CCAAT/ ENHANCER BINDING PROTEIN BETA 2 IN MAMMARY EPITHELIAL CELLS

By

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To my husband, Ron Russell,

for his unconditional love and support.

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LIST OF ABBREVIATIONS

ADH – atypical ductal hyperplasia

ALDH1 – aldehyde deydrogenase 1

AR – amphiregulin

BTC – betacellulin

C/EBP – CCαAAT/Enhancer Binding Protein

CBM – chromatin-binding motif

CDH1 – epithelial cadherin

ChIP – chromatin immunoprecipitation

CPE – cytopathic effect

CSCs – cancer stem cells

CTSB – cathepsin B

CUGBP1 – CUG repeat binding protein

CYR61 – cystein-rich, angiogenic inducer, 61

DMBA – 7,12-dimethylbenz[a]anthracene

DCIS – ductal carcinoma *in situ*

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ELISA – enzyme linked immunosorbent assay

EPG – epigen

EPR – epiregulin

ER – estrogen receptor

EMT – epithelial to mesenchymal transition

HB-EGF – heparin binding EGF-like growth factor

HME – human mammary epithelial

HMGB1– high-mobility group box 1

GFP – green fluorescent protein

ICE – interleukin-1 beta converting enzyme

IGF1R – insulin like growth factor 1

IHC – immunohistochemistry

IL33 – Interleukin 33

IL1A – interleukin-1 alpha

IL1B – interleukin-1 beta

IL1R1 – interleukin-1 receptor 1

IL1R2 – interleukin-1 receptor 2

IL1RL1 – interleukin-1 receptor like 1

IDC – invasive ductal carcinoma

LAP – liver-enriched activator protein

LIN28B – lin-28 homolog B

LIP – liver-enriched inhibitory protein

MAPK – mitogen activated protein kinase

MECs – mammary epithelial cells

NLR – NOD-like receptor

NRG – neuregulin

PYCARD – pyrin and CARD domain-containing

PI3K – phophatilinositol 3 kinase

PIN1 – peptidyl-prolyl isomerase, NIMA – interacting 1

PLC – phospholipase c

PR – progesterone receptor

RTKs – receptor tyrosine kinases

SPARC – secreted protein, acidic, cystein-rich

TDLUs – terminal ductal lobulo-aveolar units

TEBs – terminal end buds

TGF – transforming growth factor

TMS1– target of methylation induced silencing 1

TPA – tetrodecanoylphorbol

VEGF – vascular epidermal growth factor

CHAPTER I

INTRODUCTION

Breast Cancer Overview

According to the American Cancer Society, in 2009 in the United States alone it is estimated 200,000 people will be diagnosed with breast cancer and tragically 40,000 people will die from this disease. In fact breast cancer is so prevalent that 1:8 women will be diagnosed with it in their lifetime and so deadly that it remains the 2nd leading cause of cancer-related death in women. Although the overall five year survival rate is nearing 88% due to early detection and advances in treatment, women diagnosed with more advanced and/or aggressive forms of the disease have a 26% chance of surviving five years.

It is estimated that 5 - 10% of breast cancer cases are hereditary. Currently, however, the inherited mutation can be identified in only a subset of hereditary breast cancer cases. Germline mutations in BRCA1 and BRCA2 account for a majority of hereditary breast cancers in families with a high incidence (four or more) of breast and/or ovarian cancers (Miki, Swensen et al. 1994; Wooster, Bignell et al. 1995; Tavtigian, Simard et al. 1996). BRCA1 and BRCA2 have been shown to have essential roles in both DNA repair and cell-cycle checkpoint control (Scully and Livingston 2000; Jasin 2002; Venkitaraman 2002). The involvement of BRCA1 and BRCA2 in homologous recombination-based DNA double-strand break repair and s-phase DNA repair have led to the hypothesis that loss of BRCA1 or BRCA2 directly results in genomic instability and cancer progression (recently reviewed (Venkitaraman 2009)). BRCA1 and BRCA2

do not function independently in the cell, but rather are important contributors to a functional network of proteins which collectively maintain genomic stability. Mutations in the gene products of these associated proteins have been demonstrated in families with high incidences of breast cancer but who are negative for BRCA1 and BRCA2 mutations, thus expanding the number of confirmed hereditary breast cancer cases (Walsh and King 2007; Wang 2007). In total 20-30% of breast cancer patients have an immediate family member who has had breast cancer. Therefore, 70% of breast cancer patients have no family history of the disease.

Multiple other risk factors for breast cancer have been identified ranging from lifestyle risks, which can be reduced by changes in behavior, to risks which are difficult to alter (reviewed (Hulka and Moorman 2008). Obesity, use of hormone therapy following menopause, and alcohol consumption all result in an increased risk of breast cancer and can be addressed in order to reduce the risk of breast cancer. Early menarche and/or late menopause, age at first full-term pregnancy, exposure to radiation, and breast density are not easily altered to reduce risk. Many of these risk factors relate directly to the amount of estrogen (both endogenous and exogenous) to which the breast is exposed. This observation is not surprising as endogenous estrogen levels have been known to correlate with breast cancer risk for over 15 years (Bernstein and Ross 1993). Since adipose tissue is capable of producing estrogen, the link between high levels of endogenous estrogen and increased breast cancer risk is especially important in light of the growing obesity epidemic (Cleary and Grossmann 2009). In addition to increasing the risk of breast cancer, obesity is associated with increased tumor burden and higher grade tumors (Berclaz, Li et al. 2004; Cleveland, Eng et al. 2007; Demirkan, Alacacioglu

et al. 2007). Adipose tissue is also capable of producing interleukin (IL) 1, a pro-inflammatory cytokine which is known to contribute to breast cancer progression. Interestingly, the IL1 has been shown to result in increased aromatase activity, and thus increase production of estrogen, in breast cancer cell lines (Honma, Shimodaira et al. 2002).

Breast Cancer Origin and Progression

The mammary gland is a dynamic organ which undergoes remarkable growth and remodeling in response to hormonal signals at puberty and during pregnancy. A rudimentary ductal tree is formed during embryonic development which remains relatively unchanged until puberty (embryonic development of the mammary gland is reviewed (Hens and Wysolmerski 2005). At puberty estrogen stimulates the development of highly proliferative structures called terminal ductal lobulo-aveolar units (TDLUs) in humans and terminal end buds (TEBs) in mice (reviewed (Hinck and Silberstein 2005). The mammary epithelial cells (MECs) then penetrate the fatty stroma forming a system of ducts and branches. Proliferation of the epithelial cells occurs again during pregnancy in response to estrogen and progesterone resulting in an increased number of ducts and branches. Alveolar growth and differentiation occur concurrently. When the offspring are born, functional differentiation occurs and milk is synthesized and secreted. Upon weaning, a process called involution proceeds. During involution the alveolar compartment undergoes massive cell death and remodeling resulting in a virgin-like state (entire cycle reviewed (Sternlicht, Kouros-Mehr et al. 2006)). This cycle of

growth/branching, differentiation, and involution is repeated with each pregnancy. Each stage depends on a critical balance between proliferation, differentiation, and cell death.

Epithelial cells are believed to be the originating cell for ninety-nine percent of breast cancers, and it has been suggested the developmental pathways described above can be hijacked during tumorigenesis (Dickson, Creer et al. 2000; McGee, Lanigan et al. 2006; Lanigan, O'Connor et al. 2007). A single cell that accumulates multiple mutations is thought lead to breast cancer (Hanahan and Weinberg 2000; Hahn and Weinberg 2002). Both ductal and lobular epithelial cells can undergo genetic mutations resulting in the formation of cancer. Ductal carcinoma accounts for approximately eighty percent of mammary carcinomas, while lobular carcinomas make up about fifteen percent (Rosen 1996). Ductal and lobular carcinomas are distinct. Lobular carcinomas proliferate at a slower rate, are more estrogen receptor (ER) and progesterone receptor (PR) positive, and have lower vascular epidermal growth factor expression (VEGF) (Lee, Dublin et al. 1998; Kruger, Fahrenkrog et al. 1999; Coradini, Pellizzaro et al. 2002). As previously discussed, exposure to endogenous and exogenous estrogen are known risk factors for breast cancer. Estrogen is essential for the normal development of the mammary gland where it promotes proliferation of ductal epithelial cells and subsequent invasion of these cells into the surrounding stroma in a highly regulated manner. Excess estrogen, however, may contribute to the uncontrolled proliferation and invasion of epithelial cells involved in breast cancer (Lanigan, O'Connor et al. 2007).

Multiple other proteins and signaling pathways that are involved in the normal development of the mammary gland are also aberrantly activated in breast cancer. One such protein is ErbB2 (which will be discussed in detail shortly). ErbB2 is a therapeutic

target in breast cancer that also seems to play a role in normal development. TEB defects and delays in ductal penetration are apparent in mice lacking ErbB2 in MECs (Jackson-Fisher, Bellinger et al. 2004; Andrechek, White et al. 2005). However, it is currently unclear exactly how ErbB2 regulates ductal development. ErbB2 is an unusual receptor in that it has no known ligand it must interact with other ErbB family members to signal.

