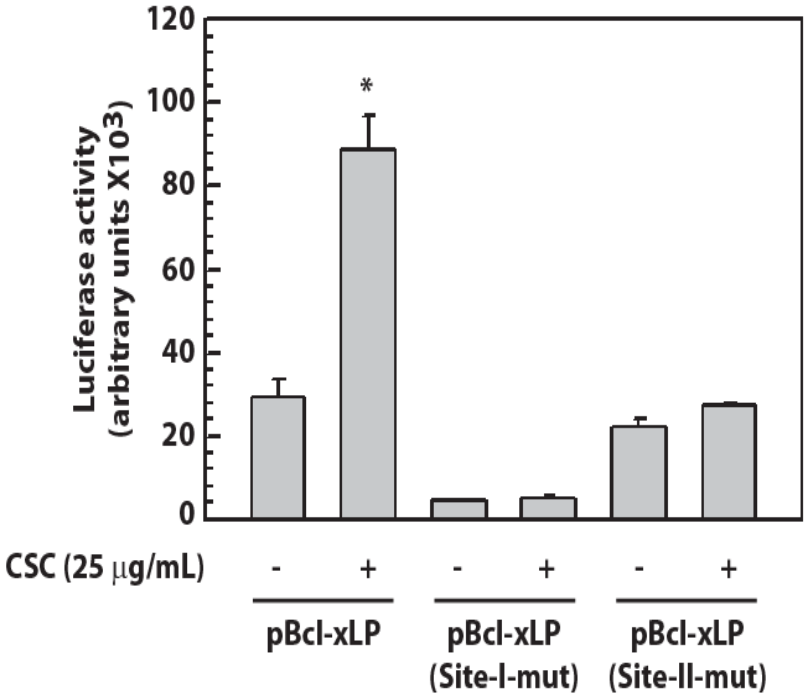


Figure 3-6. Site-directed mutagenesis of C/EBP sites on the pBcl-xLP attenuates CSC-induced promoter activity. Wild-type pBcl-xLP, C/EBP site-I mutant, or C/EBP site-II mutant were separately transfected into MCF10A cells. The transfected cells were then treated with CSC and promoter activity was analyzed with luciferase assays normalized with β-galactosidase activity. Data is the average of three replicates $\pm$ SE and representative of three independent experiments. Asterisks (*) indicate a significant difference compared to the promoter activity of the untreated cells.

CHAPTER 4
C/EBPβ REGULATES BCL-XL IN CSC-TREATED MCF10A CELLS

## Introduction

The loss of C/EBP site-I and -II reduced CSC-induced pBcl-xLP activity (Figure 3-6). C/EBPβ is one of the six C/EBP proteins and evidence suggests its role in human breast carcinogenesis, however, this role is not completely understood (Milde-Langosch et al., 2003; Zahnow, 2002). Its putative role in human breast carcinogenesis made C/EBPβ an appropriate C/EBP target protein responsible for the upregulation in *bcl-xl* in CSC-treated MCF10A cells.

## Results

### C/EBPβ is Induced by CSC Treatment in MCF10A Cells

Western analysis was used to confirm that C/EBPβ was induced by CSC treatment. The antibody used in this Western analysis was raised against the carboxy-terminal of C/EBPβ, and therefore had the ability to detect all three isoforms. Two C/EBPβ isoforms LAP1 (45 kDa) and LAP2 (42 kDa) were detected in whole cell extracts from CSC-treated MCF10A cells; however, LIP was not detected in these cells (Figure 1-4). Only LAP2 levels were significantly increased in a time and concentration-dependent manner. These experiments confirmed that C/EBPβ protein levels are induced by CSC treatment.

### C/EBPβ Site-II of the pBcl-xLP is Specific for the CSC Response in MCF10A Cells

In previous experiments, two putative C/EBP sites on the pBcl-xLP were identified as being inducible by CSC. EMSA was used to characterize these C/EBP sites as CSC-responsive sites on the pBcl-xLP. To do this, $^{32}$P-labelled double-stranded probes, identical to C/EBP site-I and site-II sequences were incubated with MCF10A nuclear extract. Two DNA-protein complexes (shifted bands I and II) were visualized with autoradiography (Figure 4-2). These bands represent nuclear proteins binding to C/EBP regulatory elements on the *bcl-xl* promoter.

For competition experiments, the appropriate unlabelled wild-type or mutant oligos were added at increasing fold excess (Figure 4-2A). For C/EBP site-I and C/EBP site-II, the unlabelled wild-type probe competed out the $^{32}$P-labelled probe, in a concentration dependent manner, as expected. However, the unlabelled C/EBP site-I mutant oligo also competed out the $^{32}$P-labelled probe at that site. This indicated one of the two scenarios: 1) the mutant is not sufficient enough to decrease binding, or 2) the binding at this site is non-specific. Specificity was tested by adding increasing concentrations of a non-specific, unlabelled GAGA probe to the reactions as indicated. The unlabelled probe also competed with the $^{32}$P-labelled wild-type probe for C/EBP site-I, confirming the second possibility that the binding at C/EBP site-I was non-specific. Conversely, the reactions with C/EBP site-II were not competed out by either the unlabelled mutant or the unlabelled non-specific GAGA probe, suggesting that MCF10A nuclear extract contained a protein that specially bound to C/EBP site-II.

The protein binding to the pBcl-xLP C/EBP site-II was identified with super-shift analysis. The three C/EBP proteins (C/EBPα, β, and δ) are expressed in mammary tissue (Gigliotti and DeWille, 1998; Sabatakos et al., 1998). To determine whether the binding was specific to either protein, antibodies specific to each were added to the reaction mixtures as indicated. No shifted bands are observed in reactions with the $^{32}$P-C/EBP site-I. $^{32}$P-C/EBP site-II reactions showed a shifted band only with the addition of anti-C/EBPβ antibody (Figure 4-2B).

It was also determined whether CSC induced C/EBPβ protein to show increased binding to the pBcl-xLP promoter in the gel-shift and super-shift assays. $^{32}$P-labelled C/EBP site-II probe was incubated with nuclear extracts isolated from untreated and CSC-treated MCF10A cells. Results showed a drastic increase in the shifted band II with extract from CSC-treated cells as compared to untreated cells (Figure 4-2C). In the reactions with untreated nuclear

extract, the addition of anti-C/EBPβ antibody resulted in a super-shift as seen in Figure 4-2B. In the reaction with CSC-treated nuclear extract, there was a shift of band I and increased binding at band II. The addition of anti-C/EBPβ antibody resulted in an additional shift of shifted band in lane 3 (without antibody) and increased binding at band II. These results indicated that CSC treatment increased the binding of C/EBPβ proteins to the pBcl-xLP *in vitro*.

**C/EBPβ Binds the Endogenous *Bcl-xl* Promoter in Response to CSC Treatment**

To confirm that C/EBPβ binds the *bcl-xl* promoter *in vivo*, ChIP analysis was performed. MCF10A cells were treated with increasing concentrations of CSC for 24h. The ChIP analysis was performed as described in Materials and Methods. PCR of the resulting DNA was performed with primers specific to the pBcl-xLP C/EBP site-II. In the untreated cells there was an initial binding of C/EBPβ to the *bcl-xl* promoter. This binding slightly decreased at 10 μg/mL of CSC treatment, increased to a level higher than that in the untreated cells at 25 μg/mL, and was sustained at 50 μg/mL of CSC treatment (Figure 4-3). This experiment mirrored the *bcl-xl* mRNA expression in Figure 3-1A. The results from the EMSA and ChIP analysis suggested that C/EBPβ binds and regulates *bcl-xl* gene expression in MC10A cells in response to CSC treatment.

**Overexpression of C/EBPβ Protein LAP2 Increases pBcl-xLP Promoter and Protein Levels in MCF10A Cells**

To demonstrate that CSC treatment increased C/EBPβ levels which bound to the *bcl-xl* promoter and regulated it expression, this condition was recapitulated by overexpression of C/EBPβ protein in MCF10A cells. To do this, each C/EBPβ isoform: hLAP1, hLAP2, and hLIP were transfected into MCF10A cells and the effect on pBcl-xLP promoter activity was measured with luciferase activity. Each C/EBPβ construct induced promoter activity. However, only hLAP2 had a significant induction when compared to the empty pCDNA3.1 vector (Figure 4-

4A). To determine the effect of these constructs on Bcl-xL protein levels, each construct was separately transfected into MC10A cells and after 48 h, the cells were harvested and processed for whole cell extract. The protein was used for Western analysis of C/EBPβ and Bcl-xL protein levels. C/EBPβ protein levels confirmed that the appropriate isoforms were overexpressed. Bcl-xL protein levels were similar in control cells and cells transfected with the empty pCDNA3.1 vector (Figure 4-4B). The overexpression of hLAP1 slightly increased Bcl-xL protein levels, while LAP2 showed the most significant increase of Bcl-xL. Conversely, hLIP expression caused a decrease in Bcl-xL protein. These results suggest that C/EBPβ, specifically LAP2, has a role in the regulation of pBcl-xLP promoter activity and protein levels in the absence of CSC treatment.

Co-transfection of C/EBPβ overexpression constructs with pBcl-xLP mutant constructs indicated that the loss of C/EBP sites on the promoter disrupted the promoter activity compared to the results from Figure 4-4 with the wild-type promoter (Figure 4-5). The presence of C/EBP site-II was required for the CSC-induced and C/EBPβ-induced upregulation of *bcl-xl* promoter activity in MCF10A cells and together, these data indicated that C/EBPβ was necessary for the upregulation of *bcl-xl* in CSC-treated MCF10A cells.

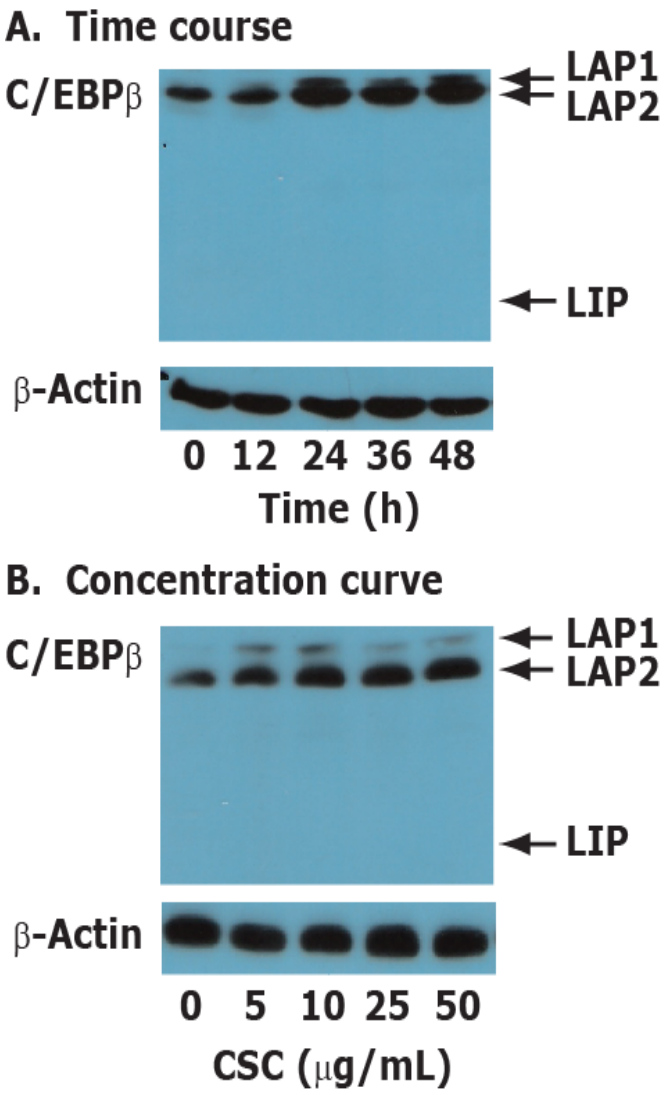


Figure 4-1. C/EBPβ protein levels are induced in MCF10A cells treated with CSC. MCF10A cells were treated with 25 μg/mL of CSC for various time points. For the concentration curve, cells were treated with increasing amounts of CSC for 24 hours. The protein was used for Western blot analysis of C/EBPβ protein. Blots were stripped and probed for β-Actin as a loading control. Data is representative of three independent experiments.

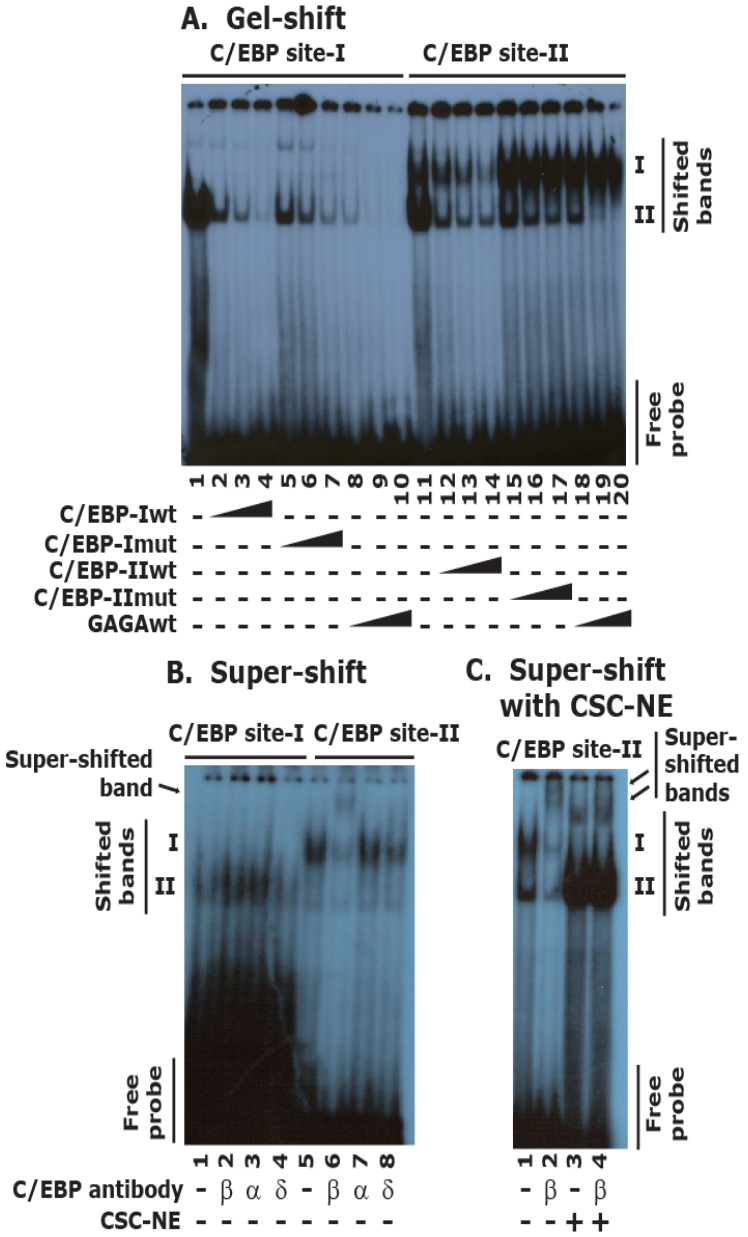


Figure 4-2. C/EBPβ binds the *bcl-xl* promoter *in vitro*. $^{32}$P-labelled C/EBP site-I or $^{32}$P-labelled C/EBP site-II oligonucleotides were incubated with MCF10A nuclear extract. (A) For competition experiments, 2.5, 5, and 10 fold excess of the appropriate unlabelled wild-type or mutant C/EBP site were added to the indicated lanes. Unlabelled GATA oligonucleotides were added to the lanes indicated to determine binding specificity. (B) Super-shift analysis of C/EBP site-II was carried out by adding antibodies specific to C/EBPβ, α, or δ to the reaction mixtures as the lanes indicate. (C) The effects of CSC treatment on super-shift analysis were analyzed by adding anti-C/EBPβ antibody to reaction mixtures using MCF10A nuclear extract from untreated or CSC-treated cells. Data is representative of three independent experiments.