Although breast cancer originates from a single cell, breast cancer tumors are highly heterogeneous (Heppner 1984; Axelson, Fredlund et al. 2005). For example, breast tumor cells exhibit different invasive and metastatic capabilities. Primary tumor growth is originally restricted by the basement membrane and at this stage is termed carcinoma *in situ*. Invasive breast cancer occurs as the tumor cells undergo epithelial to mesenchymal transition (EMT) and the basement membrane is degraded. EMT includes reduced cell-cell adhesion, upregulation of matrix metalloproteases (MMPs, including MMP-1, -2, -3, -7, and -15), and expression of vimentin (a mesenchymal filament protein) (reviewed (Hugo, Ackland et al. 2007)). Once the basement membrane is degraded the tumor cells are able to migrate into the surrounding stroma or to intravasate into blood vessels. These tumor cells are then able to cause local recurrences or travel through the blood stream to distant sites. The tumor cells can then extravasate at the distant site and may result in a secondary tumor, called a metastasis (Welch, Steeg et al. 2000). All tumor cells, however, are not capable of completing all of the steps in this process. The cells present in the tumor also differ in morphology, proliferation rate, cell-cell adhesion, and sensitivity to chemotherapy (Heppner 1984; Axelson, Fredlund et al. 2005).

Two models have been developed to explain the mechanism by which breast tumor heterogeneity arises. The first model, clonal evolution, was first proposed in 1976 in response to the observation that tumors become de-differentiated as they progress (Nowell 1976). The clonal evolution model hypothesizes that selective pressure within the tumor results in an accumulation of cells with advantageous mutations which then drive tumor progression. This natural selection is ongoing; thus any cancer cell in the population has the possibility of becoming metastatic, developing resistance to therapeutic approaches, or causing recurrence (Merlo, Pepper et al. 2006). Based on clinical data it has been proposed that breast cancer progresses through a linear sequence of pathologically defined stages: beginning as an atypical ductal hyperplasia (ADH) before progressing to a ductal carcinoma *in situ* (DCIS) and then finally becoming an invasive ductal carcinoma (IDC) (Allred, Mohsin et al. 2001). It has been suggested this progression is a result of clonal evolution. In support of this, DCIS and IDC or primary breast tumor and metastases from the same patient have similar genetic alterations with the more advanced stage having acquired additional mutations compared to their matched DCIS or IDC (Fujii, Szumel et al. 1996; Kuukasjarvi, Karhu et al. 1997).

The cancer stem cell hypothesis has also been used to explain tumor heterogeneity. The cancer stem cell hypothesis states that tumors contain a small number of cells, termed cancer stem cells (CSCs), which are responsible for tumor growth and also generate the wide variety of cell types found in the tumor due to aberrant differentiation (Reya, Morrison et al. 2001). According to this model, metastatic spread, resistance to therapeutic approaches, and recurrence are due only to the CSCs present in the tumor. These CSCs may arise from mutations to tissue-specific stem or progenitor

cells. This model is especially attractive in breast cancer since mammary stem cells have long been thought to play an important role in the dynamic growth and remodeling that occurs in the mammary gland (reviewed (Molyneux, Regan et al. 2007; Song and Miele 2007)). Early full term pregnancy may lower the risk for breast cancer by reducing the number of stem cells present in the mammary gland (Siwko, Dong et al. 2008). The existence of mammary stem cells has been supported by recent studies where a functional mouse mammary gland has been produced by a single cell (Shackleton, Vaillant et al. 2006; Stingl, Eirew et al. 2006). In addition, mammary human stem cells (or progenitor cells) cultured *in vitro* have been shown to give rise to multiple mammary cell types (Stingl, Eaves et al. 2001; Gudjonsson, Villadsen et al. 2002; Dontu, Abdallah et al. 2003). CD44, one of the markers used to isolate these normal mammary stem/progenitor cells, was subsequently used to identify breast cancer stem cells. $CD44^{+}/CD24^{low}$ cells lacking other normal cell markers were isolated from human mammary carcinomas. Injection of $CD44^{+}/CD24^{low}$ cells into immunocompromised mice resulted in heterogenic tumors, while injection of $CD44^{+}/CD24^{high}$ resulted in no tumors (Al-Hajj, Wicha et al. 2003). The $CD44^{+}/CD24^{low}$ phenotype confers mammary cells an increased metastatic potential since they are more able to survive traveling to and more able to colonize a new location (Pandit, Kennette et al. 2009). This may be due in part to higher levels of Bcl-2 and thus greater resistance to apoptosis (Madjd, Mehrjerdi et al. 2009). Recently aldehyde dehydrogenase 1 (ALDH1) activity was shown to not only be a mammary stem cell marker but also to predict poor clinical outcome (Ginestier, Hur et al. 2007). High ALDH1 activity has been found in the majority of circulating breast tumor cells and is associated with resistance to chemotherapy (Aktas, Tewes et al. 2009; Tanei, Morimoto

et al. 2009). If CSCs are responsible for the heterogeneity of breast tumors then looking at total tumor reduction by a given therapy may not correspond with patient outcome, since the small population of tumor initiating and promoting cells may survive and result in disease relapse.

Breast Cancer Subtypes

Despite the heterogeneous mixture of cells found within each breast tumor, breast cancers can be divided into several common subtypes. On the molecular level breast cancer subtypes in the clinic are usually based on the expression of the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (ErbB2, also called Her2). The expression of ER, PR, and ErbB2 is routinely detected using immunohistochemistry (IHC). ER is expressed in about seventy percent of breast cancers and is predictive of response to treatment with tamoxifen or other endocrine treatments (Allred, Harvey et al. 1998; Nadji, Gomez-Fernandez et al. 2005). PR expression is an independent prognostic factor for disease-free and overall survival (Bardou, Arpino et al. 2003). ErbB2 amplification and overexpression are found in twenty-five percent of breast cancers and is an independent marker of poor prognosis (Paik, Hazan et al. 1990; Press, Bernstein et al. 1997). ErbB2 overexpression is predictive of resistance to tamoxifen in ER+ tumors but predicts sensitivity to anthracycline-based chemotherapy (De Laurentiis, Arpino et al. 2005; Pritchard, Shepherd et al. 2006; Villman, Sjostrom et al. 2006). In addition, ErbB2 overexpression predicts sensitivity to the monoclonal antibody trastuzumab. Trastuzumab directly targets ErbB2 and results in improved response rate and survival (Cobleigh, Vogel et al.

1999; Vogel, Cobleigh et al. 2002). In fact, if trastuzumab is administered in early stages as an adjuvant therapy it can reduce the risk of recurrence by half and the mortality by a third (Piccart-Gebhart, Procter et al. 2005; Romond, Perez et al. 2005). Evaluation of tumors by IHC for ER, PR, and ErbB2 will undoubtedly continue in the clinic for some time due to their inexpensive nature combined with the prognostic and predictive value (Onitilo, Engel et al. 2009).

During the last decade extensive genomic profiling of breast tumors has been performed revealing molecular subtypes of breast cancer based on the transcriptional profiles of the tumors (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001; van 't Veer, Dai et al. 2002; Sorlie, Tibshirani et al. 2003; Sotiriou, Neo et al. 2003; Bertucci, Finetti et al. 2005; Calza, Hall et al. 2006; Kapp, Jeffrey et al. 2006). Five subtypes have consistently been proposed based on unsupervised hierarchical clustering of the genomic data. These five subtypes are independent of the microarray platform utilized and hold true for breast cancer lines as well as tumors (Ross and Perou 2001; Charafe-Jauffret, Ginestier et al. 2006; Hu, Fan et al. 2006). They are Luminal A, Luminal B, Basal-like, ErbB2+, and normal-like. ER+ tumors were defined by the expression of genes normally found in the luminal epithelium, while the subtype (A or B) depended on the expression of genes related to the activation of ER such as GATA3 and NAT1. The basal-like ER- subtype was so named because these tumors express genes normally found in the basal/myopeithelial cells. The ErbB2+ subgroup was named due to the high expression of ErbB2 and other genes present in the 17q11 chromosome region that are often co-amplified with ErbB2. The final group, termed the normal-like group may not be a group at all but represent samples where more normal then tumor cells were present (Morris and

Carey 2007). However, the molecular subtypes listed here are not without problems as demonstrated by the presence of ER+/ErbB2+ tumors which are not easily classified. In fact, unsupervised clustering can also lead to the scattering of ErbB2+ throughout the Luminal A, Luminal B, and basal-like subtypes (Neve, Chin et al. 2006).

These gene expression studies were initiated in order to better understand breast cancer with the goals of finding better prognostic markers and predicting response to treatment, likelihood of metastasis, and chance of recurrence. Because molecular subtypes defined above correlate very well with the IHC expression levels of ER, PR, and ErbB2 (Luminal A: ER/PR+, ErbB2-; Luminal B: ER/PR+, ErbB+; ErbB2: ER/PR- ,ErbB2+, and basal-like ER/PR-, ErbB2-) and genomic profiling is expensive it is unlikely that the molecular subtypes will be used in the clinic any time soon (Onitilo, Engel et al. 2009). Genomic profiling of breast cancer has lead to the development of Oncotype DX and MammaPrint which are in limited clinical use today. Oncotype DX uses a 21 gene signature to predict the likelihood of recurrence in patients with stage I or II, node negative, ER+ breast cancer (Paik, Shak et al. 2004). This information is then used to determine the likely benefit of chemotherapy following endocrine therapy. MammaPrint determines metastatic potential based on 70 genes identified by Van't Veer *et al.* (van 't Veer, Dai et al. 2002; Glas, Floore et al. 2006).

ErbB Family Members

Cells rely on receptor tyrosine kinases (RTKs) to receive external stimuli and transduce the signal into the cell. Without RTKs cells would not be able to respond to their environment in the appropriate fashion. The epidermal growth-factor receptor

(EGFR, also known as the ErbB) family of RTKs is responsible for regulating diverse biologic processes, including proliferation, differentiation, cell motility, and survival through activation of the mitogen activated protein kinase (MAPK), phospholipase c gamma (PLCgamma), and phosphatilinositol 3 kinase (PI3K) pathways (Hynes and MacDonald 2009). The ErbB family of RTKs is expressed in tissues of epithelial, mesenchymal and neuronal origin (Hynes and Lane 2005). Four closely related members make up the ErbB family: epidermal growth factor receptor (EGFR, also known as ErbB1 or Her1), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4). These receptors, upon ligand activation, undergo homodimerization and heterodimerization resulting in phosphorylation and downstream signaling. Both ErbB1 and ErbB4 are capable of binding ligands and have active tyrosine phosphorylation domains (Guy, Platko et al. 1994; Olayioye, Neve et al. 2000). ErbB3 lacks kinase activity due to substitutions within the tyrosine kinase domain and must heterodimerize with a family member to signal (Guy, Platko et al. 1994; Olayioye, Neve et al. 2000). ErbB2 has no known ligand but has potent kinase activity and is the preferred binding partner for all other family members (Graus-Porta, Beerli et al. 1997). The receptor pair along with the initiating ligand determines which pathways are activated and to what extent. There are more than ten known ligands for the ErbB family members (Figure 1). These ligands all contain an epidermal growth factor (EGF)-like domain made up of three disulfide bonded intermolecular loops (Groenen, Nice et al. 1994). The ligands begin as transmembrane bound precursors which are then proteolytically cleaved to become active (Massague and Pandiella 1993; Groenen, Nice et al. 1994).

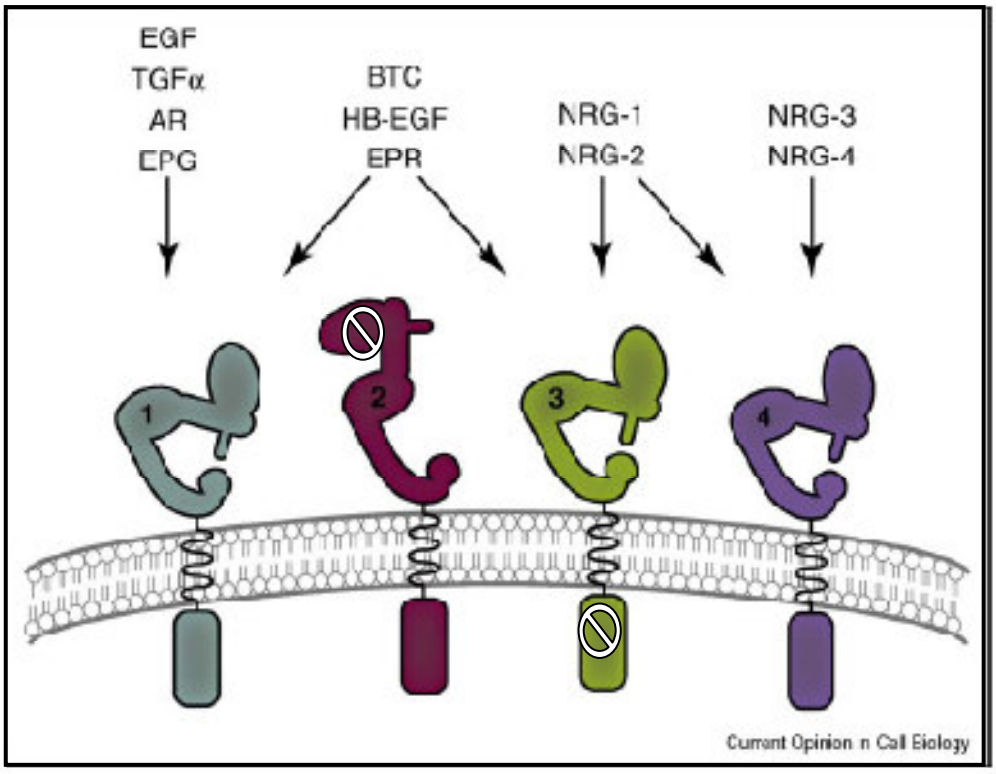


Figure 1. The ErbB family of receptors and their ligands. There are four members of the ErbB family of RTKs, EGFR (1), ErbB2 (2), ErbB3 (3), and ErbB4 (4). Upon ligand binding the receptors undergo a conformational change allowing for the formation of homodimers and heterodimers. The receptors then become phosphorylated on tyrosine residues within their cytoplasmic kinase domain, initiating downstream signaling. Four groups of ErbB ligands have been described on the basis of their receptor specificity. The first group binds EGFR exclusively and includes EGF, TGFa, AR, and EPG. Members of the second group (BTC, HB-EGF, and EPR) exhibit dual specificity for EGFR and ErbB4. The neuregulins make up the third and fourth groups on the basis of their ability to bind both ErbB3 and ErbB4 (NRG-1 and NRG-2), or ErbB4 alone (NRG-3 and NRG-4). ErbB2 does not bind any of the ErbB ligands; however, its open conformation makes it the preferred dimerization partner for all of the other ErbB receptors. Used with permission from Hynes and MacDonald, 2009.

Of these ligands, amphiregulin (AR), EGF, epigen (EPG), and transforming growth growth factor-alpha (TGFalpha) bind only to ErbB1. Betacellulin (BTC), epiregulin (EPR), and heparin-binding EGF-like growth factor (HB-EGF) bind ErbB1 and ErbB4. The neuregulin (NRG) family also interacts with the ErbB family. NRG1 and NRG2 bind both ErbB3 and ErbB4 while NRG3 and NRG4 bind only to ErbB4 (Hynes and MacDonald 2009). This complex system of receptors and ligands allows for the highly specific regulation of diverse biological responses (Yarden and Sliwkowski 2001).

ErbB RTKs are expressed in epithelial, mesenchymal, and neuronal cells and play essential roles in multiple aspects of development. Knocking-out any member of the ErbB family of RTKs results in either perinatal (ErbB1) or embryonic lethality due to defects in heart and nervous system development (Burden and Yarden 1997). Mammary transplantation studies and/or genetic rescue of null lethality have demonstrated involvement of all ErbB family members in various aspects of normal mammary gland development. ErbB1 is expressed at a high level in all cell types in the prepubescent mouse mammary gland but is found only in the stromal cells during and after puberty (Schroeder and Lee 1998). Despite the fact that ErbB1 is found only in the stromal cells transplantation studies revealed that stromal ErbB1 is essential for ductal proliferation and branching (Wiesen, Young et al. 1999). The only ErbB1 ligand present in the mammary gland during ductal morphogenesis is AR which is produced by the epithelial cells present. Activation of stromal ErbB1 following the cleavage of AR is required for ductal development through a yet to be elucidated mechanism (Luetteke, Qiu et al. 1999; Sternlicht, Sunnarborg et al. 2005). ErbB2, like ErbB1, is present at a high level in all cell types in the prepubescent mammary gland. At puberty, however, ErbB2 expression

is confined to the epithelium (Schroeder and Lee 1998). Mice which lack ErbB2 in their MECs exhibit TEB defects and delays in ductal penetration (Jackson-Fisher, Bellinger et al. 2004; Andrechek, White et al. 2005). ErbB3 and ErbB4 are expressed at low levels in adult virgin mice but are upregulated late in pregnancy and remain expressed through early lactation (Sebastian, Richards et al. 1998). NRG1, which binds both ErbB3 and ErbB4, is expressed during pregnancy and has been shown to promote both ductal and alveolar development (Jones, Jerry et al. 1996). Interestingly, ErbB3 has been shown to be important for ductal morphogenesis while ErbB4 is essential for alveolar development and functional differentiation (Tidcombe, Jackson-Fisher et al. 2003; Jackson-Fisher, Bellinger et al. 2008). In spite of what is known regarding the ErbB family of receptors in mammary gland development, many questions regarding the precise mechanisms by which they govern mammary gland development remain.

ErbB Family Members in Breast Cancer

The ErbB family has long been associated with cancer. In fact, they were originally named ErbB because of their homology to a potent oncogene transduced by an avian retrovirus (v-erbB) (Frykberg, Palmieri et al. 1983; Sealy, Moscovici et al. 1983). ErbB1 and ErbB2 are both frequently deregulated in breast cancer. On the other hand, ErbB3 and ErbB4 expression are not thought to be significantly altered in cancer versus normal tissue (Zhang, Sun et al. 1996). However, recent data calls this conclusion into question as ErbB3 and ErbB4 have been found overexpressed in a multiple tumor types including breast, melanoma, and neuroendocrine (Chiu, Masoudi et al. 2010; Ruduff and Samuels, 2010; Srirajaskanthan, Shah et al. 2010) . Almost eighty percent of head and

neck cancers display overexpression of ErbB1 (Ford and Grandis 2003). Mutations ErbB1 are known to occur and contribute to tumorigenesis in brain and non small cell lung carcinoma (Ekstrand, Sugawa et al. 1992; Paez, Janne et al. 2004). Mutations in ErbB1 have also been reported in breast and ovarian cancers; however this data remains to be substantiated (Moscatello, Holgado-Madruga et al. 1995). ErbB2 is overexpressed in a subset of breast, ovarian, gastric, and salivary cancers (Hynes and Stern 1994).