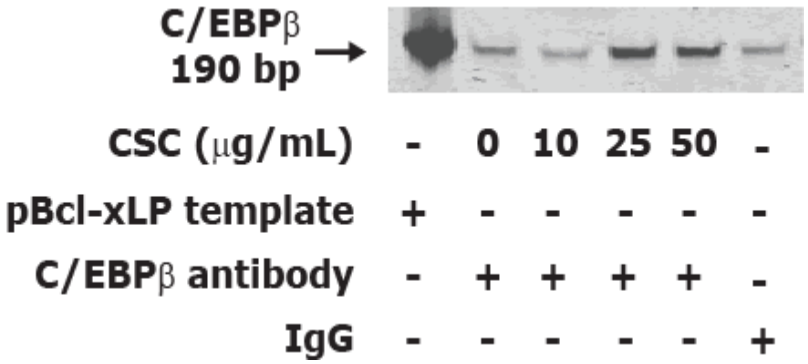


Figure 4-3. C/EBPβ is present on the *bcl-xl* promoter of MCF10A cells *in vivo*. ChIP was performed on MCF10A cells treated with increasing concentrations of CSC for 24h. An anti-C/EBPβ antibody was used to immunoprecipitate the DNA-protein complexes. PCR was performed on the isolated DNA with primers specific for C/EBP site-II on the pBcl-xLP promoter. Data is representative of three independent experiments.

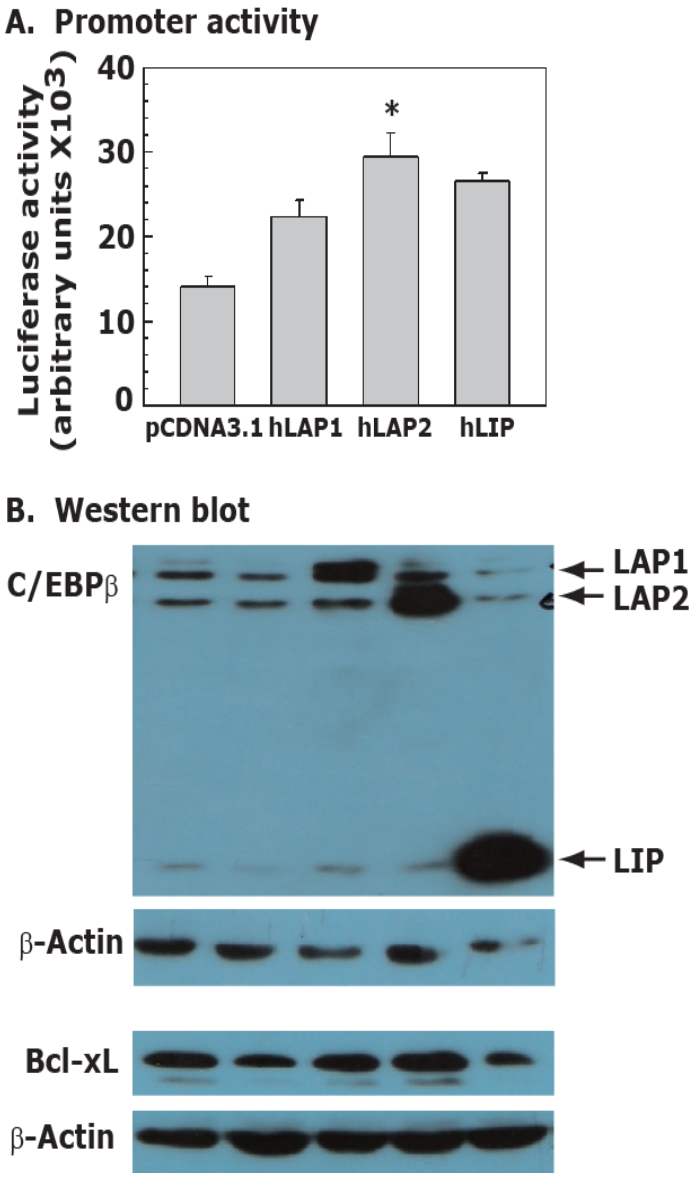


Figure 4-4. Overexpression of C/EBPβ induces pBcl-xLP promoter activity and Bcl-xL protein levels in MCF10A cells. Human C/EBPβ overexpression constructs, LAP1, LAP2, and LIP were co-transfected with the pBcl-xLP promoter into MCF10A cells. (A) Promoter activity was analyzed with luciferase assays and normalized with β-galactosidase activity. Data is the average of three replicates $\pm$ SE and representative of three independent experiments. Asterisks (*) indicate a significant difference from the promoter activity of the empty pCDNA3.1 vector. (B) Western analysis of C/EBPβ was used to confirm the overexpression of the appropriate construct and changes in Bcl-xL expression were assessed. The same whole cell extract was used to detect protein (C/EBPβ or Bcl-xL) on two blots. The blots were stripped and probed for β-Actin as a loading control. Data is representative of three independent experiments.

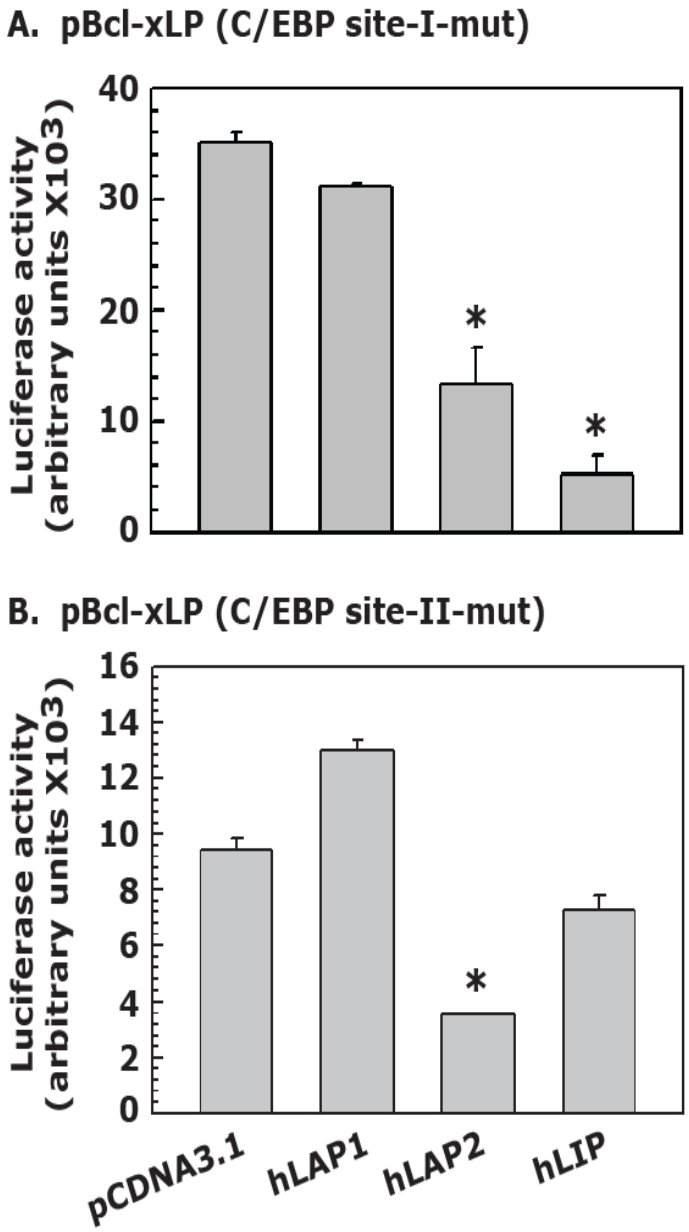


Figure 4-5. Site-directed mutagenesis of C/EBP sites on the pBcl-xLP attenuates the C/EBPβ-induced activation of the pBcl-xLP promoter. C/EBPβ overexpression constructs were co-transfected with each C/EBP mutant construct. Promoter activity was analyzed with luciferase assays and normalized with B-galactosidase activity. Data is the average of three replicates $\pm$ SE and representative of three independent experiments. Asterisks (*) indicate a significant difference from the promoter activity of the empty pCDNA3.1 vector.

# CHAPTER 5
# SUMMARY AND DISCUSSION

Breast cancer is the most common cancer women. In light of this and other studies, cigarette smoking may play a role in breast cancer development. Approximately 45 million Americans currently smoke. Even though the numbers of women smokers have decreased in the past, prevalence declined little from 1992-1998. Smoking prevalence continues to increase in women with less education and/or those below the poverty level (Centers for Disease Control and Prevention, 2007; National Center for Health Statistics, 2006). These growing groups of women are of particular interest because when they develop cancer, their accessibility to healthcare is limited. Breast cancer can arise 30-40 years from the onset of smoking and smoking for a long duration may be associated with increased breast cancer risk (Terry and Rohan, 2002). Additionally, females beginning to smoke at younger ages, increase their risk of smoking-related diseases including cancers (U.S. Department of Health and Human Services, 1994). This trend may be linked to the growing number of breast cancer cases diagnosed in the United States.

Prevention of breast cancer has been obstructed by the lack of knowledge about the etiology of the disease (Li et al., 1996a). There are two categories of breast cancer risk factors: biological risk factors and life-style risks. The primary biological risk factor is gender and increasing age. Other biological factors include, but aren't limited to genetic mutations in certain genes (BRCA1, BRCA2) and family, or personal history of breast cancer. Reproductive risk factors include menstrual periods that start early or end late in life, not having children, and childbirth after the age of 30. Behavioral factors include postmenopausal hormone replacement therapy (slight increased risk), alcohol use, obesity/high-fat diets, and lack of physical activity (American Cancer Society, 2008). Known risk factors (family history and reproductive factors)

only account for 30% of breast cancer cases (John and Kelsey, 1993). Understanding other risk factors, such as the role of smoking, is essential to developing prevention strategies and therapeutic interventions to breast carcinogenesis.

In this study, the MCF10A cell line was utilized to determine the mechanism by which cigarette smoking might be linked to breast carcinogenesis. The MCF10 series of cell lines are human breast epithelial cells that have been extensively characterized (Soule et al., 1990; Tait et al., 1990). The founding cells, MCF10M, were derived from a 36-year old parous pre-menopausal woman with extensive fibrocystic disease, but no family history or histological evidence of breast malignancy. These mortal diploid cells had finite growth in culture and were described as estrogen receptor negative (ER-). MCF10A (attached) cells are a spontaneously immortalized derivative that resulted from the culturing of MCF10M cells in medium containing low calcium. Although this cell line is immortalized, MCF10A cells have characteristics of normal cells including: the lack of tumorigenecity in nude mice (Miller et al., 1993), growth factor-dependency, and anchorage-dependent growth (Soule et al., 1990; Tait et al., 1990). MCF10A cells are considered a model for normal epithelial cells and therefore provide the opportunity to study the earliest stages of transformation and tumorigenesis (Soule et al., 1990; Tait et al., 1990). However, MCF10A cells have characteristics of luminal and basal myoepithelial cells and may represent multipotent progenitor cells (Gordon et al., 2003; Neve et al., 2006). Inner luminal (secretory) and outer basal myoepithelial cells, are the two cell types that compose the acinus (Ronnov-Jessen et al., 1996; Taylor-Papadimitriou et al., 1989) , the smallest functional unit of the mammary duct (Bissell et al., 2003; Smith et al., 1984). These cell types can be distinguished by gene expression profiling and with immunohistochemistry; luminal cells express keratins 8/18 and E-cadherin, while myoepithelial cells express keratins 5/6,

integrin-β4, and laminin (Perou et al., 2000; Ronnov-Jessen et al., 1996; Sorlie et al., 2001; Sorlie et al., 2003). Recently, studies have reported that MCF10A cells have basal-like cell characteristics (Charafe-Jauffret et al., 2006; Neve et al., 2006). Cells with basal phenotypes are more likely to undergo EMT and express more aggressive, metastatic phenotypes *in vivo* (Sarrio et al., 2008). In fact, MCF10A transiently express characteristics of EMT when cultured in low densities (Sarrio et al., 2008). Despite these characteristics, MCF10A cells cluster with other cells derived from normal mammary tissue (Lombaerts et al., 2006), display normal morphology when grown on basement membrane, and do not display a fully mesenchymal phenotype (Zajchowski et al., 2001). Using primary mammary cells as an experimental model is difficult and usually does not allow for long-term observations. The *in vitro* and *in vivo* characteristics of the MCF10A cell line indicate that these cells, as originally hypothesized (Soule et al., 1990; Tait et al., 1990), serve as the most appropriate model for normal epithelial cells in experimental studies.

MCF10A cells also provide a model to study estrogen receptor negative (ER-) cells. Approximately one-third of breast cancer patients respond to endocrine therapy, most of these have ER+ tumors (Jordan, 1993). Many breast cancers are ER-, making them refractory to anti-hormone therapy and aromatase inhibitors. These tumors are more aggressive (increased invasion and distant metastasis) than ER+ tumors (Gago et al., 1998). A significant portion of patients with ER+ cancers are initially not responsive to treatments with selective estrogen receptor modulators (SERMs) such as tamoxifen (Kumar et al., 1996). Additionally, initially responsive patients can develop resistance to anti-hormone therapy. Understanding the mechanisms by which ER- cells are transformed can also give insights to treating the patients not eligible for traditional hormone therapy.

In a previous study, CSC-mediated transformation of MCF10A cells has been described (Narayan et al., 2004). CSC has also been found to transform endocervical cells (Yang et al., 1998; Yang et al., 1997). In both cases, the expression of anti-apoptotic proteins was increased in the transformed cells (Narayan et al., 2004; Yang et al., 1998). In order to understand the mechanisms of cigarette-induced breast carcinogenesis, this study determined the transcription factor that upregulated *bcl-xl* expression in MCF10A cells after CSC treatment. Determining the role of transcription factors in CSC-mediated regulation of *bcl-xl* gene expression may give insight to this and other risk factors involved in breast carcinogenesis.

**C/EBPβ-induced Upregulation of Bcl-xL in CSC-treated MCF10A Cells**

The treatment of MCF10A cells with CSC caused the upregulation of *bcl-xl* mRNA and protein levels and the induction of Bcl-xL protein occurred at the level of transcription (Narayan et al., 2004; Figure 3-1). This observation is supported by other studies that found the induction of *bcl-xl* generally resulted from an increase in *bcl-x* promoter activity and *de novo* protein synthesis is required for the activation of *bcl-xl* transcription (Sevilla et al., 1999). This is mainly because the *bcl-xl* transcript has a short half life of about four hours (Bachelor and Bowden, 2004; Pardo et al., 2002; Sevilla et al., 1999). The increase in *bcl-xl* mRNA levels occurs through a biphasic mechanism. The base-line levels of *bcl-xl* in untreated cells ensure the survival of the cells in culture. CSC causes DNA damage in MCF10A cells (Kundu et al., 2007). After treatment with CSC, the amount of DNA damage causes the cells to respond by triggering DNA repair pathways. This is evident in the increased levels of PCNA and GADD45 protein levels previously reported. Increases in these proteins indicated active DNA repair and synthesis presumably resulting from the CSC-induced DNA damage (Narayan et al., 2004). If the DNA damage overloads the repair mechanisms, the cells undergo apoptosis, resulting in the decrease in *bcl-xl* mRNA levels after the first treatment. This decrease in *bcl-xl* levels indicated that most

treated cells die, as observed by Narayan et al. (2004). The few surviving cells were responsible for the remaining low levels of *bcl-xl* expression after the initial treatment. Cells expressing higher levels of *bcl-xl* were not removed by apoptosis. Subsequent treatments produced more DNA damage and eventually persistent mutations, possibly in tumor suppressors or oncogenes in the remaining cells. As the result of these mutations, important cell regulatory mechanisms may not have been working and *bcl-xl* expression continued to increase in these cells. Additionally, each treatment also selected for the survival of cells expressing higher levels of *bcl-xl*. As the surviving cells divided, their progeny shared the increased expression of *bcl-xl*, resulting in the time and concentration-dependent increase of *bcl-xl* mRNA levels. The survival of a few cells is all that was needed to sustain increased Bcl-xL expression because such genetic defects are clonal in nature (Tomlinson, 2001). While the time course Western blot had a biphasic response to CSC treatment, the concentration curve blot did not show this pattern. One possible explanation is that RT-PCR is more sensitive assay that can detect smaller differences in expression levels. It is also possible that there was differential regulation of *bcl-xl* mRNA and protein levels.