ErbB2, the human counterpart of v-erbB, has been especially well studied in breast cancer where it is overexpressed due to genomic amplification in 20-25% of tumors and is an independent marker of poor prognosis (Paik, Hazan et al. 1990; Press, Bernstein et al. 1997). ErbB2 overexpression likely results in increased activation of downstream signaling pathways resulting in the aberrant proliferation, migration and survival of MECs. Since ErbB2 has a high basal level of autophosphorylation, overexpression of ErbB2 may be sufficient to result in oncogenic signaling (Lonardo, Di Marco et al. 1990). Or overexpression of ErbB2 may derive its oncogenic potential from heterodimerization with other ErbB family members. This heterodimerization may or may not be ligand induced (Hynes and Lane 2005). In mouse models, ErbB2 induced mammary carcinomas exhibit elevated levels of ErbB2/ErbB3 dimerization and increased tyrosine-phosphorylation on both receptors (Siegel, Ryan et al. 1999). Co-expression of ErbB1 and/or ErbB3 is often found in ErbB2 overexpressing tumors (Wiseman, Makretsov et al. 2005). ErbB2 heterodimerization with ErbB1 has been shown to promote invasion (Zhan, Xiang et al. 2006). In contrast, ErbB2 heterodimerization with ErbB3 appears to be required for proliferation (Holbro, Beerli et al. 2003). This suggests ErbB2 amplification and overexpression is not sufficient to mediate cancer phenotypes in

MECs. ErbB4 expression in breast cancers is rarely detected and correlates with better prognosis (Vogt, Bielawski et al. 1998; Suo, Risberg et al. 2002)

Trastuzumab, marketed as Herceptin, is a monoclonal antibody to ErbB2 which was approved for the treatment of ErbB2 positive breast cancer in 1998. Trastuzumab directly binds the extracellular domain of ErbB2 and results in improved response rate and survival (Cobleigh, Vogel et al. 1999; Vogel, Cobleigh et al. 2002). In fact, in early stage breast cancer patients the use of trastuzumab as an adjuvant therapy can reduce the risk of recurrence by half and mortality by a third (Piccart-Gebhart, Procter et al. 2005; Romond, Perez et al. 2005). The use of trastuzumab in both adjuvant and metastatic settings has improved the prognosis for women diagnosed with ErbB2+ breast cancer (Hortobagyi 2001; Dahabreh, Linardou et al. 2008). Nonetheless, not every patient with demonstrated ErbB2 amplification responds to trastuzumab due to intrinsic or acquired resistance. Multiple studies have attempted to identify predictive biomarkers of trastuzumab resistance (Table 1). The majority of the proposed "biomarkers" have not been validated and none are used in the clinic (Ross, Slodkowska et al. 2009).

Table 1. Proposed Biomarkers of Trastuzumab Resistance. (modified from Ross, Slodkowksak et al. 2009)

Biomarker	Proposed Impact	Rationale	Validation status
High HER-2 gene copy number	Resistance	Higher *HER-2* gene copy number predicts greater dependency of tumor cells on the HER-2 pathway	Majority of reports favor, but large scale validation is lacking
Shedding of HER-2 protein	Resistance	Circulating shed HER-2 protein would bind infused trastuzumab and reduce therapeutic targeting	Not validated
Dimerization status	Resistance	HER-2 homo- and heterodimerization status would predict response	Not validated

Table 1. Proposed Biomarkers of Trastuzumab Resistance. (modified from Ross, Slodkowksak et al. 2009) *Continued.*

Biomarker	Proposed Impact	Rationale	Validation status
Fc receptor status and ADCC response	Resistance	Polymorphisms and other dysfunction of Fc_ receptor would reduce the ADCC response to infused trastuzumab	Not validated
PTEN deficiency/PI3K pathway activation	Resistance	Loss of PTEN expression and activation of PI3K pathway creates resistance to drugs targeting HER-2 tyrosine kinase signaling	Most highly validated Trastuzumab resistance marker
c-*MYC* amplification	Sensitivity	Coamplification of c-*MYC* and *HER-2* maypromote apoptosis in tumor cells exposed tocombination regimens of trastuzumab and cytotoxic agents	Further validation required
IGF-1R overexpression	Resistance	Activation of IGF signaling pathway overcomes inhibition of HER-2 signaling pathway	Not fully validated; conflicting studies published
MUC4 overexpression	Resistance	MUC4 expression induces steric hindrance of trastuzumab binding to p185neu (HER-2) receptor	Not validated
p95HER-2 expression	Resistance	p95HER-2 expression enables constitutive signaling of HER-2 tyrosine kinase even when p185HER-2 receptor's extracellular domain is bound by trastuzumab	Not validated
Phosphorylated HER-2 receptor	Sensitivity	Phosphorylated HER-2 receptor was identified in a subset of HER-2–positive breast cancers and associated with an higher response rate to trastuzumab monotherapy and combination therapy with cytotoxic agents	Not validated
Topoisomerase IIa amplification	Sensitivity	*Topoisomerase IIa* amplification is associated with anthracycline benefit in HER-2–positive tumors in some, but not all, studies	Not validated
Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; HER-2, human epidermal growth factor receptor 2; IGF-1R, insulin-like growth factor 1 receptor; miRNA, micro-RNA; MUC4, mucin 4; PI3K, phosphatidylinositol 3_ kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten.			

It has long been proposed that overexpression of ErbB1 might confer resistance to trastuzumab (Smith 1993). Currently there are no large-scale clinical studies which validate this observation (Ross, Slodkowska et al. 2009). In fact, a recent study found no

significant correlation between ErbB1 expression in ErbB2+ human breast tumors and resistance to trastuzumab (Gori, Sidoni et al. 2009). Multiple preclinical studies, however, support this hypothesis and also implicate ErbB3 in resistance to trastuzumab. Acquired resistance to trastuzumab by human breast cancer cells grown *in vitro* in the presence of low levels of trastuzumab results in upregulation of ErbB1 and ErbB3 (Wang, Xiang et al. 2008; Narayan, Wilken et al. 2009). These studies go on to show activation of ErbB1 and ErbB3 via known ligands is essential for their ability to confer resistance to trastuzumab. Upregulation of ErbB1 is also exhibited when the BT474 human breast cancer line is grown *in vivo* in athymic nude mice under low concentrations of trastuzumab until they develop resistance (Ritter, Perez-Torres et al. 2007). Again these cells rely on EGF ligands for the activation of ErbB1 and resistance to trastuzumab. The recent use of tykerb, marketed under the name lapatinib, in the clinic also indicates ErbB1 is likely to play a role in trastuzumab resistance (at least in a subset of ErbB2+ breast cancers). Tykerb is a small molecule that is capable of inhibiting both the tyrosine kinase activity of both ErbB1 and ErbB2, which has demonstrated clinical efficacy in trastuzumab resistant tumors (Medina and Goodin 2008). Although tykerb works in a subset of patients, intrinsic and acquired resistance to tykerb has already been documented (Paul, Trovato et al. 2008). Interestingly, the transcription factor CCAAT/Enhancer Binding Protein beta2 (C/EBPbeta2) enables MECs the ability to grow and survive independent of ErbB signaling (addressed in Chapter 2 and (Bundy, Wells et al. 2005)). As C/EBPbeta2 confers ErbB independent growth, I looked specifically at the ability of C/EBPbeta2 to mediate resistance to trastuzumab (detailed in Chapter 3).

Inflammation in Breast Cancer

Inflammation plays an important role in the development and progression of breast cancer. The idea that inflammation contributes to cancer development goes back to 1863 when Rudolf Virchow, a German doctor and scientist, suggested that cancer arises at sites of chronic inflammation (Balkwill and Mantovani 2001). Since that time a multitude of studies have confirmed that chronic inflammation contributes to cancer development and progression (Coussens and Werb 2002; Clevers 2004; Philip, Rowley et al. 2004; Balkwill, Charles et al. 2005). In breast cancer, markers of inflammation have been suggested to have prognostic value (Albuquerque, Price et al. 1995; Al Murri, Bartlett et al. 2006; Al Murri, Wilson et al. 2007). Research demonstrates inflammation contributes to the development of breast cancer via the continual aberrant activation of humoral immunity, the recruitment of Th2 cells, and polarization of innate immune cells (DeNardo and Coussens 2007). These changes result in a pro-tumorgenic and pro-angiogenic environment (DeNardo and Coussens 2007).