The cloned human *bcl-xl* promoter (pBcl-xLP) was responsive to CSC treatment when transfected into MCF10A cells (Figure 3-3). Two pBcl-xLP promoter constructs were cloned. The lack of promoter activity when the longest construct, pBcl-xLP (-145,+707), was transfected into MCF10A cells indicated that uncharacterized repressive *cis*-elements might have been present which were absent on the pBcl-xLP (-54,+647) construct (Figure 3-4B). Reductions in promoter activity also resulted from the removal of uncharacterized repressive *cis*-elements on pBcl-xLP (-28,+282). Promoter deletion studies suggested that two C/EBP *cis*-elements were responsible for the CSC-induced increase in *bcl-xl* promoter activity (Figure 3-4). C/EBPβ was

investigated as the target C/EBP protein family member. C/EBPβ is critical for mammary gland development (Robinson et al., 1998; Seagroves et al., 1998) and is increased in rodent and human breast cancer (Dearth et al., 2001; Eaton et al., 2001; Milde-Langosch et al., 2003; Zahnow et al., 1997). Studies indicate that C/EBPβ can induce a survival phenotype in intravascular cells, possibly by its anti-apoptotic activity (Shimizu et al., 2007). In subsequent experiments, protein levels of C/EBPβ were also induced by CSC treatment (Figure 4-1).

Site-directed mutagenesis confirmed that CSC-induced pBcl-xLP activity was attenuated in the absence of either C/EBP site (Figure 3-6). However, EMSA confirmed that C/EBPβ bound only to the *bcl-xl* promoter at C/EBP site-II *in vitro*. The presence of protein binding at C/EBP site-I suggested that a protein bound to the *bcl-xl* promoter at that site, but this binding was not specific to a C/EBP protein (Figure 4-2A, B). This is not surprising because C/EBP site-I is not a true C/EBP consensus site on the pBcl-xLP promoter and C/EBP site-II is 100% identical to the core C/EBP consensus sequence. The use of MCF10A nuclear extract in this assay meant that many proteins were available to bind the C/EBP site-I. The data suggested that an unidentified transcription factor bound to the pBcl-xLP at C/EBP site-I in response to CSC treatment. In parallel, C/EBPβ bound the *bcl-xl* promoter at C/EBP site-II *in vivo*, as shown by ChIP assay (Figure 4-3). This binding had a biphasic pattern similar to that seen in the mRNA analysis (Figure 3-1A), indicating that *bcl-xl* mRNA levels correspond with C/EBPβ-binding to and regulating the *bcl-xl* promoter. Overexpression studies confirmed that C/EBPβ-induced pBcl-xLP promoter and Bcl-xL protein levels (Figure 4-4) and site-directed mutagenesis showed that C/EBP-binding sites on the pBcl-xLP were necessary for C/EBPβ to properly regulate pBcl-xLP activity (Figure 4-5).

Only C/EBPβ-LAP2 protein levels were induced in time and concentration-dependent manner following CSC treatment (Figure 4-1). Additionally, only LAP2 significantly induced pBcl-xLP activity and Bcl-xL protein levels (Figure 4-4). Site-directed mutagenesis of C/EBP site-II on the pBcl-xLP results in the lowest promoter activity when LAP2 was co-transfected with the mutant construct (Figure 4-5B), supporting the hypothesis that C/EBPB-LAP2 binds to the pBcl-xLP at C/EBP site-II. Although C/EBPβ-LAP2 has not been fully implicated in human breast carcinogenesis, studies support its role in the disease. LAP2 is the most prevalent form of C/EBPβ in human breast cancer cells (Eaton et al., 2001). Increased levels of C/EBPβ-LAP2 have also been implicated in the transformation human breast epithelial cells. MCF10A cells infected with a LAP2-expressing virus became anchorage independent, expressed markers of epithelial-mesenchymal transition (EMT), and acquired invasive phenotypes (Bundy and Sealy, 2003). EMT is a mechanism that is necessary for developmental processes such as gastrulation and neural crest formation (Thiery, 2003). During EMT, cells of epithelial origin loss their characteristics and acquire mesenchymal phenotypes with increased migratory behavior and display loss of intercellular adhesion (E-cadherins), downregulation of epithelial markers (cytokeratins), and upregulation of mesenchymal markers (vimentin). Therefore, aberrant EMT plays a role in tumor invasion and metastasis (Gupta and Massague, 2006; Savagner, 2001; Thiery, 2002; Thiery and Sleeman, 2006; Thompson et al., 2005).

Additional studies found that MCF10A cells transfected with the same LAP2 virus gained epidermal growth factor (EGF)-independent growth and had disruption of normal acinar structure when grown on basement membrane (Bundy et al., 2005). Many of the characteristics displayed by MCF10A cells overexpressing LAP2 are considered hallmarks of cancer cells (Hanahan and Weinberg, 2000). The LAP-2-induced disruption of the polarized architecture in

MCF10A cells is similar to that induced by the activation of Erb2 (HER2) receptor expression in these cells. HER2 is a transmembrane receptor tyrosine kinase (Stern et al., 1986) that is overexpressed in breast cancer (Slamon et al., 1984), has roles in tamoxifen resistance (Benz et al., 1992; Leitzel et al., 1995; Wright et al., 1992), and is associated with poor clinical prognosis (Slamon et al., 1989). In MCF10A cells, activation of HER2 signaling in acini reinitiated proliferation and induced complex multi-acinar structures with filled lumen (Debnath et al., 2002; Muthuswamy et al., 2001), a process considered to be carcinogenic (Muthuswamy et al., 2001). Interestingly, the overexpression of HER2 is correlated with the upregulation of Bcl-xL protein in MCF-7 breast cancer cells and breast ductal carcinoma *in situ* tissues (Kumar et al., 1996; Siziopikou and Khan, 2005), indicating a link between LAP-2, HER2, and Bcl-xL expression. This and other studies suggest that aberrant expression of C/EBPβ isoforms, especially LAP2, contributes to breast carcinogenesis (Grimm and Rosen, 2003).

The expression and role of LIP varies by cell type. Although MCF10A cells did not express the LIP isoform (Figure 3-1), the overexpression of LIP has differential effects on *bcl-xl* promoter and protein activity. LIP expression slightly induces pBcl-xLP activity (Figure 4-4A). LIP heterodimers can act as dominant negative transcriptional regulators (Descombes and Schibler, 1991). However, LIP has also been shown to increase transcriptional activation of some genes (Hsieh et al., 1998). In MCF10A cells, LIP overexpression could have activated the transcription of pBcl-xLP into the cells. Possible mechanisms include the inhibition of genes that repress *bcl-xl* or activation of genes that increase *bcl-xl* transcription (Dearth et al., 2001). This study indicates that when in excess, LIP may bind the pBcl-xLP at C/EBP site-I. In Figure 5-4A (disruption of C/EBP site-I), when LIP is overexpressed, the pBcl-xLP activity is at its lowest level compared to the other overexpression constructs. This binding was not detected in

EMSA experiments (Figure 4-2) because LIP is not endogenously expressed in MCF10A cells (Figure 3-1). Conversely, LIP overexpression decreases Bcl-xL protein levels in MCF10A cells, by decreasing protein levels of LAP2 (Figure 4-4B). This and other studies have shown that LIP decreases LAP1 and LAP2 protein expression in MCF10A cells (Bundy et al., 2005) but the mechanism by which LIP decreases LAP2 expression remains unclear.

**Induction of C/EBPβ by CSC Treatment**

The mechanism by which C/EBPβ is induced by CSC continues to be unclear. It is possible that the protein undergoes post-translational modifications in response to CSC treatment. C/EBPβ is highly modified in breast cancer cells (Eaton et al., 2001). Gel-shift analysis with nuclear extract from CSC-treated cells showed a slower migrating band before the addition of antibody, when compared to analysis with untreated nuclear extract (Figure 4-2C). This higher band could be the result of a modification that slows band migration. Post-translational modifications are required for the activation of C/EBPβ and phosphorylation readily occurs on the C/EBPβ protein. Phosphorylation functions to increase C/EBPβ transcriptional activity and efficiency (Nakajima et al., 1993; Trautwein et al., 1993). Dual phosphorylation at Thr188 by MAP kinase and then at Ser184 or Thr179 by glycogen synthase kinase β (GSK3β) causes a change in confirmation that renders the leucine zipper of the monomeric protein available for the dimerization that is required for DNA-binding activity (Tang et al., 2005) and renders the basic region accessible to bind regulatory elements (Kim et al., 2007). The protein is also phosphorylated by mitogen-activated protein (MAP) kinase (Nakajima et al., 1993; Trautwein et al., 1993) and by protein kinase C (PKC) on Ser105 in the transactivation domain (Trautwein et al., 1993). Differential phosphorylation of C/EBPβ may account for its participation in a wide variety of biological effects (Piwien-Pilipuk et al., 2002). It has been speculated that C/EBPβ has negative regulatory regions that can also be phosphorylated.

Therefore, the protein may be present in cells as a repressed transcription factor that becomes activated upon phosphorylation (Kowenz-Leutz et al., 1994; Williams et al., 1995).

C/EBPβ can also be acetylated (Cesena et al., 2007; Duong et al., 2002; Joo et al., 2004). The acetylation of proteins was first detected in histones and is considered a mechanism allowing DNA to become accessible to transcription regulatory machinery (Allfrey et al., 1964; Roth et al., 2001). C/EBPβ has been shown to interact with the coactivator, p300 (Mink et al., 1997), that has acetyltransferase activity (Ogryzko et al., 1996) and with the acetyltransferase, cyclic AMP (cAMP) response-element-binding protein (Duong et al., 2002; Kovacs et al., 2003). Recently, a novel acetylation site at Lys-39, which is activated by growth hormone (GH), was identified. Mutations in this site decreased the ability of the protein to mediate transcriptional activation of target genes, *c-fos* and *c/ebpα* (Cesena et al., 2007). The effect acetylation has on C/EBPβ activity is context specific. The association of Stat5 with histone deacetylase (HDAC) deacetylated C/EBPβ and promoted transcription of the target gene *Id-1* (Xu et al., 2003).

CSC treatment could also affect the localization of C/EBPβ protein in MCF10A cells by post-translational modifications. The localization of the protein probably contributes to its function (Eaton et al., 2001). C/EBPβ is localized primarily in the cytoplasm in primary human mammary epithelial cells, but shifts to the nucleus where it can more readily act on target genes, in breast cancer cell lines (Eaton et al., 2001). Phosphorylation has also been shown to affect the subcellular distribution of C/EBPβ. Relocalization of C/EBP proteins to an active, nuclear state is mediated by cAMP or Ca2+-dependent protein kinases (Metz and Ziff, 1991). The nuclear import of C/EBPβ allowed for the transcriptional activation of β-casein in mouse primary mammary epithelial cells (Kim et al., 2002). CSC treatment may activate signal transduction pathways that affect the translation of C/EBPβ isoforms. PKR and mTOR affect the translation

of C/EBPβ isoforms and aberrant translational control of these kinases inhibited differentiation and induced mammary epithelial cell transformation (Calkhoven et al., 2000). Ras and PI3K signaling also targets C/EBPβ (Bundy and Sealy, 2003).

**The Potential Role of C/EBPβ in CSC-induced Breast Carcinogenesis**

C/EBPβ expression is a critical component in the control of mammary epithelial cell proliferation and differentiation in the functional mammary gland (Robinson et al., 1998; Seagroves et al., 1998). It stands to reason that overexpression of the protein could lead to hyper proliferation of mammary epithelial cells and eventually breast carcinogenesis. However, the mechanisms by which C/EBPβ influences breast carcinogenesis in general, are not well established. The acquired metastatic properties of C/EBPβ may be partially regulated by enhanced survival of cells. The overexpression of C/EBPβ in rat pancreatic tumor cells resulted in increased levels of Bcl-xL (Shimizu et al., 2007). The current study indicates that the anti-apoptotic activity of C/EBPβ may occur through the upregulation of Bcl-xL. The involvement of C/EBPβ in breast carcinogenesis probably involves interactions with other proteins. The role of C/EBPβ in cell cycle progression is dependent on its interactions with Rb and cyclin D1. Rb interacts with C/EBPβ, however, the implications of these interactions are not fully understood (Charles et al., 2001; Chen et al., 1996a; Chen et al., 1996b). LAP2 selectively activates the cyclin D1 promoter (Eaton et al., 2001). The *cyclin D1* gene, plays a role in cell cycle progression, is amplified in 15-20% of breast cancers, and the protein or mRNA is overexpressed in about 50% of breast cancers (Bartkova et al., 1994; Buckley et al., 1993). It has been suggested that the inability of LAP1 to activate the cyclin D1 promoter is due to the lack of the required protein-protein interactions (Eaton et al., 2001).

C/EBPβ protein interacts with proteins to open chromatin for access to transcription factors and RNAPII. LAP1 recruits SWI/SNF complexes to activate gene promoters (Kowenz-Leutz and Leutz, 1999). SWI/SNF is a chromatin remodeling complex that opens chromatin for RNA polymerase II loading and is required for eukaryotic transcription (Narlikar et al., 2002). C/EBPβ along with Runx2 recruits SWI/SNF to the bone-specific osteocalcin gene to recruit RnAP II (Villagra et al., 2006). The oncogenic transcription factor, myb, and C/EBPβ work together to open chromatin at the (myb-inducible myelomoncytic-1) *mim-1* promoter. C/EBPβ alone partially activated promoter activity, but Myb was required for full transcriptional activation. This study was the first to identify C/EBPβ in the initial steps of localized chromatin opening at a relevant target region (Plachetka et al., 2008). From these studies it is reasonable to hypothesize that increased levels of C/EBPβ play a role in carcinogenesis by interacting with other proteins to open chromatin and induce transcription of oncogenic genes.

**The Relationship between C/EBPβ, Bcl-xL, and Breast Carcinogenesis**

The results of this study indicate that C/EBPβ is at least one of the transcription factors that regulates the induction of *bcl-xl* mRNA and protein levels in CSC-treated MCF10A cells. This discovery places the *bcl-xl* promoter as a novel target gene of transcription factor, C/EBPβ. The following model is proposed as a starting point to uncovering the role of C/EBPβ in the upregulation of *bcl-xl* in MCF10A cells treated with CSC (Figure 5-1). When human breast epithelial cells are exposed to CSC, cells are damaged and most undergo apoptosis. In the few surviving cells the C/EBPβ protein levels are activated by an unknown mechanism. This activation triggers the dimerization of two C/EBPβ LAP2 monomers. LAP2 homodimers then bind C/EBP site-II on the *bcl-xl* promoter and transcriptionally activate the mRNA and subsequent protein expression levels of Bcl-xL. Since Bcl-xL is by definition an anti-apoptotic protein (Boise et al., 1993) it is expected that increased levels of Bcl-xL impede the apoptotic

pathway, allowing for the accumulation of DNA damage (Mendez et al., 2005; Mendez et al., 2001). When genes involved in DNA repair or the apoptotic pathway are also altered, the accumulation of DNA damage can lead to cell cycle deregulation. Disruption of apoptotic pathways, may also allow for damaged cells to survive and acquire the characteristics of transformed cells. Bcl-xL expression has roles in breast carcinogenesis. Tumor cells overexpressing Bcl-xL can adapt to new microenvironments (Espana et al., 2004; Fernandez et al., 2002; Mendez et al., 2006; Rubio et al., 2001), have increased potential to metastasize (Fernandez et al., 2002; Rubio et al., 2001), and are also more prone to be resistant to chemotherapy and radiation therapy (Cherbonnel-Lasserre et al., 1996; Datta et al., 1995; Fernandez et al., 2002; Simonian et al., 1997). All of these factors contribute to the initiation and promotion of breast carcinogenesis.

The relationship between the C/EBPβ and Bcl-xL is supported by several lines of evidence. This study indicates that C/EBPβ is required for CSC-induced regulation of *bcl-xl* in MCF10A cells. The proteins are required for proper mammary gland development (Robinson et al., 1998; Seagroves et al., 1998; Seagroves et al., 2000; Walton et al., 2001). Bcl-xL and C/EBPβ are both expressed in stages of mammary gland development characterized by rapidly proliferating cells such as lactation and pregnancy and are decreased during the apoptotic involution phase (Gigliotti and DeWille, 1998; Heermeier et al., 1996; Robinson et al., 1998; Sabatakos et al., 1998). Additionally, both are overexpressed in human breast cancer (Eaton et al., 2001; Krajewski et al., 1999) and are associated with cancer progression, and more invasive tumors that display higher histological grades (Eaton and Sealy, 2003; Milde-Langosch et al., 2003; Olopade et al., 1997). This data suggests that it is likely that Bcl-xL and C/EBPβ cooperate during human breast tumorigenesis. The role of C/EBPβ in inducing Bcl-xL

expression, along with other studies, also indicates that LAP2 is the primary C/EBPβ isoform involved in breast carcinogenesis. The current study not only provides insight to the mechanism of cigarette smoke-induced breast epithelial cell transformation and carcinogenesis, it adds to the literature that supports the link between cigarette smoking and increased breast cancer risk. The results of the present study can therefore be used to determine chemotherapeutic targets to decrease aberrant *bcl-xl* expression during breast carcinogenesis especially that which is induced by exposure to cigarette smoke.

As with other proteins, there are a many factors that can regulate *bcl-xl* activity and other transcription factors may still have a role in *bcl-xl* regulation. Studies have identified four major classes of transcription factors that regulate the *bcl-xl* gene: Ets, Rel/ Nuclear factor kappa B (NF-κB), STAT, and AP1 (Grad et al., 2000; Sevilla et al., 2001). One of the first studies aimed at identifying transcription factors regulating the *bcl-xl* promoter identified Ets2. Ets2, a member of the Ets transcription factor family, is a nuclear proto-oncogene with sequence identity to the v-ETS protein of the gag-Myb-Ets fusion protein of the E26 avian retrovirus (Boulukos et al., 1988; Ghysdael et al., 1986; Watson et al., 1985; Watson et al., 1988). Ets inhibits colony-stimulating factor 1 (CSF-1)-induced apoptosis macrophages by upregulating *bcl-xl* transcription (Sevilla et al., 1999). Ets proteins are deregulated in a number of cancers (Boyd and Farnham, 1999) and are implicated in the regulation of matrix metalloproteinase expression, which offers a potential connection to control of cell survival and metastasis (Westermarck and Kahari, 1999). The NFκB family of transcription factors is involved in the regulation of inflammation, stress and apoptosis (Beg et al., 1995; Sonenshein, 1997). NF-κB has been repeatedly shown to regulation *bcl-xl* expression (Chen et al., 2000; Chen et al., 1999; Glasgow et al., 2001; Glasgow et al., 2000; Tsukahara et al., 1999). The two proteins may form a positive feedback loop,

because Bcl-xL can affect upstream NF-κB activation (Badrichani et al., 1999). The relationship between NF-κB and *bcl-xl* raise the possibility that activity of pro-survival genes may contribute to oncogenesis when NF-κB is aberrantly expressed (Chen et al., 2000). Signal transducer and activators of transcription (STATs) play roles in growth factor, cytokine, or hormone-mediated cellular signal transduction (Darnell, 1997). Members of this protein family have been shown to regulate *bcl-xl* (Grad et al., 2000) and evidence suggests that STATs contribute to oncogenesis by modulating Bcl-xL levels (Bromberg et al., 1999; Grandis et al., 2000; Karni et al., 1999). AP1 complexes have roles in proliferation and differentiation pathways (Bannister, 1997). AP1 complexes consist of the oncogenes, Fos and Jun heterodimers or Jun homodimers, that bind to the AP1 DNA binding sites and have been shown to regulate Bcl-xL expression (Jacobs-Helber et al., 1998; Sevilla et al., 1999).

Despite these transcription factors, it is important to note that when MCF10A cells are treated with CSC, C/EBPβ is the primary transcription factor responsible for increased Bcl-xL expression. Similar to the other transcription factors that regulate Bcl-xL, C/EBPβ seems to have a role in carcinogenesis. The regulation of the *bcl-xl* gene is most likely dependent on cell type and stimuli (Grad et al., 2000).

## Future and Directions

As with most scientific investigations, this study leads to other questions and experiments that will provide a complete picture of the CSC-induced transformation of MCF10A cells. The present study focused specifically on the CSC-induced upregulation of *bcl-xl* in MCF10A cells and more studies are needed to confirm this mechanism in other cell types and situations. Determining the protein that binds to C/EBP site-I on the pBcl-xLP will shed light on the transformation of MCF10A cells treated with CSC, and identify another protein that may be used as a therapeutic target. The determination of the mechanism by which CSC induces C/EBPβ is

also of up most importance. The present study suggests C/EBPβ may be post-translationally upregulated by CSC treatment. It is also possible that C/EBPβ can be regulated on the transcriptional level. Future experiments should focus on which, if any modifications are induced by CSC treatment. Determining the types and sites of modifications can give a clearer picture of CSC-induced C/EBPβ and subsequent increased Bcl-xL expression in MCF10A cells.

Although the C/EBPβ hLIP overexpression construct allowed for an endogenous knockdown system, siRNA can be used to more efficiently rid cells of C/EBPβ expression and determine whether Bcl-xL levels are still responsive to CSC treatment. Whether C/EBPβ is directly involved in the CSC-induced transformation of MCF10A cells can also be determined with a siRNA knockdown system. Characteristics of transformation can be compared between MCF10A cells treated with CSC in the presence or absence of C/EBPβ siRNA. An important question left unanswered by C/EBPβ-LAP2 overexpression studies (Bundy et al., 2005; Bundy and Sealy, 2003) is if LAP2 can cause MCF10A cells to become tumorigenic in a mouse model system. Establishing this relationship will indicate that C/EBPβ, especially LAP2 has a role in breast carcinogenesis.

Bcl-xL is associated with decreased apoptosis in tumors, resistance to chemotherapy, and poor clinical outcome (Taylor et al., 1999). Several strategies to decrease Bcl-xL expression have been developed. One example is the use of *bcl-xl* antisense oligonucleotides (Ackermann et al., 1999; Dibbert et al., 1998; Espana et al., 2004; Pollman et al., 1998). Newer oligonucleotides have been developed that are specific to *bcl-xl* and do not target *bcl-x* pre-mRNA or *bcl-xs* (Simoes-Wust et al., 2000). Bcl-xS expression has also been used as therapeutic agent against Bcl-xL (Ealovega et al., 1996). A pharmacological intervention that simultaneously decreases Bcl-xL and increases Bcl-xS is also an important anti-tumor treatment

(Reed, 1995; Yang and Korsmeyer, 1996). Studies have also developed probes that bind to the 5' of *bcl-x* mRNA and force the translation of Bcl-xS protein instead of Bcl-xL (Mercatante et al., 2001; Taylor et al., 1999). Strategies that keep Bcl-xL deaminated also have therapeutic potential (Weintraub et al., 2004) because suppression of deamination occurs during carcinogenesis (Takehara and Takahashi, 2003; Zhao et al., 2004). More recently, ABT-737, a small molecule inhibitor of Bcl-xL as well as Bcl-2 and Bcl-w was discovered. It binds to the BH3 binding groove of these anti-apoptotic proteins, enhancing the death signal by keeping them from interacting with endogenous BH3-only proteins. The molecule regressed established tumors and improved survival and cure rates in mouse models (Oltersdorf et al., 2005). However, interventions targeting C/EBPβ expression are limited. The implications of this study include identifying C/EBPβ as a potential oncogene and sparking research into therapeutics aimed at decreasing its expression in cancer cells or tumors. C/EBPβ may also become a valuable molecular tool used to determine patient response to therapy and prognosis (Milde-Langosch et al., 2003; Zahnow et al., 1997). Studies focusing on the regulation of the protein will no doubt be critical to developing such therapeutic interventions.

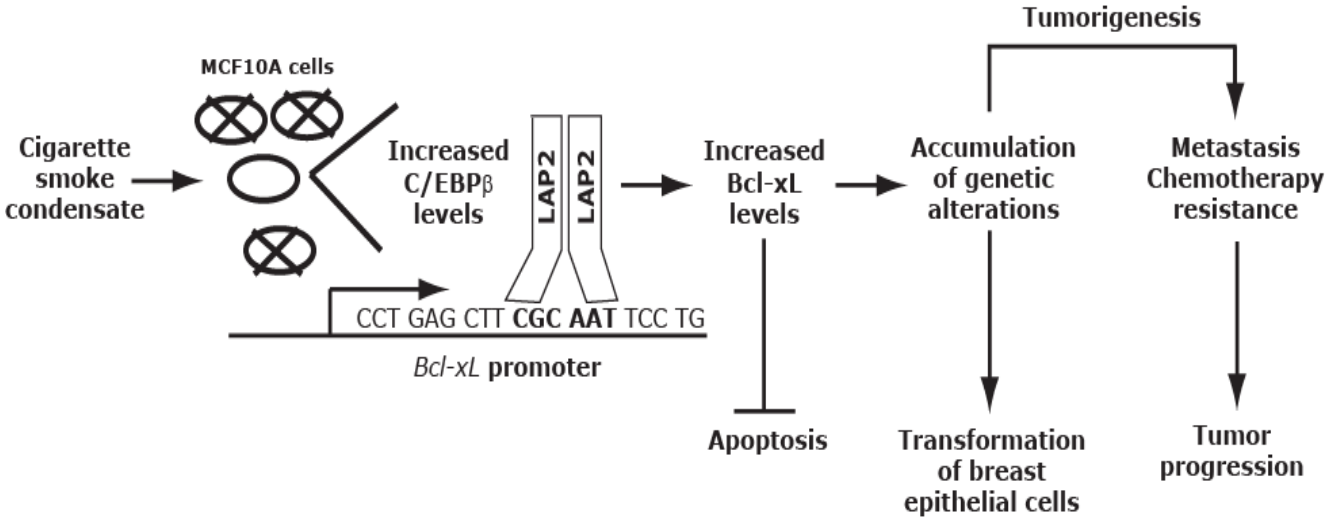


Figure 5-1. Model of CSC-Induced C/EBPβ upregulation of Bcl-xL in MCF10A cells. Exposure of CSC to MCF10A cells causes DNA damage and most cells die. The surviving cells display increased levels of C/EBPβ by an unknown mechanism. C/EBPβ-LAP2 homodimers form and bind to C/EBP site-II on the *bcl-xl* promoter, positively activating its transcription. Increased levels of Bcl-xL protein prevents damaged cells from being removed by apoptosis. Persistent DNA damage in these cells leads genetic alterations, transformation of normal epithelial cells, and eventually breast carcinogenesis. During carcinogenesis, Bcl-xL expression is linked to metastasis and resistance to chemotherapy which affect tumor progression.

LIST OF REFERENCES

Ackermann, E.J., Taylor, J.K., Narayana, R., and Bennett, C.F. (1999). The role of antiapoptotic Bcl-2 family members in endothelial apoptosis elucidated with antisense oligonucleotides. J Biol Chem *274*, 11245-11252.

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J *9*, 1897-1906.

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., and McEwan, R.N. (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res *47*, 3239-3245.

Allan, D.J., Howell, A., Roberts, S.A., Williams, G.T., Watson, R.J., Coyne, J.D., Clarke, R.B., Laidlaw, I.J., and Potten, C.S. (1992). Reduction in apoptosis relative to mitosis in histologically normal epithelium accompanies fibrocystic change and carcinoma of the premenopausal human breast. J Pathol *167*, 25-32.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A *51*, 786-794.

Ambrosone, C.B., Freudenheim, J.L., Graham, S., Marshall, J.R., Vena, J.E., Brasure, J.R., Michalek, A.M., Laughlin, R., Nemoto, T., Gillenwater, K.A., and Shields, P.G. (1996). Cigarette smoking, N-acetyltransferase 2 genetic polymorphisms, and breast cancer risk. JAMA *276*, 1494-1501.

Ambrosone, C.B., Kropp, S., Yang, J., Yao, S., Shields, P.G., and Chang-Claude, J. (2008). Cigarette smoking, N-acetyltransferase 2 genotypes, and breast cancer risk: pooled analysis and meta-analysis. Cancer Epidemiol Biomarkers Prev *17*, 15-26.

American Cancer Society (2008). Cancer Facts & Figures 2008. (Atlanta, American Cancer Society).

Ames, B.N., McCann, J., and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res *31*, 347-364.

Annis, M.G., Soucie, E.L., Dlugosz, P.J., Cruz-Aguado, J.A., Penn, L.Z., Leber, B., and Andrews, D.W. (2005). Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. EMBO J *24*, 2096-2103.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G.*, et al.* (1997). Inhibition of Bax channel-forming activity by Bcl-2. Science *277*, 370-372.

Armstrong, R. (1997). Glutathione-S-transferases, Vol 3 (New York, NY: Elsevier Science).

Artandi, S.E., Chang, S., Lee, S.L., Alson, S., Gottlieb, G.J., Chin, L., and DePinho, R.A. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature *406*, 641-645.

Bachelor, M.A., and Bowden, G.T. (2004). Ultraviolet A-induced modulation of Bcl-XL by p38 MAPK in human keratinocytes: post-transcriptional regulation through the 3'-untranslated region. J Biol Chem *279*, 42658-42668.

Badrichani, A.Z., Stroka, D.M., Bilbao, G., Curiel, D.T., Bach, F.H., and Ferran, C. (1999). Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. J Clin Invest *103*, 543-553.

Baer, M., and Johnson, P.F. (2000). Generation of truncated C/EBPbeta isoforms by in vitro proteolysis. J Biol Chem *275*, 26582-26590.

Baldwin, B.R., Timchenko, N.A., and Zahnow, C.A. (2004). Epidermal growth factor receptor stimulation activates the RNA binding protein CUG-BP1 and increases expression of C/EBPbeta-LIP in mammary epithelial cells. Mol Cell Biol *24*, 3682-3691.

Ban, J., Eckhart, L., Weninger, W., Mildner, M., and Tschachler, E. (1998). Identification of a human cDNA encoding a novel Bcl-x isoform. Biochem Biophys Res Commun *248*, 147-152.

Band, P.R., Le, N.D., Fang, R., and Deschamps, M. (2002). Carcinogenic and endocrine disrupting effects of cigarette smoke and risk of breast cancer. Lancet *360*, 1044-1049.

Baron, J.A., La Vecchia, C., and Levi, F. (1990). The antiestrogenic effect of cigarette smoking in women. Am J Obstet Gynecol *162*, 502-514.

Baron, J.A., Newcomb, P.A., Longnecker, M.P., Mittendorf, R., Storer, B.E., Clapp, R.W., Bogdan, G., and Yuen, J. (1996). Cigarette smoking and breast cancer. Cancer Epidemiol Biomarkers Prev *5*, 399-403.

Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M., and Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. Int J Cancer *57*, 353-361.

Bartsch, H., Terracini, B., Malaveille, C., Tomatis, L., Wahrendorf, J., Brun, G., and Dodet, B. (1983). Quantitative comparison of carcinogenicity, mutagenicity and electrophilicity of 10 direct-acting alkylating agents and of the initial O6:7-alkylguanine ratio in DNA with carcinogenic potency in rodents. Mutat Res *110*, 181-219.

Bannister, A., Kouzarides, T. (1997). Structure/function and oncogenic conversion of Fos and Jun (Birkhauser. Basel [from 118]).

Baron, J.A., La Vecchia, C., and Levi, F. (1990). The antiestrogenic effect of cigarette smoking in women. Am J Obstet Gynecol *162*, 502-514.

Barry, M.A., Behnke, C.A., and Eastman, A. (1990). Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol *40*, 2353-2362.

Beckmann, M.W., Niederacher, D., Schnurch, H.G., Gusterson, B.A., and Bender, H.G. (1997). Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Med *75*, 429-439.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167-170.

Bennett, W.P., Alavanja, M.C., Blomeke, B., Vahakangas, K.H., Castren, K., Welsh, J.A., Bowman, E.D., Khan, M.A., Flieder, D.B., and Harris, C.C. (1999). Environmental tobacco smoke, genetic susceptibility, and risk of lung cancer in never-smoking women. J Natl Cancer Inst *91*, 2009-2014.

Benz, C.C., Scott, G.K., Sarup, J.C., Johnson, R.M., Tripathy, D., Coronado, E., Shepard, H.M., and Osborne, C.K. (1992). Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat *24*, 85-95.

Bissell, M.J., Rizki, A., and Mian, I.S. (2003). Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol *15*, 753-762.

Biswas, R.S., Cha, H.J., Hardwick, J.M., and Srivastava, R.K. (2001). Inhibition of drug-induced Fas ligand transcription and apoptosis by Bcl-XL. Mol Cell Biochem *225*, 7-20.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R., and Salvesen, G.S. (2003). A unified model for apical caspase activation. Mol Cell *11*, 529-541.

Bode, A.M., and Dong, Z. (2005). Signal transduction pathways in cancer development and as targets for cancer prevention. Prog Nucleic Acid Res Mol Biol *79*, 237-297.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597-608.

Boise, L.H., and Thompson, C.B. (1997). Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc Natl Acad Sci U S A *94*, 3759-3764.

Bonfil, R.D., Reddel, R.R., Ura, H., Reich, R., Fridman, R., Harris, C.C., and Klein-Szanto, J.P. (1989). Invasive and metastatic potential of a v-Ha-ras-transformed human bronchial epithelial cell line. J Natl Cancer Inst *81*, 587-594.

Boulukos, K.E., Pognonec, P., Begue, A., Galibert, F., Gesquiere, J.C., Stehelin, D., and Ghysdael, J. (1988). Identification in chickens of an evolutionarily conserved cellular ets-2 gene (c-ets-2) encoding nuclear proteins related to the products of the c-ets proto-oncogene. EMBO J *7*, 697-705.