Interleukin 1beta production and processing

Interleukin 1 beta (IL1B) is a key regulator of inflammation whose activity is tightly regulated. Both IL1B and the related protein interleukin 1 alpha (IL1A) are synthesized as 31 kD proforms that are cleaved by proteases to mature 17kD forms which can then be secreted. ProIL1B is processed by the IL1B-converting enzyme (ICE, also called caspase-1), while proIL1A is cleaved by calpain (Apte and Voronov 2008). IL1A is capable of binding to the interleukin 1 receptor 1 (IL1R1) and inducing a biological response in both the mature form and the uncleaved proform but only the mature form of IL1B binds to IL1R1 (Mosley, Urdal et al. 1987). For this reason, proIL1B has been

considered biologically inactive to date. Cleavage of proIL1B, and thus activation of IL1B, by caspase-1 is a complex process which has been extensively reviewed (Mosley, Urdal et al. 1987; Petrilli, Papin et al. 2005; Ogura, Sutterwala et al. 2006; Petrilli, Dostert et al. 2007; Netea, van de Veerdonk et al. 2008; Franchi, Eigenbrod et al. 2009; Martinon, Mayor et al. 2009). In short, a large multi-protein complex called the inflammasome is required for the activation of caspase-1 (which is itself synthesized as an inactive precursor) (Figure 2). Although multiple inflammasome complexes have now been identified, each is thought to be activated by NOD-like receptors (NLRs) upon detection of danger signals in the form of microbial products and/or endogenous stress signals. Perhaps the most well studied inflammasome is the NALP3 inflammasome, named for its NLR family member. NAPL3 senses a variety of danger signals ranging from viral RNA to bacterial toxins to endogenous signals such as monosodium urate crystals and ATP. Upon sensing the danger signal, NALP3 becomes activated and recruits the adaptor proteins PYCARD (Pyrin and CARD domain-containing) and CARDINAL along with caspase-1 (Martinon, Burns et al. 2002). This leads to the activation of caspase-1 resulting in the cleavage and activation of IL1B. The NALP1 inflammasome is also known to lead to the activation of IL1B via activation of caspase-1. The NALP1 inflammasome is composed of NALP1, PYCARD, and caspase-5 (Dowds, Masumoto et al. 2004). Interestingly, PYCARD was first identified during a screen for extensive promoter methylation in breast cancer samples (Conway, McConnell et al. 2000; Levine, Stimson-Crider et al. 2003). In fact, PYCARD was originally called TMS1 (target of methylation induced silencing). The status of the inflammasome has not been extensively investigated in

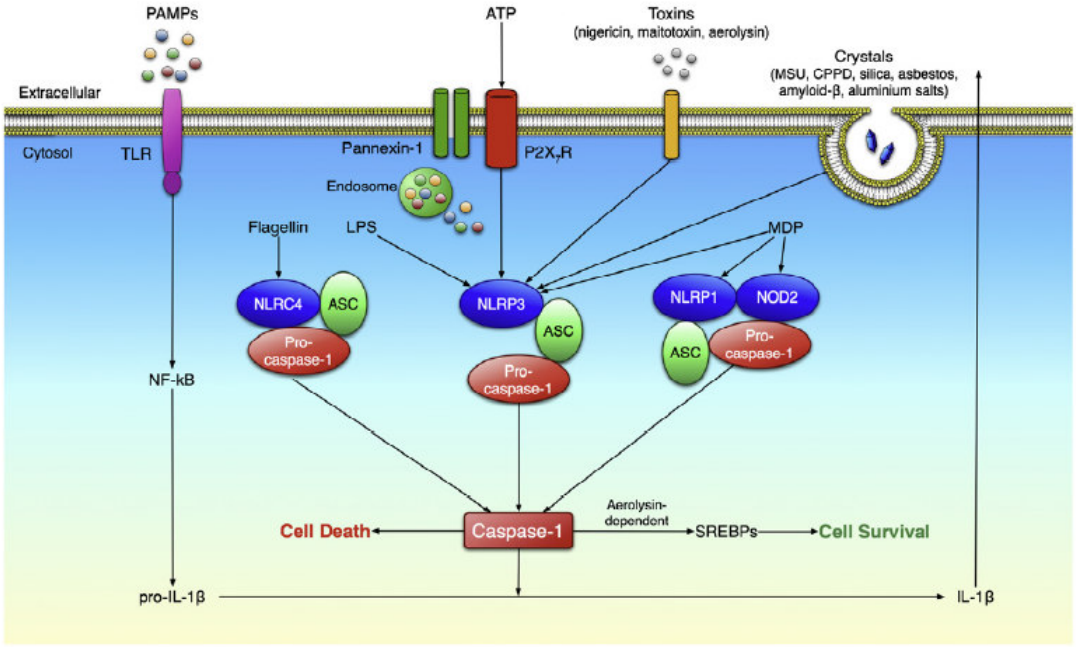


Figure 2. Activation of the Inflammasome. The inflammasome consists of the nucleotide-binding domain and leucine-rich repeat containing protein (NLR), ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain; PYCARD), and pro-caspase-1. ASC bridges NLR and pro-caspase-1. The name of each inflammasome is designated by the NLR within it, such as the NLRP1 inflammasome, the NLRP3 inflammasome, and the NLRC4 inflammasome. Extracellular PAMPs promote pro-IL-1b synthesis by engaging the TLR-NF-kB pathway. They can also be delivered into host cell cytosol via pores formed by ATP- P2X7R-activated pannexin-1. Unlike extracellular PAMPs, different cytosolic PAMPs are able to activate distinct inflammasomes. For example, cytosolic flagellin and LPS activate the NLRC4 and NLRP3 inflammasomes, respectively, whereas cytosolic MDP activates NLRP3 and NLRP1 inflammasomes. NOD2 and NLRP1 associate with each other in response to MDP. DAMPs, such as ATP, toxins, and crystals, can induce NLRP3 inflammasome activation. ATP-P2X7R-mediated activation of pannexin-1 leads to NLRP3 inflammasome activation. The mechanisms of how other DAMPs activate inflammasomes are summarized in Figure 2. Inflammasome activation triggers the processing of pro-caspase-1 into its mature form, caspase-1. Active caspase-1 induces cell death and the processing of proinflammatory cytokine pro-IL-1b into IL-1b, which is secreted out of the cell. As an exception, however, aerolysin-induced caspase-1 activation upregulates SREBPs, which promote cell survival. PAMPs, pathogen associated molecular patterns; DAMPs, danger associated molecular patterns; P2X7R, a purinergic receptor; LPS, lipopolysaccharide; MDP, muramyl dipeptide; SREBPs, sterol regulatory element binding proteins. Used with permission from Yu and Finlay 2008.

MECs. It has been shown MECs upregulate IL1B mRNA in response to LPS and retinoic acid, however, these studies did not address whether IL1B protein was induced and/or cleaved (Rabot 2001; Liu and Gudas 2002). Since the PYCARD promoter is aberrantly methylated and silenced in 46% of breast cancer cell lines and 30-40% of breast tumor tissues (Conway, McConnell et al. 2000; Levine, Stimson-Crider et al. 2003), this suggests that the inflammasome, and thus cleavage of proIL1B, may be compromised in a subset of breast cancers.

Interleukin 1beta in Breast Cancer

Many studies highlight the importance of IL1B in cancer, including breast cancer. Secreted IL1B is known to play a key role in carcinogenesis, tumor invasiveness and tumor-host interactions (recently reviewed (Apte and Voronov 2008)). IL1B has been found to be expressed in human breast carcinoma, and is expressed by the malignant cells as well as the microenviroment (Jin, Yuan et al. 1997; Kurtzman, Anderson et al. 1999; Pantschenko, Pushkar et al. 2003). In fact, IL1B has been demonstrated to be present in 90% of invasive breast carcinomas by ELISA and to correlate with high tumor grade (Levine, Stimson-Crider et al. 2003). These studies did not distinguish between secreted IL1B and proIL1B. Secreted IL1B promotes tumor progression and decreases immune surveillance by suppressing the cell-mediated immunity (Bunt, Sinha et al. 2006). Secreted IL1 can result in growth factor independence. Intriguingly, a recent study demonstrated that the aggressive breast cancer cell line SUM149 can proliferate in the presence of secreted mature IL1A when EGFR activity is inhibited (Streicher, Willmarth et al. 2007). This IL-1 mediated EGF independence occurred through ‘classical’ IL1R1

signaling and was blocked by the addition of the interleukin-1 receptor antagonist (Streicher, Willmarth et al. 2007). The ability of secreted mature IL1B to cause proliferation when EGFR is inhibited was not directly addressed in their study, although mature IL1B was upregulated along with mature IL1A by amphiregulin signaling through EGFR. This upregulation of mature IL1B and mature IL1A was specific to activation of EGFR signaling by amphiregulin and did not occur with EGF stimulation. Secreted IL1B stabilizes IL-8 mRNA in breast cancer cells. Expression of IL-8 in breast cancer correlates with angiogenesis, cell invasion, and metastasis (Suswam, Nabors et al. 2005).