Boyd, K.E., and Farnham, P.J. (1999). Identification of target genes of oncogenic transcription factors. Proc Soc Exp Biol Med *222*, 9-28.

Bremnes, Y., Ursin, G., Bjurstam, N., and Gram, I.T. (2007). Different measures of smoking exposure and mammographic density in postmenopausal Norwegian women: a cross-sectional study. Breast Cancer Res *9*, R73.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E., Jr. (1999). Stat3 as an oncogene. Cell *98*, 295-303.

Buckley, M.F., Sweeney, K.J., Hamilton, J.A., Sini, R.L., Manning, D.L., Nicholson, R.I., deFazio, A., Watts, C.K., Musgrove, E.A., and Sutherland, R.L. (1993). Expression and amplification of cyclin genes in human breast cancer. Oncogene *8*, 2127-2133.

Bundy, L., Wells, S., and Sealy, L. (2005). C/EBPbeta-2 confers EGF-independent growth and disrupts the normal acinar architecture of human mammary epithelial cells. Mol Cancer *4*, 43.

Bundy, L.M., and Sealy, L. (2003). CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. Oncogene *22*, 869-883.

Burchell, B., McGurk, K, Brierley, CH, Clarke, DJ (1997). UDPglucuronosyltransferases, Vol 3 (New York, NY: Elsevier Science).

Cai, J., Yang, J., and Jones, D.P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta *1366*, 139-149.

Calaf, G., and Russo, J. (1993). Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis *14*, 483-492.

Calkhoven, C.F., Muller, C., and Leutz, A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes Dev *14*, 1920-1932.

Campos, L., Rouault, J.P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J.P., and Guyotat, D. (1993). High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood *81*, 3091-3096.

Cao, Z., Umek, R.M., and McKnight, S.L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev *5*, 1538-1552.

Castle, V.P., Heidelberger, K.P., Bromberg, J., Ou, X., Dole, M., and Nunez, G. (1993). Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am J Pathol *143*, 1543-1550.

Cavalieri, E.L., Higginbotham, S., RamaKrishna, N.V., Devanesan, P.D., Todorovic, R., Rogan, E.G., and Salmasi, S. (1991). Comparative dose-response tumorigenicity studies of dibenzo[alpha,l]pyrene versus 7,12-dimethylbenz[alpha]anthracene, benzo[alpha]pyrene and two dibenzo[alpha,l]pyrene dihydrodiols in mouse skin and rat mammary gland. Carcinogenesis *12*, 1939-1944.

Centers for Disease Control and Prevention (2007). Cigarette Smoking Among Adults – United States, 2004. In MMWR Morb Mortal Wkly Rep, pp. 1157-1161.Chambers, A.F., Naumov, G.N., Varghese, H.J., Nadkarni, K.V., MacDonald, I.C., and Groom, A.C. (2001). Critical steps in hematogenous metastasis: an overview. Surg Oncol Clin N Am *10*, 243-255, vii.

Cesena, T.I., Cardinaux, J.R., Kwok, R., and Schwartz, J. (2007). CCAAT/enhancer-binding protein (C/EBP) beta is acetylated at multiple lysines: acetylation of C/EBPbeta at lysine 39 modulates its ability to activate transcription. J Biol Chem *282*, 956-967.

Chambers, A.F., Naumov, G.N., Varghese, H.J., Nadkarni, K.V., MacDonald, I.C., and Groom, A.C. (2001). Critical steps in hematogenous metastasis: an overview. Surg Oncol Clin N Am *10*, 243-255, vii.

Chang, B.S., Minn, A.J., Muchmore, S.W., Fesik, S.W., and Thompson, C.B. (1997). Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. EMBO J *16*, 968-977.

Change, S. (1966). In vitro transformation of human epithelial cells. Biochem Biophys Acta *823*, 161-194.

Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., and Bertucci, F. (2006). Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene *25*, 2273-2284.

Charles, A., Tang, X., Crouch, E., Brody, J.S., and Xiao, Z.X. (2001). Retinoblastoma protein complexes with C/EBP proteins and activates C/EBP-mediated transcription. J Cell Biochem *83*, 414-425.

Chen, C., Edelstein, L.C., and Gelinas, C. (2000). The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol *20*, 2687-2695.

Chen, P.L., Riley, D.J., Chen, Y., and Lee, W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev *10*, 2794-2804.

Chen, P.L., Riley, D.J., Chen-Kiang, S., and Lee, W.H. (1996). Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc Natl Acad Sci U S A *93*, 465-469.

Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. (1997). Geometric control of cell life and death. Science *276*, 1425-1428.

Cheng, E.H., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. Nature *379*, 554-556.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell *8*, 705-711.

Chepiga, T.A., Morton, M.J., Murphy, P.A., Avalos, J.T., Bombick, B.R., Doolittle, D.J., Borgerding, M.F., and Swauger, J.E. (2000). A comparison of the mainstream smoke chemistry and mutagenicity of a representative sample of the US cigarette market with two Kentucky reference cigarettes (K1R4F and K1R5F). Food Chem Toxicol *38*, 949-962.

Cherbonnel-Lasserre, C., Gauny, S., and Kronenberg, A. (1996). Suppression of apoptosis by Bcl-2 or Bcl-xL promotes susceptibility to mutagenesis. Oncogene *13*, 1489-1497.

Chinnaiyan, A.M. (1999). The apoptosome: heart and soul of the cell death machine. Neoplasia *1*, 5-15.

Chinnaiyan, A.M., and Dixit, V.M. (1996). The cell-death machine. Curr Biol *6*, 555-562.

Chinnaiyan, A.M., and Dixit, V.M. (1997). Portrait of an executioner: the molecular mechanism of FAS/APO-1-induced apoptosis. Semin Immunol *9*, 69-76.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chipuk, J.E., Bhat, M., Hsing, A.Y., Ma, J., and Danielpour, D. (2001). Bcl-xL blocks transforming growth factor-beta 1-induced apoptosis by inhibiting cytochrome c release and not by directly antagonizing Apaf-1-dependent caspase activation in prostate epithelial cells. J Biol Chem *276*, 26614-26621.

Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G., and Lutz, R.J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J *14*, 5589-5596.

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. Cell *79*, 13-21.

Cory, S., and Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer *2*, 647-656.

Cory, S., Huang, D.C., and Adams, J.M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene *22*, 8590-8607.

Cramer, W.A., Heymann, J.B., Schendel, S.L., Deriy, B.N., Cohen, F.S., Elkins, P.A., and Stauffacher, C.V. (1995). Structure-function of the channel-forming colicins. Annu Rev Biophys Biomol Struct *24*, 611-641.

Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G.J., Poli, V., Hanson, R.W., and Reshef, L. (1997). Role of the isoforms of CCAAT/enhancer-binding protein in the initiation of phosphoenolpyruvate carboxykinase (GTP) gene transcription at birth. J Biol Chem *272*, 26306-26312.

Cuzick, J., Routledge, M.N., Jenkins, D., and Garner, R.C. (1990). DNA adducts in different tissues of smokers and non-smokers. Int J Cancer *45*, 673-678.

Darnell, J.E., Jr. (1997). STATs and gene regulation. Science *277*, 1630-1635.

Datta, R., Manome, Y., Taneja, N., Boise, L.H., Weichselbaum, R., Thompson, C.B., Slapak, C.A., and Kufe, D. (1995). Overexpression of Bcl-XL by cytotoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. Cell Growth Differ *6*, 363-370.

Davis, B.R., Whitehead, J.K., Gill, M.E., Lee, P.N., Butterworth, A.D., and Roe, F.J. (1975). Response of rat lung to inhaled tobacco smoke with or without prior exposure to 3,4-benzpyrene (BP) given by intratracheal instillation. Br J Cancer *31*, 469-484.

Davis, D., Vaught, A, Tso,TC, Bush, LP (1984). Analysis of a new low yield research cigarette (Lexington, KY: Tobacco and Health Institute).

Dearth, L.R., Hutt, J., Sattler, A., Gigliotti, A., and DeWille, J. (2001). Expression and function of CCAAT/enhancer binding proteinbeta (C/EBPbeta) LAP and LIP isoforms in mouse mammary gland, tumors and cultured mammary epithelial cells. J Cell Biochem *82*, 357-370.

Debnath, J., Mills, K.R., Collins, N.L., Reginato, M.J., Muthuswamy, S.K., and Brugge, J.S. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell *111*, 29-40.

DeMarini, D.M. (2004). Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat Res *567*, 447-474.

Dertinger, S.D., Nazarenko, D.A., Silverstone, A.E., and Gasiewicz, T.A. (2001). Aryl hydrocarbon receptor signaling plays a significant role in mediating benzo[a]pyrene- and cigarette smoke condensate-induced cytogenetic damage in vivo. Carcinogenesis *22*, 171-177.

Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990). LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes Dev *4*, 1541-1551.

Descombes, P., and Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell *67*, 569-579.

Deverman, B.E., Cook, B.L., Manson, S.R., Niederhoff, R.A., Langer, E.M., Rosova, I., Kulans, L.A., Fu, X., Weinberg, J.S., Heinecke, J.W.*, et al.* (2002). Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. Cell *111*, 51-62.

Dibbert, B., Daigle, I., Braun, D., Schranz, C., Weber, M., Blaser, K., Zangemeister-Wittke, U., Akbar, A.N., and Simon, H.U. (1998). Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte-macrophage colony-stimulating factor and interleukin-5. Blood *92*, 778-783.

DiPaolo, J.A. (1983). Relative difficulties in transforming human and animal cells in vitro. J Natl Cancer Inst *70*, 3-8.

Doll, R., Peto, R (1981). The Causes of Cancer (New York, NY: Oxford Press).

Donahue, T.F., Cigan, A.M., Pabich, E.K., and Valavicius, B.C. (1988). Mutations at a Zn(II) finger motif in the yeast eIF-2 beta gene alter ribosomal start-site selection during the scanning process. Cell *54*, 621-632.

Donepudi, M., Mac Sweeney, A., Briand, C., and Grutter, M.G. (2003). Insights into the regulatory mechanism for caspase-8 activation. Mol Cell *11*, 543-549.

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell *102*, 33-42.

Duong, D.T., Waltner-Law, M.E., Sears, R., Sealy, L., and Granner, D.K. (2002). Insulin inhibits hepatocellular glucose production by utilizing liver-enriched transcriptional inhibitory protein to disrupt the association of CREB-binding protein and RNA polymerase II with the phosphoenolpyruvate carboxykinase gene promoter. J Biol Chem *277*, 32234-32242.

Egan, K.M., Stampfer, M.J., Hunter, D., Hankinson, S., Rosner, B.A., Holmes, M., Willett, W.C., and Colditz, G.A. (2002). Active and passive smoking in breast cancer: prospective results from the Nurses' Health Study. Epidemiology *13*, 138-145.

Ealovega, M.W., McGinnis, P.K., Sumantran, V.N., Clarke, M.F., and Wicha, M.S. (1996). bcl-xs gene therapy induces apoptosis of human mammary tumors in nude mice. Cancer Res *56*, 1965-1969.

Espana, L., Fernandez, Y., Rubio, N., Torregrosa, A., Blanco, J., and Sierra, A. (2004). Overexpression of Bcl-xL in human breast cancer cells enhances organ-selective lymph node metastasis. Breast Cancer Res Treat *87*, 33-44.

Eaton, E.M., Hanlon, M., Bundy, L., and Sealy, L. (2001). Characterization of C/EBPbeta isoforms in normal versus neoplastic mammary epithelial cells. J Cell Physiol *189*, 91-105.

el-Bayoumy, K., Chae, Y.H., Upadhyaya, P., Rivenson, A., Kurtzke, C., Reddy, B., and Hecht, S.S. (1995). Comparative tumorigenicity of benzo[a]pyrene, 1-nitropyrene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine administered by gavage to female CD rats. Carcinogenesis *16*, 431-434.

Fang, W., Rivard, J.J., Mueller, D.L., and Behrens, T.W. (1994). Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. J Immunol *153*, 4388-4398.

Farrow, S.N., and Brown, R. (1996). New members of the Bcl-2 family and their protein partners. Curr Opin Genet Dev *6*, 45-49.

Fearon, E. R. and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759-767.

Fernandez, Y., Gu, B., Martinez, A., Torregrosa, A., and Sierra, A. (2002). Inhibition of apoptosis in human breast cancer cells: role in tumor progression to the metastatic state. Int J Cancer *101*, 317-326.

Fernandez, Y., Espana, L., Manas, S., Fabra, A., and Sierra, A. (2000). Bcl-xL promotes metastasis of breast cancer cells by induction of cytokines resistance. Cell Death Differ *7*, 350-359.

Fiebig, A.A., Zhu, W., Hollerbach, C., Leber, B., and Andrews, D.W. (2006). Bcl-XL is qualitatively different from and ten times more effective than Bcl-2 when expressed in a breast cancer cell line. BMC Cancer *6*, 213.

Fink, A.K., and Lash, T.L. (2003). A null association between smoking during pregnancy and breast cancer using Massachusetts registry data (United States). Cancer Causes Control *14*, 497-503.

Firozi, P.F., Bondy, M.L., Sahin, A.A., Chang, P., Lukmanji, F., Singletary, E.S., Hassan, M.M., and Li, D. (2002). Aromatic DNA adducts and polymorphisms of CYP1A1, NAT2, and GSTM1 in breast cancer. Carcinogenesis *23*, 301-306.

Finucane, D.M., Bossy-Wetzel, E., Waterhouse, N.J., Cotter, T.G., and Green, D.R. (1999). Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J Biol Chem *274*, 2225-2233.

Frisch, S.M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol *124*, 619-626.

Gago, F.E., Tello, O.M., Diblasi, A.M., and Ciocca, D.R. (1998). Integration of estrogen and progesterone receptors with pathological and molecular prognostic factors in breast cancer patients. J Steroid Biochem Mol Biol *67*, 431-437.

Gery, S., Tanosaki, S., Bose, S., Bose, N., Vadgama, J., and Koeffler, H.P. (2005). Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer. Clin Cancer Res *11*, 3184-3190.

Ghysdael, J., Gegonne, A., Pognonec, P., Boulukos, K., Leprince, D., Dernis, D., Lagrou, C., and Stehelin, D. (1986). Identification in chicken macrophages of a set of proteins related to, but distinct from, the chicken cellular c-ets-encoded protein p54c-ets. EMBO J *5*, 2251-2256.

Gibson, L., Holmgreen, S.P., Huang, D.C., Bernard, O., Copeland, N.G., Jenkins, N.A., Sutherland, G.R., Baker, E., Adams, J.M., and Cory, S. (1996). bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene *13*, 665-675.

Gigliotti, A.P., and DeWille, J.W. (1998). Lactation status influences expression of CCAAT/enhancer binding protein isoform mRNA in the mouse mammary gland. J Cell Physiol *174*, 232-239.

Gigliotti, A.P., Johnson, P.F., Sterneck, E., and DeWille, J.W. (2003). Nulliparous CCAAT/enhancer binding proteindelta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperlasia. Exp Biol Med (Maywood) *228*, 278-285.

Giovannucci, E., Colditz, G.A., Stampfer, M.J., Hunter, D., Rosner, B.A., Willett, W.C., and Speizer, F.E. (1994). A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. women. J Natl Cancer Inst *86*, 192-199.

Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A., Kearney, J., and Willett, W.C. (1994). A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men. J Natl Cancer Inst *86*, 183-191.

Giovannucci, E., and Martinez, M.E. (1996). Tobacco, colorectal cancer, and adenomas: a review of the evidence. J Natl Cancer Inst *88*, 1717-1730.

Gisselsson, D. (2003). Chromosome instability in cancer: how, when, and why? Adv Cancer Res *87*, 1-29.

Glasgow, J.N., Qiu, J., Rassin, D., Grafe, M., Wood, T., and Perez-Pol, J.R. (2001). Transcriptional regulation of the BCL-X gene by NF-kappaB is an element of hypoxic responses in the rat brain. Neurochem Res *26*, 647-659.

Glasgow, J.N., Wood, T., and Perez-Polo, J.R. (2000). Identification and characterization of nuclear factor kappaB binding sites in the murine bcl-x promoter. J Neurochem *75*, 1377-1389.

Gonzalez-Garcia, M., Garcia, I., Ding, L., O'Shea, S., Boise, L.H., Thompson, C.B., and Nunez, G. (1995). bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc Natl Acad Sci U S A *92*, 4304-4308.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L.H., Thompson, C.B., and Nunez, G. (1994). bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development *120*, 3033-3042.

Goode, E.L., Ulrich, C.M., and Potter, J.D. (2002). Polymorphisms in DNA repair genes and associations with cancer risk. Cancer Epidemiol Biomarkers Prev *11*, 1513-1530.

Gordon, L.A., Mulligan, K.T., Maxwell-Jones, H., Adams, M., Walker, R.A., and Jones, J.L. (2003). Breast cell invasive potential relates to the myoepithelial phenotype. Int J Cancer *106*, 8-16.

Grad, J.M., Zeng, X.R., and Boise, L.H. (2000). Regulation of Bcl-xL: a little bit of this and a little bit of STAT. Curr Opin Oncol *12*, 543-549.

Grandis, J.R., Drenning, S.D., Zeng, Q., Watkins, S.C., Melhem, M.F., Endo, S., Johnson, D.E., Huang, L., He, Y., and Kim, J.D. (2000). Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci U S A *97*, 4227-4232.

Green, D.R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626-629.

Griffith, R.B., and Hancock, R. (1985). Simultaneous mainstream-sidestream smoke exposure systems I. Equipment and procedures. Toxicology *34*, 123-138.

Grillot, D.A., Gonzalez-Garcia, M., Ekhterae, D., Duan, L., Inohara, N., Ohta, S., Seldin, M.F., and Nunez, G. (1997). Genomic organization, promoter region analysis, and chromosome localization of the mouse bcl-x gene. J Immunol *158*, 4750-4757.

Grimm, S.L., and Rosen, J.M. (2003). The role of C/EBPbeta in mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia *8*, 191-204.

Gross, A., McDonnell, J.M., and Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*, 1899-1911.

Gu, B., Espana, L., Mendez, O., Torregrosa, A., and Sierra, A. (2004). Organ-selective chemoresistance in metastasis from human breast cancer cells: inhibition of apoptosis, genetic variability and microenvironment at the metastatic focus. Carcinogenesis *25*, 2293-2301.

Guengerich, F.P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol *14*, 611-650.

Hamajima, N., Hirose, K., Tajima, K., Rohan, T., Calle, E.E., Heath, C.W., Jr., Coates, R.J., Liff, J.M., Talamini, R., Chantarakul, N.*, et al.* (2002). Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. Br J Cancer *87*, 1234-1245.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

He, L., Perkins, G.A., Poblenz, A.T., Harris, J.B., Hung, M., Ellisman, M.H., and Fox, D.A. (2003). Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. Proc Natl Acad Sci U S A *100*, 1022-1027.

Hecht, S.S. (1999). Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst *91*, 1194-1210.

Hecht, S.S. (2002). Tobacco smoke carcinogens and breast cancer. Environ Mol Mutagen *39*, 119-126.

Hecht, S.S. (2003). Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer *3*, 733-744.

Heermeier, K., Benedict, M., Li, M., Furth, P., Nunez, G., and Hennighausen, L. (1996). Bax and Bcl-xs are induced at the onset of apoptosis in involuting mammary epithelial cells. Mech Dev *56*, 197-207.

Hirai, Y., Radisky, D., Boudreau, R., Simian, M., Stevens, M.E., Oka, Y., Takebe, K., Niwa, S., and Bissell, M.J. (2001). Epimorphin mediates mammary luminal morphogenesis through control of C/EBPbeta. J Cell Biol *153*, 785-794.

Hoffmann, D., Hoffmann, I., and El-Bayoumy, K. (2001). The less harmful cigarette: a controversial issue. a tribute to Ernst L. Wynder. Chem Res Toxicol *14*, 767-790.

Hoffmann, D., and Wynder, E.L. (1971). A study of tobacco carcinogenesis. XI. Tumor initiators, tumor accelerators, and tumor promoting activity of condensate fractions. Cancer *27*, 848-864.

Hoffmann, D., and Wynder, E.L. (1968). Selective reduction of the tumorigenicity of tobacco smoke. Experimental approaches. Natl Cancer Inst Monogr *28*, 151-172.

Hsieh, C.C., Xiong, W., Xie, Q., Rabek, J.P., Scott, S.G., An, M.R., Reisner, P.D., Kuninger, D.T., and Papaconstantinou, J. (1998). Effects of age on the posttranscriptional regulation of CCAAT/enhancer binding protein alpha and CCAAT/enhancer binding protein beta isoform synthesis in control and LPS-treated livers. Mol Biol Cell *9*, 1479-1494.

Hsu, S.Y., Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A.J. (1997). Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc Natl Acad Sci U S A *94*, 12401-12406.

Hsu, T.C., Cherry, L.M., Bucana, C., Shirley, L.R., and Gairola, C.G. (1991). Mitosis-arresting effects of cigarette smoke condensate on human lymphoid cell lines. Mutat Res *259*, 67-78.

Hsu, Y.T., and Youle, R.J. (1998). Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J Biol Chem *273*, 10777-10783.

Hu, Y., Benedict, M.A., Wu, D., Inohara, N., and Nunez, G. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci U S A *95*, 4386-4391.

Huang, D.C., Adams, J.M., and Cory, S. (1998). The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. EMBO J *17*, 1029-1039.

International Agency for Research on Cancer (1972-2000). IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans.

International Agency for Research on Cancer (1985). IARC monographs on the Evaluation of Carcinogenic Risks to Humans (Lyon, France, International Agency for Cancer Research), p. 15.

International Agency for Research on Cancer (2004). Tobacco smoking and tobacco smoke. In IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans (Lyon, France).

Jaattela, M., Benedict, M., Tewari, M., Shayman, J.A., and Dixit, V.M. (1995). Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. Oncogene *10*, 2297-2305.

Jacobs-Helber, S.M., Wickrema, A., Birrer, M.J., and Sawyer, S.T. (1998). AP1 regulation of proliferation and initiation of apoptosis in erythropoietin-dependent erythroid cells. Mol Cell Biol *18*, 3699-3707.

Jaiswal, A.S., Aneja, R., Connors, S.K., Joshi, H.C., Multani, A.S., Pathak, S., Narayan, S. (2008). Increased mitotic arrest and apoptosis of cigarette smoke condensate-transformed versus normal human breast epithelial cells in response to 9-Br-Noscapine. In unpublished data.

Jalas, J.R., Hecht, S.S., and Murphy, S.E. (2005). Cytochrome P450 enzymes as catalysts of metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco specific carcinogen. Chem Res Toxicol *18*, 95-110.

Jeong, S.Y., Gaume, B., Lee, Y.J., Hsu, Y.T., Ryu, S.W., Yoon, S.H., and Youle, R.J. (2004). Bcl-x(L) sequesters its C-terminal membrane anchor in soluble, cytosolic homodimers. EMBO J *23*, 2146-2155.

John, E.M., and Kelsey, J.L. (1993). Radiation and other environmental exposures and breast cancer. Epidemiol Rev *15*, 157-162.

Johnson, K.C., Hu, J., and Mao, Y. (2000). Passive and active smoking and breast cancer risk in Canada, 1994-97. Cancer Causes Control *11*, 211-221.

Johnson, P.F. (1993). Identification of C/EBP basic region residues involved in DNA sequence recognition and half-site spacing preference. Mol Cell Biol *13*, 6919-6930.

Joo, M., Park, G.Y., Wright, J.G., Blackwell, T.S., Atchison, M.L., and Christman, J.W. (2004). Transcriptional regulation of the cyclooxygenase-2 gene in macrophages by PU.1. J Biol Chem *279*, 6658-6665.

Jordan, V.C. (1993). Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol *110*, 507-517.

Karrison, T.G., Ferguson, D.J., and Meier, P. (1999). Dormancy of mammary carcinoma after mastectomy. J Natl Cancer Inst *91*, 80-85.

Kaufmann, S.H. (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res *49*, 5870-5878.

Kelekar, A., and Thompson, C.B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol *8*, 324-330.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kim, J.W., Tang, Q.Q., Li, X., and Lane, M.D. (2007). Effect of phosphorylation and S-S bond-induced dimerization on DNA binding and transcriptional activation by C/EBPbeta. Proc Natl Acad Sci U S A *104*, 1800-1804.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A *68*, 820-823.

Korsmeyer, S.J. (1992). Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood *80*, 879-886.

Kowenz-Leutz, E., Twamley, G., Ansieau, S., and Leutz, A. (1994). Novel mechanism of C/EBP beta (NF-M) transcriptional control: activation through derepression. Genes Dev *8*, 2781-2791.

Kovacs, K.A., Steinmann, M., Magistretti, P.J., Halfon, O., and Cardinaux, J.R. (2003). CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation. J Biol Chem *278*, 36959-36965.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., and Craig, R.W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A *90*, 3516-3520.

Krajewska, M., Moss, S.F., Krajewski, S., Song, K., Holt, P.R., and Reed, J.C. (1996). Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. Cancer Res *56*, 2422-2427.

Krajewski, S., Krajewska, M., Turner, B.C., Pratt, C., Howard, B., Zapata, J.M., Frenkel, V., Robertson, S., Ionov, Y., Yamamoto, H.*, et al.* (1999). Prognostic significance of apoptosis regulators in breast cancer. Endocr Relat Cancer *6*, 29-40.

Kumar, R., Mandal, M., Lipton, A., Harvey, H., and Thompson, C.B. (1996). Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin Cancer Res *2*, 1215-1219.

Kumar, R., Medina, D., and Sukumar, S. (1990). Activation of H-ras oncogenes in preneoplastic mouse mammary tissues. Oncogene *5*, 1271-1277.

Kundu, C.N., Balusu, R., Jaiswal, A.S., Gairola, C.G., and Narayan, S. (2007). Cigarette smoke condensate-induced level of adenomatous polyposis coli blocks long-patch base excision repair in breast epithelial cells. Oncogene *26*, 1428-1438.

Kuribayashi, K., Mayes, P.A., and El-Deiry, W.S. (2006). What are caspases 3 and 7 doing upstream of the mitochondria? Cancer Biol Ther *5*, 763-765.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science *243*, 1681-1688.

Landschulz, W.H., Johnson, P.F., Adashi, E.Y., Graves, B.J., and McKnight, S.L. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev *2*, 786-800.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science *240*, 1759-1764.

Lash, T.L., and Aschengrau, A. (2002). A null association between active or passive cigarette smoking and breast cancer risk. Breast Cancer Res Treat *75*, 181-184.

Lekstrom-Himes, J., and Xanthopoulos, K.G. (1998). Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem *273*, 28545-28548.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S., and Korsmeyer, S.J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183-192.

Li, C., Fox, C.J., Master, S.R., Bindokas, V.P., Chodosh, L.A., and Thompson, C.B. (2002). Bcl-X(L) affects Ca(2+) homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors. Proc Natl Acad Sci U S A *99*, 9830-9835.

Li, D., Wang, M., Dhingra, K., and Hittelman, W.N. (1996a). Aromatic DNA adducts in adjacent tissues of breast cancer patients: clues to breast cancer etiology. Cancer Res *56*, 287-293.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, M., Hu, J., Heermeier, K., Hennighausen, L., and Furth, P.A. (1996b). Apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways. Cell Growth Differ *7*, 13-20.

Li, M., Hu, J., Heermeier, K., Hennighausen, L., and Furth, P.A. (1996c). Expression of a viral oncoprotein during mammary gland development alters cell fate and function: induction of p53-independent apoptosis is followed by impaired milk protein production in surviving cells. Cell Growth Differ *7*, 3-11.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Lin, E.Y., Orlofsky, A., Wang, H.G., Reed, J.C., and Prystowsky, M.B. (1996). A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood *87*, 983-992.

Liotta, L.A. (1984). Tumor invasion and metastases: role of the basement membrane. Warner-Lambert Parke-Davis Award lecture. Am J Pathol *117*, 339-348.

Liu, Q.Y., and Stein, C.A. (1997). Taxol and estramustine-induced modulation of human prostate cancer cell apoptosis via alteration in bcl-xL and bak expression. Clin Cancer Res *3*, 2039-2046.

Liu, R., Page, C., Beidler, D.R., Wicha, M.S., and Nunez, G. (1999). Overexpression of Bcl-x(L) promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. Am J Pathol *155*, 1861-1867.

Lohmann, C.M., League, A.A., Clark, W.S., Lawson, D., DeRose, P.B., and Cohen, C. (2000). Bcl-2: bax and bcl-2: Bcl-x ratios by image cytometric quantitation of immunohistochemical expression in ovarian carcinoma: correlation with prognosis. Cytometry *42*, 61-66.

London, E. (1992). Diphtheria toxin: membrane interaction and membrane translocation. Biochim Biophys Acta *1113*, 25-51.

Luo, L.Z., Werner, K.M., Gollin, S.M., and Saunders, W.S. (2004). Cigarette smoke induces anaphase bridges and genomic imbalances in normal cells. Mutat Res *554*, 375-385.

MacCarthy-Morrogh, L., Wood, L., Brimmell, M., Johnson, P.W., and Packham, G. (2000). Identification of a novel human BCL-X promoter and exon. Oncogene *19*, 5534-5538.

MacCarthy, J., Basara, MI, Palon, DF, Funcht, LT (1988). The role of cell adhesion proteins, laminum and fibronectiv in the movement of malignant and metastatic cells. Cancer Metastases Rev *4*, 12-152.

MacMahon, B. (1990). Cigarette smoking and cancer of the breast (Oxford, United Kingdom: Oxford University Press).

Manna, S.K., Haridas, V., and Aggarwal, B.B. (2000). Bcl-x(L) suppresses TNF-mediated apoptosis and activation of nuclear factor-kappaB, activation protein-1, and c-Jun N-terminal kinase. J Interferon Cytokine Res *20*, 725-735.

Martin, S.S., and Leder, P. (2001). Human MCF10A mammary epithelial cells undergo apoptosis following actin depolymerization that is independent of attachment and rescued by Bcl-2. Mol Cell Biol *21*, 6529-6536.

Martin, S.S., and Vuori, K. (2004). Regulation of Bcl-2 proteins during anoikis and amorphosis. Biochim Biophys Acta *1692*, 145-157.

Maung, Z.T., MacLean, F.R., Reid, M.M., Pearson, A.D., Proctor, S.J., Hamilton, P.J., and Hall, A.G. (1994). The relationship between bcl-2 expression and response to chemotherapy in acute leukaemia. Br J Haematol *88*, 105-109.

Maurer, C.A., Friess, H., Buhler, S.S., Wahl, B.R., Graber, H., Zimmermann, A., and Buchler, M.W. (1998). Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. Dig Dis Sci *43*, 2641-2648.

McClintock, B. (1942). The Fusion of Broken Ends of Chromosomes Following Nuclear Fusion. Proc Natl Acad Sci U S A *28*, 458-463.

McConkey, D.J., Greene, G., and Pettaway, C.A. (1996). Apoptosis resistance increases with metastatic potential in cells of the human LNCaP prostate carcinoma line. Cancer Res *56*, 5594-5599.

McCormick, J.J., and Maher, V.M. (1989). Malignant transformation of mammalian cells in culture, including human cells. Environ Mol Mutagen *14 Suppl 16*, 105-113.

Medema, J.P., Scaffidi, C., Krammer, P.H., and Peter, M.E. (1998). Bcl-xL acts downstream of caspase-8 activation by the CD95 death-inducing signaling complex. J Biol Chem *273*, 3388-3393.

Mei, J., Hu, H., McEntee, M., Plummer, H., 3rd, Song, P., and Wang, H.C. (2003). Transformation of non-cancerous human breast epithelial cell line MCF10A by the tobacco-specific carcinogen NNK. Breast Cancer Res Treat *79*, 95-105.

Mendez, O., Fernandez, Y., Peinado, M.A., Moreno, V., and Sierra, A. (2005). Anti-apoptotic proteins induce non-random genetic alterations that result in selecting breast cancer metastatic cells. Clin Exp Metastasis *22*, 297-307.

Mendez, O., Martin, B., Sanz, R., Aragues, R., Moreno, V., Oliva, B., Stresing, V., and Sierra, A. (2006). Underexpression of transcriptional regulators is common in metastatic breast cancer cells overexpressing Bcl-xL. Carcinogenesis *27*, 1169-1179.

Mensing, H., Albini, A., Krieg, T., Pontz, B.F., and Muller, P.K. (1984). Enhanced chemotaxis of tumor-derived and virus-transformed cells to fibronectin and fibroblast-conditioned medium. Int J Cancer *33*, 43-48.

Metz, R., and Ziff, E. (1991). cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to trans-locate to the nucleus and induce c-fos transcription. Genes Dev *5*, 1754-1766.