Dual Function Cytokines

Although IL1B is thought to be active only when cleaved into the 17kD form and subsequently secreted, several other cytokines have been shown to have intracellular nuclear functions in their proform. These dual function cytokines include two members of the interleukin 1 family, IL1A and IL33, and the high-mobility group box 1 protein (HMGB1). Secreted IL1A binds to IL1R1 resulting in activation of signaling leading to inflammation (Suswam, Nabors et al. 2005). Nuclear IL1A has been detected under multiple different conditions and can result in proinflammatory excitability by binding histone acetyltransferases leading to decompaction of chromatin (Maier, Statuto et al. 1994; McMahon, Garfinkel et al. 1997; Hu, Wang et al. 2003; Pollock, Turck et al. 2003; Werman, Werman-Venkert et al. 2004; Kawaguchi, Nishimagi et al. 2006; Cheng, Shivshankar et al. 2008). Secreted IL33 signals through the IL1/Toll Like Receptor (TLR) family member interleukin 1 receptor like 1 (IL1RL1; also called ST2); this results in the induction of T helper type 2 responses (Coyle, Lloyd et al. 1999; Townsend, Fallon

et al. 2000; Schmitz, Owyang et al. 2005). Nuclear IL33 associates with the chromatin via the H2A-H2B acidic pocket resulting in increased chromatin compaction and transcriptional repression (Carriere, Roussel et al. 2007; Roussel, Erard et al. 2008). Signaling of extracellular HMGB1 through the receptor for advanced glycation endproducts (RAGE) controls the migratory response of multiple cell types (Rauvala and Rouhiainen 2007). Nuclear HMGB1 binds to linker DNA causing a conformational change which prevents nucleasome compaction by histone 1 (Cato, Stott et al. 2008). Thus, all of these dual function cytokines mediate changes in the chromatin landscape resulting in increased or decreased transcription.

CCAAT/ Enhancer Binding Protein Beta (C/EBPbeta)

In the present study, I demonstrate proIL1B is upregulated by the transcription factor C/EBPbeta2 in MECs, and that proIL1B may have a nuclear function (Chapter 2). C/EBPbeta is a basic-leucine zipper transcription factor that plays an essential role in mammary gland development (Robinson, Johnson et al. 1998; Seagroves, Krnacik et al. 1998).

C/EBP family

The CCAAT/ enhancer binding protein (C/EBP) family are members of the basic leucine zipper superfamily. Members of this superfamily utilize their basic domain to interact in a sequence specific manner with DNA, while their leucine zipper allows them to homodimerize or heterodimerize with other members of the family (Landschulz, Johnson et al. 1988). The C/EBP family is encoded by six genes and includes

C/EBPalpha, C/EBPbeta, C/EBPgamma, C/EBPdelta, C/EBPepsilon, and C/EBPzeta (Lekstrom-Himes and Xanthopoulos 1998; Ramji and Foka 2002). C/EBPdelta, C/EBPgamma, and C/EBPzeta generate single polypeptides while alternative splicing and/or alternative translation initiation result in the production of multiple protein isoforms of C/EBPalpha, C/EBPbeta, and C/EBPepsilon (Ramji and Foka 2002). The C-terminal region, which contains the DNA binding and dimerization motifs, are highly conserved within the family. This allows the C/EBP family to heterodimerize with one another and to bind similar DNA target sequences (Agre, Johnson et al. 1989; Vinson, Sigler et al. 1989; Hurst 1995). The C/EBP family was the first to demonstrate a lack of rigid sequence specificity for DNA binding when they were shown to bind RTTGCGYAAY (where R is A or G and Y is C or T) (Osada, Yamamoto et al. 1996; McKnight 2001). Although the N-terminal transactivation domain is not highly conserved among the family members it does have short motifs which are conserved. These short conserved regions play a role in interactions with the basal transcriptional apparatus (TBP/TFIIB), transcriptional co-activators (CBP/p300) and in the case of C/EBPalpha and C/EBPbeta the SWI/SNF complex (Nerlov and Ziff 1995; Mink, Haenig et al. 1997; Kowenz-Leutz and Leutz 1999; Pedersen, Kowenz-Leutz et al. 2001). In addition, a consensus site for sumolyation has been identified in the N-terminal region of multiple family members (Kim, Cantwell et al. 2002; Eaton and Sealy 2003). The physiological effects of these interactions/modifications are not completely understood and appear to be highly context dependent (for a review see (Nerlov 2008)).

The expression patterns and the effects of knocking out individual C/EBP family members in mice have been well studied (reviewed (Lekstrom-Himes and Xanthopoulos

1998)). C/EBPalpha is normally expressed in the liver, adipose, intestine, lung, adrenal gland, placenta, ovary, and peripheral blood mononuclear cells. As a result, knocking out C/EBPalpha results in perinatal lethality and defects in hepatocyte proliferation, carbohydrate and lipid metabolism, and myeloid differentiation. C/EBPbeta expression is normally detected in the liver, intestine, lung, adipose, ovary, lymphocytes, macrophages, and the mammary gland. Thus, C/EBPbeta knockout results in defective carbohydrate and lipid metabolism. It also results in a compromised immune system, female sterility, and impaired mammary gland development. C/EBPdelta is expressed in the liver, lung, adipose, and intestine and C/EBPdelta knockout mice have defective lipid storage and neurologic defects. C/EBPgamma is ubiquitously expressed. Knockout of C/EBPgamma is perinatally lethal and also impairs the development of natural killer cells. C/EBPepsilon, on the other hand, is expressed only in myeloid and lymphoid cells, thus its knockout results in immunodeficiency and reduced myeloid proliferation. Finally, C/EBPzeta is ubiquitously expressed and knocking it out results in resistance to apoptosis induced by the endoplasmic reticulum. Thus, studies using null mice demonstrate that all C/EBP family members play important roles in normal development (Lekstrom-Himes and Xanthopoulos 1998).

C/EBPbeta

Although C/EBPbeta is intronless, three different proteins can be made from its mRNA due to the presence of alternative translation initiation sites (Figure 3). (Descombes and Schibler 1991; Timchenko, Welm et al. 1999). The first two isoforms, C/EBPbeta1 and C/EBPbeta2, are transcriptional transactivators and differ by only 23 N-

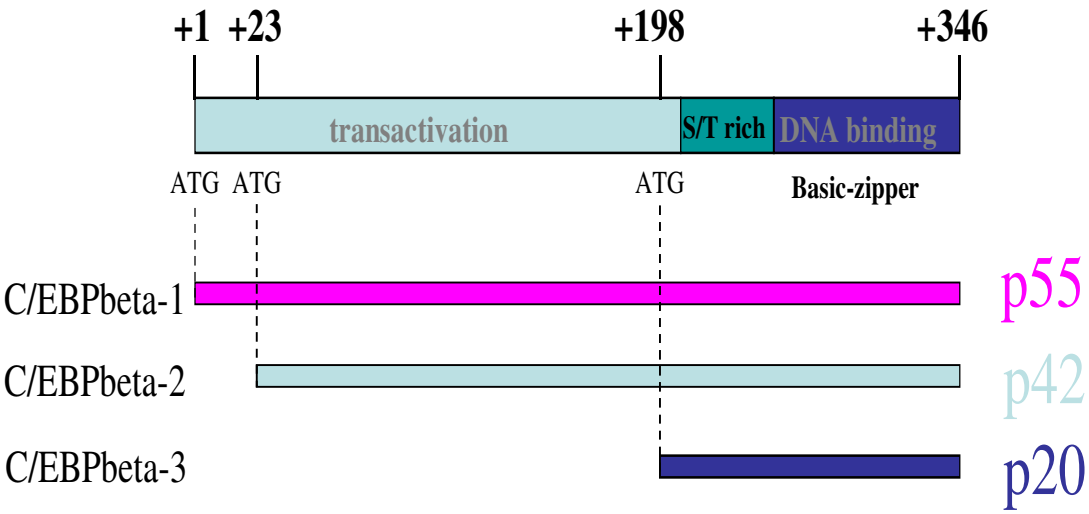


Figure 3. C/EBPbeta isoforms. Although C/EBPbeta is an intronless gene, three different proteins can be produced from its mRNA due to the presence of alternative translation initiation sites.

terminal amino acids. C/EBPbeta3 lacks the first 198 amino acids and thus functions as a transcriptional repressor. In other words, the full length isoform C/EBPbeta1 begins at the first in-frame methionine and consists of the entire 345 amino acids in the human (Akira, Isshiki et al. 1990). C/EBPbeta1 is 297 amino acids in the rat and mouse (Cao, Umek et al. 1991; Descombes and Schibler 1991). C/EBPbeta2 begins at the second in-frame methionine which is 23 amino acids (or in rat/ mouse 21 amino acids) downstream from the first. The final in-frame ATG is at position 198 in the human protein and this is where C/EBPbeta3 translation begins (Descombes and Schibler 1991). It is important to note that C/EBPbeta was isolated independently by multiple groups working in different systems and is therefore known by multiple names in the literature. These include: liver-enriched activator protein (LAP), LAP*, and liver-enriched inhibitory protein (LIP) due to their roles in liver generation (Descombes, Chojkier et al. 1990); nuclear factor- (NF) interleukin 6 (IL6) due to the ability to activate the IL6 promoter in a human glioblastoma line (Akira, Isshiki et al. 1990); and NF-M for its ability to cause proliferation of avian myelomocytes via induction of the myelomocytic growth factor (Katz, Kowenz-Leutz et al. 1993). LAP/LIP, NF-IL6, and NF-M were later renamed C/EBPbeta based on their homology to C/EBPalpha which had been previously cloned.

The exact mechanism by which a cell regulates which isoform of C/EBPbeta is produced is not currently known. The simplest explanation is that leaky ribosomal scanning results in the expression of all three C/EBPbeta isoforms (Descombes and Schibler 1991). It is, however, unlikely to be this simple since expression of different C/EBPbeta isoforms have been linked to specific biological outcomes (Lee, Miau et al.