Milde-Langosch, K., Loning, T., and Bamberger, A.M. (2003). Expression of the CCAAT/enhancer-binding proteins C/EBPalpha, C/EBPbeta and C/EBPdelta in breast cancer: correlations with clinicopathologic parameters and cell-cycle regulatory proteins. Breast Cancer Res Treat *79*, 175-185.

Miller, F.R., Soule, H.D., Tait, L., Pauley, R.J., Wolman, S.R., Dawson, P.J., and Heppner, G.H. (1993). Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst *85*, 1725-1732.

Mink, S., Haenig, B., and Klempnauer, K.H. (1997). Interaction and functional collaboration of p300 and C/EBPbeta. Mol Cell Biol *17*, 6609-6617.

Minn, A.J., Velez, P., Schendel, S.L., Liang, H., Muchmore, S.W., Fesik, S.W., Fill, M., and Thompson, C.B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature *385*, 353-357.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., and Reed, J.C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799-1805.

Montgomery, E., Wilentz, R.E., Argani, P., Fisher, C., Hruban, R.H., Kern, S.E., and Lengauer, C. (2003). Analysis of anaphase figures in routine histologic sections distinguishes chromosomally unstable from chromosomally stable malignancies. Cancer Biol Ther *2*, 248-252.

Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., and et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science *267*, 1506-1510.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D., Chang, B.S., Thompson, C.B., Wong, S.L.*, et al.* (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature *381*, 335-341.

Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J., and Brugge, J.S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol *3*, 785-792.

Nagaraj, N.S., Beckers, S., Mensah, J.K., Waigel, S., Vigneswaran, N., and Zacharias, W. (2006). Cigarette smoke condensate induces cytochromes P450 and aldo-keto reductases in oral cancer cells. Toxicol Lett *165*, 182-194.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc Natl Acad Sci U S A *90*, 2207-2211.
Nakayama, T., Kaneko, M., Kodama, M., and Nagata, C. (1985). Cigarette smoke induces DNA single-strand breaks in human cells. Nature *314*, 462-464.

Narayan, S., Jaiswal, A.S., Kang, D., Srivastava, P., Das, G.M., and Gairola, C.G. (2004). Cigarette smoke condensate-induced transformation of normal human breast epithelial cells in vitro. Oncogene *23*, 5880-5889.

Narlikar, G.J., Fan, H.Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.

National Cancer Institute (2007). Women's Health Report, Fiscal Years 2005-2006.

National Center for Health Statistics (2006). Health, United States, 2006 with Chartbook on Trends in the Health of Americans. (Hyattsville, MD, Public Health Service).

Nebert, D.W., Dalton, T.P., Okey, A.B., and Gonzalez, F.J. (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J Biol Chem *279*, 23847-23850.

Newton, K., Harris, A.W., Bath, M.L., Smith, K.G., and Strasser, A. (1998). A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J *17*, 706-718.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F.*, et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515-527.

O'Connor, L., and Strasser, A. (1999). The Bcl-2 protein family. Results Probl Cell Differ *23*, 173-207.

Obana, H., Hori, S., Kashimoto, T., and Kunita, N. (1981). Polycyclic aromatic hydrocarbons in human fat and liver. Bull Environ Contam Toxicol *27*, 23-27.

Ochieng, J., Basolo, F., Albini, A., Melchiori, A., Watanabe, H., Elliott, J., Raz, A., Parodi, S., and Russo, J. (1991). Increased invasive, chemotactic and locomotive abilities of c-Ha-ras-transformed human breast epithelial cells. Invasion Metastasis *11*, 38-47.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Olopade, O.I., Adeyanju, M.O., Safa, A.R., Hagos, F., Mick, R., Thompson, C.B., and Recant, W.M. (1997). Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. Cancer J Sci Am *3*, 230-237.

Oltersdorf, T., Elmore, S.W., Shoemaker, A.R., Armstrong, R.C., Augeri, D.J., Belli, B.A., Bruncko, M., Deckwerth, T.L., Dinges, J., Hajduk, P.J.*, et al.* (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature *435*, 677-681.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Opferman, J.T., and Korsmeyer, S.J. (2003). Apoptosis in the development and maintenance of the immune system. Nat Immunol *4*, 410-415.

O'Rourke, J., Yuan, R., and DeWille, J. (1997). CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem *272*, 6291-6296.

O'Rourke, J.P., Newbound, G.C., Hutt, J.A., and DeWille, J. (1999). CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. J Biol Chem *274*, 16582-16589.

Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. (1996). DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J Biol Chem *271*, 3891-3896.

Osada, H., and Takahashi, T. (2002). Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. Oncogene *21*, 7421-7434.

Ossipow, V., Descombes, P., and Schibler, U. (1993). CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci U S A *90*, 8219-8223.

Packham, G., White, E.L., Eischen, C.M., Yang, H., Parganas, E., Ihle, J.N., Grillot, D.A., Zambetti, G.P., Nunez, G., and Cleveland, J.L. (1998). Selective regulation of Bcl-XL by a Jak kinase-dependent pathway is bypassed in murine hematopoietic malignancies. Genes Dev *12*, 2475-2487.

Palmer, J.R., and Rosenberg, L. (1993). Cigarette smoking and the risk of breast cancer. Epidemiol Rev *15*, 145-156.

Pan, G., O'Rourke, K., and Dixit, V.M. (1998). Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol Chem *273*, 5841-5845.

Pardo, O.E., Arcaro, A., Salerno, G., Raguz, S., Downward, J., and Seckl, M.J. (2002). Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. J Biol Chem *277*, 12040-12046.

Park, I.W., Wistuba, II, Maitra, A., Milchgrub, S., Virmani, A.K., Minna, J.D., and Gazdar, A.F. (1999). Multiple clonal abnormalities in the bronchial epithelium of patients with lung cancer. J Natl Cancer Inst *91*, 1863-1868.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C., Jr., and Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature *371*, 762-767.

Pecci, A., Viegas, L.R., Baranao, J.L., and Beato, M. (2001). Promoter choice influences alternative splicing and determines the balance of isoforms expressed from the mouse bcl-X gene. J Biol Chem *276*, 21062-21069.

Pelkonen, O., Vahakangas, K., and Nebert, D.W. (1980). Binding of polycyclic aromatic hydrocarbons to DNA: comparison with mutagenesis and tumorigenesis. J Toxicol Environ Health *6*, 1009-1020.

Perera, F.P., Estabrook, A., Hewer, A., Channing, K., Rundle, A., Mooney, L.A., Whyatt, R., and Phillips, D.H. (1995). Carcinogen-DNA adducts in human breast tissue. Cancer Epidemiol Biomarkers Prev *4*, 233-238.

Peter, M.E., Kischkel, F.C., Scheuerpflug, C.G., Medema, J.P., Debatin, K.M., and Krammer, P.H. (1997). Resistance of cultured peripheral T cells towards activation-induced cell death involves a lack of recruitment of FLICE (MACH/caspase 8) to the CD95 death-inducing signaling complex. Eur J Immunol *27*, 1207-1212.

Pollman, M.J., Hall, J.L., Mann, M.J., Zhang, L., and Gibbons, G.H. (1998). Inhibition of neointimal cell bcl-x expression induces apoptosis and regression of vascular disease. Nat Med *4*, 222-227.

Seto, R., Lopez, A.D., Boreham, J., Thun, M., Heath, C., Jr., and Doll, R. (1996). Mortality from smoking worldwide. Br Med Bull *52*, 12-21.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A.*, et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Petrakis, N.L. (1977). Breast secretory activity in nonlactating women, postpartum breast involution, and the epidemiology of breast cancer. Natl Cancer Inst Monogr *47*, 161-164.

Petrakis, N.L. (1977). Genetic factors in the etiology of breast cancer. Cancer *39*, 2709-2715.

Petrakis, N.L., Gruenke, L.D., Beelen, T.C., Castagnoli, N., Jr., and Craig, J.C. (1978). Nicotine in breast fluid of nonlactating women. Science *199*, 303-305.

Petrakis, N.L., Maack, C.A., Lee, R.E., and Lyon, M. (1980). Mutagenic activity in nipple aspirates of human breast fluid. Cancer Res *40*, 188-189.

Petrakis, N.L., Mason, L., Lee, R., Sugimoto, B., Pawson, S., and Catchpool, F. (1975). Association of race, age, menopausal status, and cerumen type with breast fluid secretion in nonlactating women, as determined by nepple aspiration. J Natl Cancer Inst *54*, 829-834.

Petros, A.M., Medek, A., Nettesheim, D.G., Kim, D.H., Yoon, H.S., Swift, K., Matayoshi, E.D., Oltersdorf, T., and Fesik, S.W. (2001). Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A *98*, 3012-3017.

Phillips, D.H., and Garte, S. (2008). Smoking and breast cancer: is there really a link? Cancer Epidemiol Biomarkers Prev *17*, 1-2.

Piwien-Pilipuk, G., MacDougald, O., and Schwartz, J. (2002). Dual regulation of phosphorylation and dephosphorylation of C/EBPbeta modulate its transcriptional activation and DNA binding in response to growth hormone. J Biol Chem *277*, 44557-44565.

Plachetka, A., Chayka, O., Wilczek, C., Melnik, S., Bonifer, C., and Klempnauer, K.H. (2008). C/EBPbeta induces chromatin opening at a cell-type-specific enhancer. Mol Cell Biol *28*, 2102-2112.

Poli, V., Mancini, F.P., and Cortese, R. (1990). IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell *63*, 643-653.

Porter, D., Lahti-Domenici, J., Keshaviah, A., Bae, Y.K., Argani, P., Marks, J., Richardson, A., Cooper, A., Strausberg, R., Riggins, G.J.*, et al.* (2003). Molecular markers in ductal carcinoma in situ of the breast. Mol Cancer Res *1*, 362-375.

Poruchynsky, M.S., Wang, E.E., Rudin, C.M., Blagosklonny, M.V., and Fojo, T. (1998). Bcl-xL is phosphorylated in malignant cells following microtubule disruption. Cancer Res *58*, 3331-3338.

Polyak, K., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1996). Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. Am J Pathol *149*, 381-387.

Ramji, D.P., and Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J *365*, 561-575.

Raught, B., Gingras, A.C., James, A., Medina, D., Sonenberg, N., and Rosen, J.M. (1996). Expression of a translationally regulated, dominant-negative CCAAT/enhancer-binding protein beta isoform and up-regulation of the eukaryotic translation initiation factor 2alpha are correlated with neoplastic transformation of mammary epithelial cells. Cancer Res *56*, 4382-4386.

Raught, B., Liao, W.S., and Rosen, J.M. (1995). Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence beta-casein gene expression. Mol Endocrinol *9*, 1223-1232.

Reed, J.C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-776.

Repesh, L.A. (1989). A new in vitro assay for quantitating tumor cell invasion. Invasion Metastasis *9*, 192-208.

Reynolds, P., Hurley, S., Goldberg, D.E., Anton-Culver, H., Bernstein, L., Deapen, D., Horn-Ross, P.L., Peel, D., Pinder, R., Ross, R.K.*, et al.* (2004). Active smoking, household passive smoking, and breast cancer: evidence from the California Teachers Study. J Natl Cancer Inst *96*, 29-37.

Rithidech, K., Chen, B.T., Mauderly, J.L., Whorton, E.B., Jr., and Brooks, A.L. (1989). Cytogenetic effects of cigarette smoke on pulmonary alveolar macrophages of the rat. Environ Mol Mutagen *14*, 27-33.

Robinson, G.W., Johnson, P.F., Hennighausen, L., and Sterneck, E. (1998). The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev *12*, 1907-1916.

Ronnov-Jessen, L., Petersen, O.W., and Bissell, M.J. (1996). Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev *76*, 69-125.

Roth, S.Y., Denu, J.M., and Allis, C.D. (2001). Histone acetyltransferases. Annu Rev Biochem *70*, 81-120.

Rouayrenc, J.F., Boise, L.H., Thompson, C.B., Privat, A., and Patey, G. (1995). Presence of the long and the short forms of Bcl-X in several human and murine tissues. C R Acad Sci III *318*, 537-540.

Routledge, M.N., Garner, R.C., Jenkins, D., and Cuzick, J. (1992). 32P-postlabelling analysis of DNA from human tissues. Mutat Res *282*, 139-145.

Rubio, N., Espana, L., Fernandez, Y., Blanco, J., and Sierra, A. (2001). Metastatic behavior of human breast carcinomas overexpressing the Bcl-x(L) gene: a role in dormancy and organospecificity. Lab Invest *81*, 725-734.

Rudin, N. (1997). Transformation. In Dictionary of Modern Biology (Hauppauge, NY, Barron's Educational Series, Inc.), p. 371.

Russo, J., and Russo, I.H. (1980). Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res *40*, 2677-2687.

Russo, J., and Russo, I.H. (1987). Biological and molecular bases of mammary carcinogenesis. Lab Invest *57*, 112-137.

Russo, J., and Russo, I.H. (2001). The pathway of neoplastic transformation of human breast epithelial cells. Radiat Res *155*, 151-154.

Russo, J., Tay, L.K., and Russo, I.H. (1982). Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat *2*, 5-73.

Sarrio, D., Rodriguez-Pinilla, S.M., Hardisson, D., Cano, A., Moreno-Bueno, G., and Palacios, J. (2008). Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res *68*, 989-997.

Sabatakos, G., Davies, G.E., Grosse, M., Cryer, A., and Ramji, D.P. (1998). Expression of the genes encoding CCAAT-enhancer binding protein isoforms in the mouse mammary gland during lactation and involution. Biochem J *334 ( Pt 1)*, 205-210.

Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S., Montal, M., and Reed, J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci U S A *94*, 5113-5118.

Schneider, T.J., Grillot, D., Foote, L.C., Nunez, G.E., and Rothstein, T.L. (1997). Bcl-x protects primary B cells against Fas-mediated apoptosis. J Immunol *159*, 4834-4839.

Schott, A.F., Apel, I.J., Nunez, G., and Clarke, M.F. (1995). Bcl-XL protects cancer cells from p53-mediated apoptosis. Oncogene *11*, 1389-1394.

Schreier, M.H., and Staehelin, T. (1973). Initiation of eukaryotic protein synthesis: (Met-tRNA f -40S ribosome) initiation complex catalysed by purified initiation factors in the absence of mRNA. Nat New Biol *242*, 35-38.

Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G., and et al. (1995). Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. EMBO J *14*, 1932-1941.

Seagroves, T.N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G.J., and Rosen, J.M. (1998). C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev *12*, 1917-1928.

Seagroves, T.N., Lydon, J.P., Hovey, R.C., Vonderhaar, B.K., and Rosen, J.M. (2000). C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. Mol Endocrinol *14*, 359-368.

Seto, M., Jaeger, U., Hockett, R.D., Graninger, W., Bennett, S., Goldman, P., and Korsmeyer, S.J. (1988). Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J *7*, 123-131.

Sevilla, L., Aperlo, C., Dulic, V., Chambard, J.C., Boutonnet, C., Pasquier, O., Pognonec, P., and Boulukos, K.E. (1999). The Ets2 transcription factor inhibits apoptosis induced by colony-stimulating factor 1 deprivation of macrophages through a Bcl-xL-dependent mechanism. Mol Cell Biol *19*, 2624-2634.

Shapiro, D.J., Sharp, P.A., Wahli, W.W., and Keller, M.J. (1988). A high-efficiency HeLa cell nuclear transcription extract. DNA *7*, 47-55.

Sheridan, C., Kishimoto, H., Fuchs, R.K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C.H., Goulet, R., Jr., Badve, S., and Nakshatri, H. (2006). CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res *8*, R59.

Shimizu, Y., Kishimoto, T., Ohtsuka, M., Kimura, F., Shimizu, H., Yoshidome, H., and Miyazaki, M. (2007). CCAAT/enhancer binding protein-beta promotes the survival of intravascular rat pancreatic tumor cells via antiapoptotic effects. Cancer Sci *98*, 1706-1713.

Shin, S.I., Freedman, V.H., Risser, R., and Pollack, R. (1975). Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc Natl Acad Sci U S A *72*, 4435-4439.

Shu, H.P., and Bymun, E.N. (1983). Systemic excretion of benzo(a)pyrene in the control and microsomally induced rat: the influence of plasma lipoproteins and albumin as carrier molecules. Cancer Res *43*, 485-490.

Simoes-Wust, A.P., Olie, R.A., Gautschi, O., Leech, S.H., Haner, R., Hall, J., Fabbro, D., Stahel, R.A., and Zangemeister-Wittke, U. (2000). Bcl-xl antisense treatment induces apoptosis in breast carcinoma cells. Int J Cancer *87*, 582-590.