1996; Kowenz-Leutz and Leutz 1999; Eaton, Hanlon et al. 2001; Bundy and Sealy 2003; Bundy, Wells et al. 2005; Marcinkowska, Garay et al. 2006). While no model has been proposed for the specific generation of C/EBPbeta1 or C/EBPbeta2, two mechanisms for enhanced C/EBPbeta3 expression have been proposed. The first involves the binding of CUG triplet repeat binding protein (CUGBP1) to the 5' end of C/EBPbeta mRNA. CUGBP1 binds at two distinct sites between the first and second translation initiation start sites in the C/EBPbeta mRNA and results in increased C/EBPbeta3 translation *in vitro* and *in vivo* (Timchenko, Welm et al. 1999). Timchenko et al. hypothesized CUGBP1 binding may stabilize a mRNA conformation which favors translation initiation at the third translation initation start site. The second proposed mechanism is the generation of C/EBPbeta3 by specific proteolysis of the larger C/EBPbeta isoforms (Welm, Timchenko et al. 1999; Baer and Johnson 2000). Specific cleavage resulting in C/EBPbeta3 has been shown *in vivo*. In the liver C/EBPbeta3 is generated by proteolysis in a C/EBPalpha dependent manner, and it is likely this cleavage occurs in other tissues where C/EBPbeta and C/EBPalpha are co-expressed (Welm, Timchenko et al. 1999). *In vitro* cleavage of the larger C/EBPbeta isoforms can also occur and care must be taken to avoid such proteolysis during sample preparation. In addition, the method of sample preparation calls into question several studies regarding expression of C/EBPbeta3 (Baer and Johnson 2000).

Although different by only 23 N-terminal amino acids, evidence is accumulating that C/EBPbeta1and C/EBPbeta2 are not biologically equivalent (Kowenz-Leutz and Leutz 1999; Eaton, Hanlon et al. 2001; Eaton and Sealy 2003). C/EBPbeta1 and C/EBPbeta2 were originally considered to functionally redundant; however, multiple

studies have now demonstrated C/EBPbeta1 and C/EBPbeta2 have distinct functional roles. C/EBPbeta1, but not C/EBPbeta2, has been shown to cooperate with NFkappaB in the synergistic activation of the alpha1 acid glycoprotein promoter (Lee, Miau et al. 1996). In addition, C/EBPbeta1, but not C/EBPbeta2, is able to synergistically activate multiple myeloid specific genes in cooperation with c-Myb. C/EBPbeta1 contributed to gene activation via recruitment of the SWI/SNF chromatin remodeling complex (Kowenz-Leutz and Leutz 1999). In both of these studies, the N-terminal amino acids absent from C/EBPbeta2 but present in C/EBPbeta1 were shown to be necessary but not sufficient. Sumoylation of C/EBPbeta1, but not C/EBPbeta2 by SUMO-2 and SUMO-3 has been demonstrated and again relies on the N-terminal amino acids present in C/EBPbeta1 but not C/EBPbeta2 (Eaton and Sealy 2003). Mutation of Lysine 173, the site of sumoylation, alters the functional activity of C/EBPbeta1 as demonstrated by expression at the cyclin D1 promoter (Eaton and Sealy 2003). On the other hand, C/EBPbeta2, but not C/EBPbeta1, is expressed in human breast cancer lines and tissues (Eaton, Hanlon et al. 2001). In fact, forced expression of C/EBPbeta2, but not C/EBPbeta1, in a normal MEC line model results in a variety of cancer phenotypes (Bundy and Sealy 2003; Bundy, Wells et al. 2005).

C/EBPbeta null mice

C/EBPbeta null mice exhibit a plethora of dramatic phenotypes. C/EBPbeta was originally described in hepatic and lymphatic cells and knockout studies confirmed the importance of C/EBPbeta in these systems. C/EBPbeta deficiency results in fifty percent perinatal lethality due to the lack of activation of phosphoenoypyruvate carboxykinase

(PEPCK) at birth resulting in extreme hypoglycemia (Croniger, Trus et al. 1997). The C/EBPbeta null mice which survive are sensitive to fasting-induced hypoglycemis due to aberrant cAMP regulation in the liver (Arizmendi, Liu et al. 1999; Liu, Croniger et al. 1999; Croniger, Millward et al. 2001). Liver regeneration is impared in C/EBPbeta null mice following partial hepatectomy, which also causes them to become hypoglycemic while wild type mice are not (Greenbaum, Li et al. 1998). C/EBPbeta was found to be essential in machrophage-mediated bacterial killing as evidenced by increased susceptibility to *listera monocyogenes* and *salmonella typherium* (Tanaka, Akira et al. 1995). C/EBPbeta null mice also have increased sensitivity to *candida albicans* due to lymphoproliferative defects and a reduced T-helper cell response (Screpanti, Romani et al. 1995).

C/EBPbeta null mice revealed other important roles for C/EBPbeta. C/EBPbeta null mice are sterile due to impaired granulose cell development resulting in the absence of the corpora lutea (Sterneck, Tessarollo et al. 1997). Implantation of wild type ovaries allows C/EBPbeta null mice to become pregnant and give birth to live offspring. The C/EBPbeta null mice, however, did not lactate (Sterneck, Tessarollo et al. 1997). Interestingly, C/EBPbeta null mice are completely resistant to skin tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) application followed by 12-o-tetrodecanoylphorbol (TPA) (Zhu, Yoon et al. 2002). Almost one hundred percent of tumors initiated in this way have mutations in Ha-ras (Balmain and Brown 1988; Moser, Robinette et al. 1993). Expression of v-Ha-ras in the C/EBPbeta null mice also demonstrated the importance of C/EBPbeta in formation of these tumors, supporting earlier studies indicating C/EBPbeta is a downstream effector of ras (Nakajima, Kinoshita

et al. 1993; Kowenz-Leutz, Twamley et al. 1994; Hanlon and Sealy 1999; Zhu, Yoon et al. 2002).

Studies demonstrate C/EBPbeta plays an essential role in mammary gland development. Mammary epithelial cell proliferation and differentiation are severely impaired in C/EBPbeta-null mice (Robinson, Johnson et al. 1998; Seagroves, Krnacik et al. 1998). Virgin, post pubescent C/EBPbeta null mice have enlarged ducts with reduced branching compared to their wild type litter mates. Since C/EBPbeta null mice are sterile, pregnancy associated changes in the mammary gland can only be studied when wild type ovaries or estrogen and progesterone pellets are implanted simulating pregnancy. Although some ductal branching occurred with both techniques neither restored the wild type level of branching indicating endocrine defects were only partially responsible for the abnormal ductal development in these mice (Robinson, Johnson et al. 1998; Seagroves, Krnacik et al. 1998). Both groups went on to demonstrate that transplantation of the C/EBPbeta null MECs into a wild type background did not rescue the phenotype. In addition to the defects noted in ductal morphogenesis, functional differentiation did not occur in C/EBPbeta null mice and the mammary glands failed to produce milk. In fact, there was no whey acidic protein or beta-casein found in the glands of C/EBPbeta null mice (Robinson, Johnson et al. 1998; Seagroves, Krnacik et al. 1998). This result confirmed previous studies which had shown C/EBPbeta-mediated activation of the beta-casein promoter (Robinson, McKnight et al. 1995). It is interesting to note that the mammary glands of C/EBPbeta null mice have defects in proliferation/invasion and differentiation. This may be due to functional differences in the C/EBPbeta isoforms.

Role of C/EBPbeta2 in breast cancer

Histological studies indicate CCAAT/Enhancer binding protein-beta (C/EBPbeta), is involved in the progression of human breast cancer and is predictive of poor prognosis (Milde-Langosch, Loning et al. 2003). Importantly, C/EBPbeta2 has been found to be expressed at high levels in 70% of invasive mammary carcinomas but is not detected in normal tissue obtained from reduction mammoplasties (Eaton, Hanlon et al. 2001). Additionally C/EBPbeta2 overexpression in MCF10A cells, an immortalized but not transformed mammary epithelial cell line, results in multiple cancer phenotypes. These phenotypes include anchorage independence, an invasive phenotype, epidermal growth factor independence, altered acinar architecture in 3D-culture models, and epithelial to mesenchymal transition (EMT) (Bundy and Sealy 2003; Bundy, Wells et al. 2005).

The cancer phenotypes mediated by C/EBPbeta2 overexpression in MCF10A cells are remarkably similar to those that result when ErbB2 is overexpressed/activated in MCF10A cells or immortalized human mammary epithelial (HME) cells. Activation of ErbB2 in MCF10A and HME cells results in anchorage independence, an invasive phenotype, epidermal growth factor independence, and altered architecture in 3D-culture models (Ignatoski, Lapointe et al. 1999; Ignatoski, Maehama et al. 2000; Muthuswamy, Li et al. 2001). C/EBPbeta2 may be a key downstream effector of ErbB signaling in MECs. In support of this, C/EBPbeta2 is downstream of multiple signaling pathways, including those that activate the ERK and RSK kinases (Wegner, Cao et al. 1992; Nakajima, Kinoshita et al. 1993; Buck, Poli et al. 1999; Hanlon, Sturgill et al. 2001; Shuman, Sebastian et al. 2004). ERK and RSK kinases are often activated by the ErbB

family of receptor tyrosine kinases in breast cancer via the Shc- and/or Grb2-activated Ras-Raf-MAPK pathways and phosphatidylinositol-3-kinase (PI3 K) pathways (Prenzel, Fischer et al. 2001; Yarden and Sliwkowski 2001). It has been demonstrated that C/EBPbeta is necessary for Ras transformation in multiple cell types (Zhu, Yoon et al. 2002; Wessells, Yakar et al. 2004). For example, C/EBPbeta null mice are completely resistant to carcinogen-induced skin tumors involving mutant Ras (Zhu, Yoon et al. 2002). Taken together these data suggest C/EBPbeta2 is a key downstream effector of ErbB signaling in MEC.