Simonian, P.L., Grillot, D.A., and Nunez, G. (1997). Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood *90*, 1208-1216.
Sivko, G.S., and DeWille, J.W. (2004). CCAAT/Enhancer binding protein delta (c/EBPdelta) regulation and expression in human mammary epithelial cells: I. "Loss of function" alterations in the c/EBPdelta growth inhibitory pathway in breast cancer cell lines. J Cell Biochem *93*, 830-843.

Siziopikou, K.P., and Khan, S. (2005). Correlation of HER2 gene amplification with expression of the apoptosis-suppressing genes bcl-2 and bcl-x-L in ductal carcinoma in situ of the breast. Appl Immunohistochem Mol Morphol *13*, 14-18.

Slamon, D.J., deKernion, J.B., Verma, I.M., and Cline, M.J. (1984). Expression of cellular oncogenes in human malignancies. Science *224*, 256-262.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707-712.

Smale, S.T., and Baltimore, D. (1989). The "initiator" as a transcription control element. Cell *57*, 103-113.

Smith, C.J., and Hansch, C. (2000). The relative toxicity of compounds in mainstream cigarette smoke condensate. Food Chem Toxicol *38*, 637-646.

Smith, C.J., Livingston, S.D., and Doolittle, D.J. (1997). An international literature survey of "IARC Group I carcinogens" reported in mainstream cigarette smoke. Food Chem Toxicol *35*, 1107-1130.

Smith, C.J., Perfetti, T.A., Garg, R., and Hansch, C. (2003). IARC carcinogens reported in cigarette mainstream smoke and their calculated log P values. Food Chem Toxicol *41*, 807-817.

Smith, C.J., Perfetti, T.A., Rumple, M.A., Rodgman, A., and Doolittle, D.J. (2000). "IARC group 2A Carcinogens" reported in cigarette mainstream smoke. Food Chem Toxicol *38*, 371-383.

Smith, C.J., Perfetti, T.A., Rumple, M.A., Rodgman, A., and Doolittle, D.J. (2001). "IARC Group 2B carcinogens" reported in cigarette mainstream smoke. Food Chem Toxicol *39*, 183-205.

Smith, H.S., Wolman, S.R., and Hackett, A.J. (1984). The biology of breast cancer at the cellular level. Biochim Biophys Acta *738*, 103-123.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., *et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., *et al.* (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A *100*, 8418-8423.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Jr., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., and Brooks, S.C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res *50*, 6075-6086.

Srinivasan, A., Li, F., Wong, A., Kodandapani, L., Smidt, R., Jr., Krebs, J.F., Fritz, L.C., Wu, J.C., and Tomaselli, K.J. (1998). Bcl-xL functions downstream of caspase-8 to inhibit Fas- and tumor necrosis factor receptor 1-induced apoptosis of MCF7 breast carcinoma cells. J Biol Chem *273*, 4523-4529.

Stern, D.F., Heffernan, P.A., and Weinberg, R.A. (1986). p185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol Cell Biol *6*, 1729-1740.

Sterneck, E., Tessarollo, L., and Johnson, P.F. (1997). An essential role for C/EBPbeta in female reproduction. Genes Dev *11*, 2153-2162.

Stingl, J., and Caldas, C. (2007). Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer *7*, 791-799.

Strasser, A., Harris, A.W., Huang, D.C., Krammer, P.H., and Cory, S. (1995). Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J *14*, 6136-6147.

Streuli, C.H., and Gilmore, A.P. (1999). Adhesion-mediated signaling in the regulation of mammary epithelial cell survival. J Mammary Gland Biol Neoplasia *4*, 183-191.

Sullivan, S. (1984). The Reference and Research Cigarette Series (Lexington, KY: University of Kentucky Printing Services).

Sundfeldt, K., Ivarsson, K., Carlsson, M., Enerback, S., Janson, P.O., Brannstrom, M., and Hedin, L. (1999). The expression of CCAAT/enhancer binding protein (C/EBP) in the human ovary in vivo: specific increase in C/EBPbeta during epithelial tumour progression. Br J Cancer *79*, 1240-1248.

Tait, L., Soule, H.D., and Russo, J. (1990). Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res *50*, 6087-6094.

Tahirov, T.H., Inoue-Bungo, T., Morii, H., Fujikawa, A., Sasaki, M., Kimura, K., Shiina, M., Sato, K., Kumasaka, T., Yamamoto, M.*, et al.* (2001). Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta. Cell *104*, 755-767.

Tahirov, T.H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M., Kimura, K., Takata, S., Fujikawa, A., Morii, H.*, et al.* (2002). Mechanism of c-Myb-C/EBP beta cooperation from separated sites on a promoter. Cell *108*, 57-70.

Takehara, T., and Takahashi, H. (2003). Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. Cancer Res *63*, 3054-3057.

Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell *80*, 353-361.

Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. EMBO J *16*, 7432-7443.

Tang, Q.Q., Gronborg, M., Huang, H., Kim, J.W., Otto, T.C., Pandey, A., and Lane, M.D. (2005). Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. Proc Natl Acad Sci U S A *102*, 9766-9771.

Tanko, L.B., and Christiansen, C. (2004). An update on the antiestrogenic effect of smoking: a literature review with implications for researchers and practitioners. Menopause *11*, 104-109.

Taylor, J.K., Zhang, Q.Q., Wyatt, J.R., and Dean, N.M. (1999). Induction of endogenous Bcl-xS through the control of Bcl-x pre-mRNA splicing by antisense oligonucleotides. Nat Biotechnol *17*, 1097-1100.

Taylor-Papadimitriou, J., Stampfer, M., Bartek, J., Lewis, A., Boshell, M., Lane, E.B., and Leigh, I.M. (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium. J Cell Sci *94 ( Pt 3)*, 403-413.

Terry, P.D., Miller, A.B., and Rohan, T.E. (2002). Cigarette smoking and breast cancer risk: a long latency period? Int J Cancer *100*, 723-728.

Terry, P.D., and Rohan, T.E. (2002). Cigarette smoking and the risk of breast cancer in women: a review of the literature. Cancer Epidemiol Biomarkers Prev *11*, 953-971.

Thiery, J.P. (2003). Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol *15*, 740-746.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Timchenko, L.T., Iakova, P., Welm, A.L., Cai, Z.J., and Timchenko, N.A. (2002). Calreticulin interacts with C/EBPalpha and C/EBPbeta mRNAs and represses translation of C/EBP proteins. Mol Cell Biol *22*, 7242-7257.

Tomlinson, I.P. (2001). Mutations in normal breast tissue and breast tumours. Breast Cancer Res *3*, 299-303.

Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. (1993). Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature *364*, 544-547.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C., and Croce, C.M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science *226*, 1097-1099.

Tsukahara, T., Kannagi, M., Ohashi, T., Kato, H., Arai, M., Nunez, G., Iwanaga, Y., Yamamoto, N., Ohtani, K., Nakamura, M., and Fujii, M. (1999). Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax. J Virol *73*, 7981-7987

U.S. Department of Human and Health Services (1994). Preventing Tobacco use among yound people: A report of hte surgeon general.

U.S. Department of Human and Health Services (2004). The Health Consequences of Smoking – A Report of the Surgeon General. (Rockville, MD, Public Health Service, Centers for Disease Control and Prevention, Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health).

U.S. Department of Human and Health Services (2006). The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General. (Rockville, MD, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health).

Vander Heiden, M.G., Chandel, N.S., Schumacker, P.T., and Thompson, C.B. (1999). Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol Cell *3*, 159-167.

Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T., and Thompson, C.B. (1997). Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell *91*, 627-637.

Van Dyke, M.W., Roeder, R.G., and Sawadogo, M. (1988). Physical analysis of transcription preinitiation complex assembly on a class II gene promoter. Science *241*, 1335-1338.

Vaux, D.L., Cory, S., and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature *335*, 440-442.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell *102*, 43-53.

Villagra, A., Cruzat, F., Carvallo, L., Paredes, R., Olate, J., van Wijnen, A.J., Stein, G.S., Lian, J.B., Stein, J.L., Imbalzano, A.N., and Montecino, M. (2006). Chromatin remodeling and transcriptional activity of the bone-specific osteocalcin gene require CCAAT/enhancer-binding protein beta-dependent recruitment of SWI/SNF activity. J Biol Chem *281*, 22695-22706.

Vineis, P., Veglia, F., Benhamou, S., Butkiewicz, D., Cascorbi, I., Clapper, M.L., Dolzan, V., Haugen, A., Hirvonen, A., Ingelman-Sundberg, M*., et al.* (2003). CYP1A1 T3801 C polymorphism and lung cancer: a pooled analysis of 2451 cases and 3358 controls. Int J Cancer *104*, 650-657.

Vinson, C.R., Hai, T., and Boyd, S.M. (1993). Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. Genes Dev *7*, 1047-1058.

Vinson, C.R., Sigler, P.B., and McKnight, S.L. (1989). Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science *246*, 911-916.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. N Engl J Med *319*, 525-532.

Wang, T.C., Cardiff, R.D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E.V. (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature *369*, 669-671.

Watson, D.K., McWilliams, M.J., Lapis, P., Lautenberger, J.A., Schweinfest, C.W., and Papas, T.S. (1988). Mammalian ets-1 and ets-2 genes encode highly conserved proteins. Proc Natl Acad Sci U S A *85*, 7862-7866.

Watson, D.K., McWilliams-Smith, M.J., Nunn, M.F., Duesberg, P.H., O'Brien, S.J., and Papas, T.S. (1985). The ets sequence from the transforming gene of avian erythroblastosis virus, E26, has unique domains on human chromosomes 11 and 21: both loci are transcriptionally active. Proc Natl Acad Sci U S A *82*, 7294-7298.

Weintraub, S.J., Manson, S.R., and Deverman, B.E. (2004). Resistance to antineoplastic therapy. The oncogenic tyrosine kinase-Bcl-x(L) axis. Cancer Cell *5*, 3-4.

Weis, L., and Reinberg, D. (1992). Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. FASEB J *6*, 3300-3309.

Wells, A. (2000). Smoking and cancer in Women. Journal of Women's Cancer *2*, 55-66.

Welm, A.L., Timchenko, N.A., and Darlington, G.J. (1999). C/EBPalpha regulates generation of C/EBPbeta isoforms through activation of specific proteolytic cleavage. Mol Cell Biol *19*, 1695-1704.

Westermarck, J., and Kahari, V.M. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J *13*, 781-792.

Whitehead, J.K., and Rothwell, K. (1969). The mouse skin carcinogenicity of cigarette smoke condensate: fractionated by solvent partition methods. Br J Cancer *23*, 840-857.

Williams, S.C., Baer, M., Dillner, A.J., and Johnson, P.F. (1995). CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. EMBO J *14*, 3170-3183.

Williams, S.C., Cantwell, C.A., and Johnson, P.F. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev *5*, 1553-1567.

Wistuba, II, Mao, L., and Gazdar, A.F. (2002). Smoking molecular damage in bronchial epithelium. Oncogene *21*, 7298-7306.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J.R., Farndon, J., Cairns, J., Harris, A.L., and Horne, C.H. (1992). Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer *65*, 118-121.

Wynder, E., Hoffman, D (1967). Tobacco and Tobacco Smoke (New York, NY: Academic Press).

Wynder, E.L., and Wright, G. (1957). A study of tobacco carcinogenesis. I. The primary fractions. Cancer *10*, 255-271.

Xiong, W., Hsieh, C.C., Kurtz, A.J., Rabek, J.P., and Papaconstantinou, J. (2001). Regulation of CCAAT/enhancer-binding protein-beta isoform synthesis by alternative translational initiation at multiple AUG start sites. Nucleic Acids Res *29*, 3087-3098.

Xu, M., Nie, L., Kim, S.H., and Sun, X.H. (2003). STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPbeta. EMBO J *22*, 893-904.

Yang, X., Hao, Y., Pater, M.M., Tang, S.C., and Pater, A. (1998). Enhanced expression of anti-apoptotic proteins in human papillomavirus-immortalized and cigarette smoke condensate-transformed human endocervical cells: correlation with resistance to apoptosis induced by DNA damage. Mol Carcinog *22*, 95-101.

Yang, X., Nakao, Y., Pater, M.M., Tang, S.C., and Pater, A. (1997). Expression of cellular genes in HPV16-immortalized and cigarette smoke condensate-transformed human endocervical cells. J Cell Biochem *66*, 309-321.

Yefenof, E., Picker, L.J., Scheuermann, R.H., Tucker, T.F., Vitetta, E.S., and Uhr, J.W. (1993). Cancer dormancy: isolation and characterization of dormant lymphoma cells. Proc Natl Acad Sci U S A *90*, 1829-1833.

Yin, X.M., Oltvai, Z.N., and Korsmeyer, S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature *369*, 321-323.

Zahnow, C.A. (2002). CCAAT/enhancer binding proteins in normal mammary development and breast cancer. Breast Cancer Res *4*, 113-121.

Zahnow, C.A., Cardiff, R.D., Laucirica, R., Medina, D., and Rosen, J.M. (2001). A role for CCAAT/enhancer binding protein beta-liver-enriched inhibitory protein in mammary epithelial cell proliferation. Cancer Res *61*, 261-269.

Zahnow, C.A., Younes, P., Laucirica, R., and Rosen, J.M. (1997). Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. J Natl Cancer Inst *89*, 1887-1891.

Zajchowski, D.A., Bartholdi, M.F., Gong, Y., Webster, L., Liu, H.L., Munishkin, A., Beauheim, C., Harvey, S., Ethier, S.P., and Johnson, P.H. (2001). Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res *61*, 5168-5178.

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell *87*, 619-628.

Zhao, R., Yang, F.T., and Alexander, D.R. (2004). An oncogenic tyrosine kinase inhibits DNA repair and DNA-damage-induced Bcl-xL deamidation in T cell transformation. Cancer Cell *5*, 37-49.

Zhivotovsky, B., and Kroemer, G. (2004). Apoptosis and genomic instability. Nat Rev Mol Cell Biol *5*, 752-762.

Zhou, H., Hou, Q., Chai, Y., and Hsu, Y.T. (2005). Distinct domains of Bcl-XL are involved in Bax and Bad antagonism and in apoptosis inhibition. Exp Cell Res *309*, 316-328.

Zhu, C., Mills, K.D., Ferguson, D.O., Lee, C., Manis, J., Fleming, J., Gao, Y., Morton, C.C., and Alt, F.W. (2002). Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. Cell *109*, 811-821.

Zimmermann, A., and Keller, H.U. (1987). Locomotion of tumor cells as an element of invasion and metastasis. Biomed Pharmacother *41*, 337-344.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev *15*, 1481-1486.

Zoratti, M., and Szabo, I. (1995). The mitochondrial permeability transition. Biochim Biophys Acta *1241*, 139-176.

# BIOGRAPHICAL SKETCH

The product of a military family, Shahnjayla Connors was born in Siegen, (West) Germany. She is the only daughter of Rodney and Sharon Connors. While attending Warner Robins High School, Shahnjayla was an active member in Mu Alpha Theta Math Club and the Beta Club. She was also active in Girl Scouting and earned her Girl Scout Silver Award and Gold Award.

After graduating from high school in 1999, Shahnjayla pursued a B.S. in biology from Georgia Southern University, where she was a member of the University Honors Program. The end of her sophomore year, Shahnjayla was named a Ronald E. McNair Postbaccalaureate Achievement Program Scholar and was exposed to her first research experience. She completed a summer project on the population genetics of *Ixodes scapularis*, the tick vector of Lyme disease. This research project confirmed her love of biological research and she participated in several other research projects during her undergraduate studies. Shahnjayla continued her study of *Ixodes scapularis* and identified spiroplasma bacteria from the gut of horseflies as independent study projects. She also traveled to Iowa and participated in a project characterizing programmed cell death in the neurons of the nematode, *Caenorhabditis elegans*. She was able to present her work two national McNair conferences and several research symposiums at her college.

After graduating from Georgia Southern in May of 2003, Shahnjayla entered the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida and completed her doctoral research on the upregulation of *bcl-xl* in human breast epithelial cells treated with cigarette smoke condensate in the laboratory of Satya Narayan, Ph.D. She has presented her doctoral research at several departmental and national meetings. During her doctoral studies, she also served as a McNair Peer Advisor for the University of Florida.

Shahnjayla received her Ph.D. in Medical Sciences in August 2008. She is currently a postdoctoral associate working in the area of cancer disparities and pursuing a Master in Public Health.