Purpose of this Study

To gain insight into the cancer phenotypes acquired by MCF10A upon C/EBPbeta2 overexpression, I characterized global changes in gene expression. Interestingly, C/EBPbeta2 overexpression in MCF10A cells dramatically upregulated (more then 30 fold) IL1B, IL1 receptor 2 (IL1R2), and IL1 receptor like 1 (IL1RL1). Although C/EBPbeta has been shown to bind the IL1B promoter and upregulate expression of IL1B in myeloid derived cells, the isoform of C/EBPbeta was not addressed in these studies (Yang, Wara-Aswapati et al. 2000). I used chromatin immunoprecipitation (ChIP) to demonstrate C/EBPbeta2 is capable of binding the IL1B promoter. Surprisingly I found that the proIL1B present in MCF10A-C/EBPbeta2 cells is not cleaved and is localized to the nucleus where it is tightly associated with the chromatin. I used multiple bioinformatic approaches to examine the possibility that nuclear proIL1B may function via chromatin remodeling like the other dual function

cytokines: IL1A, IL33, and HMGB1. Next, I demonstrated that nuclear IL1B can be detected in the nucleus of human breast cancer samples.

In order to better understand the role of C/EBPbeta2 in trastuzumab resistance, the level of C/EBPbeta2 in trastuzumab sensitive and resistant cell lines was assayed. To determine if C/EBPbeta2 could mediate resistance to trastuzumab it was overexpressed in ErbB2 overexpressing lines sensitive to trastuzumab. Growth assays were then preformed to determine the effects on trastuzumab sensitivity. The presence of proIL1B in the C/EBPbeta2 overexpressing ErbB2+ cell lines was then determined. The results demonstrate C/EBPbeta2 may result in resistance to trastuzumab via multiple mechanisms, although the details still remain to be elucidated.

CHAPTER II

C/EBPbeta2 REGULATION OF GENE EXPRESSION IN MCF10A CELLS: A ROLE FOR THE PROFORM OF INTERLEUKIN 1 BETA

Introduction

Chronic inflammation has been associated with the development and progression of cancer for some time (Balkwill and Mantovani 2001; Coussens and Werb 2002; Clevers 2004; Balkwill, Charles et al. 2005). Interleukin 1beta (IL1B) is a central mediator of inflammation. IL1B is produced as a 31 kD proform (proIL1B) which must be cleaved by caspase-1 to produce the mature, biologically active 17 kD form (Mosley, Urdal et al. 1987). The transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPbeta) is essential for mammary gland growth and development and has been associated with poor prognosis in breast cancer (Robinson, Johnson et al. 1998; Seagroves, Krnacik et al. 1998; Milde-Langosch, Loning et al. 2003). Overexpression of C/EBPbeta2 (one of three C/EBPbeta isoforms) in MCF10A cells results in a variety of cancer phenotypes including EMT and ErbB independence (Bundy and Sealy 2003; Bundy, Wells et al. 2005). Here, I characterize C/EBPbeta2 mediated changes in gene expression in MCF10A cells.

Interestingly, IL1B is dramatically upregulated in MCF10A-C/EBPbeta2 cells but is not cleaved to the mature 17 kD form. Although proIL1B has previously been considered to be biologically inactive, I demonstrate that proIL1B is not only localized to the nucleus but is also tightly associated with the chromatin. Furthermore, I show that proIL1B is bound at specific locations in the genome. Bioinformatic analysis of proIL1B

chromatin binding locations show proIL1B is poised to play a role in the cancer phenotypes observed in MCF10A-C/EBPbeta2 cells. Moreover, immunohistochemical analysis reveals nuclear IL1B in human breast cancer samples.

The data presented in this chapter provides the first evidence that proIL1B may have a biological function and indicates IL1B may be a dual function cytokine exhibiting both secreted and nuclear functions.

Materials and Methods

Cell culture and treatments

The MCF10A human mammary cell line was obtained from the American Type Culture Collection (ATCC) in Manassas, VA. Cells were maintained as previously described (Bundy and Sealy 2003). Briefly, cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 containing 2.5 mM L-glutamine and supplemented with 5% horse serum (Sigma, St. Louis MO), 10 micrograms/ml recombinant human insulin (Invitrogen Corp., Grand Island NY), 0.5 micrograms/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 50 U/ml penicillin, and 50 micrograms/ml streptomycin.

Establishment of MCF10A cells overexpressing T7 epitope tagged C/EBPbeta2 was also described previously (Bundy and Sealy 2003). In short, MCF10A cells were retrovirally infected with LZRS-His-C/EBPbeta2 in the presence of 8µg/ml polybrene. At 3-5 hours after infection, growth media was added bringing the polybrene concentration to 4 µg/ml. After 18-20 hours of incubation, the cells were placed in fresh growth media and maintained as detailed above. In the MCF10A cell cultures infected

with LZRS-His-C/EBPbeta2 a subpopulation of viable nonadherent cells began to accumulate 4-5 days post infection. Subcultures were then established by collecting the floating cells contained in the conditioned media by centrifugation at 500 x g for 5 min at RT and plating these cells in fresh media. The cells were maintained in the same manner as the MCF10A, except that in studies looking at EGF independent growth EGF was not added to the media.

Genomic Profiling

RNA was submitted to the Vanderbilt Microarray Shared Resource for quality assurance and microarray analysis. The resulting data has been deposited in the Gene Expression Omnibus (GEO). In short, after confirming RNA quality, biotinylated complementary RNA was prepared, fragmented, and hybridized to Affymetrix GeneChip U133 PLUS 2.0 arrays. Total RNA was isolated from MCF10A cells stably overexpressing C/EBPbeta2 and parental MCF10A cells using the RNeasy Mini kit and RNase-Free DNase kit (Qiagen). Streptavidin coupled with phycoerythrin was used to detect and visualize hybridized complementary RNA using a GeneChip Scanner 3000 7G Plus 2. GeneChip Operating System (GCOS, Affymetrix, Santa Clara, CA) was used to grid images and generate .CEL and .CHP files for further analysis. Three independent replicates were performed to allow for statistical analysis. CEL files were imported in GeneSpring 7.0 (Agilent Technologies) and transformed by RMA (Robust Multichip Analysis). All probesets showing at least a 2 fold change in one of the C/EBPbeta2 overexpressing MCF10A cells compared to parental MCF10A were tested with a Welch's t-test and a p-value cutoff of 0.05. This restriction tested 3,721 genes. As a result 443 genes were found to be differentially expressed upon C/EBPbeta2

overexpression in MCF10A cells. Of these differentially expressed genes, 86 were found to be upregulated 2 fold or more while 121 were found to be downregulated 2 fold or more.

Real-time PCR

RNA was extracted as described above. cDNA was synthesized with the high capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems). Taqman real-time PCR was then performed to determine the relative levels of targets, using GAPDH as the internal control. The reaction was performed in a total volume of 20µl using a real-time PCR instrument (StepOnePlus, Applied Biosystems).

Whole cell lysates, cell fractionation, and immunoblot analysis

Whole cell extracts were prepared from 100 mm dishes of 80-90% confluent MCF10A cells or MCF10A cells overexpressing C/EBPbeta2 by scraping into chilled phosphate-buffered saline with 100uM NaVandadate and collected by centrifugation as described previously (Bundy and Sealy 2003). The pellet was then resuspended in saline tris EDTA (STE; 100 mM NaCl, 10 mM Tris pH 7.5, and 1 mM EDTA) with protease and phosphatase inhibitors, as described previously (Bundy and Sealy 2003). Nuclear extracts were also prepared from 80-90% confluent cultures but were scraped into a chilled phosphate-buffered saline containing 1 mM EDTA. After they were collected by centrifugation, the cells were resuspended in 10 volumes 10 mM HEPES pH 8, 0.5M sucrose, 50 mM NaCl, 1mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM

spermine, 0.5% Triton X-100, 7 mM beta-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 µg of leupeptin per liter, 0.1µM pepstatin, 1 ng/ml aprotinin and phosphatase inhibitors (Buffer A). After vigorous vortexing, nuclei were collected by centrifugation at 1000 X *g* for 5 min. This was process was continued as the sample was subjected to increasingly high ionic strength in a stepwise manner beginning at 0.1 M NaCl and ending at 1.0 M NaCl. An equal volume of 2X Laemmeli sample butter was added to whole cell lystates, cytoplasmic fractions, and nuclear fractions and they were boiled for 5 minutes. Protein Assay Reagent (BioRad Laboratores, Hercules, CA, USA) was used as per the manufacturer's instructions to ensure equal amounts of protein were loaded onto a 10% SDS-PAGE. After separation by electrophoresis the proteins were transferred to an Immobilon P filter. The filters were blocked in 5% nonfat dry milk (NFDM) in TBS-T (TBS with 0.1% Tween 20). Primary antibodies anti-IL1B (detailed below), anti-TFIID (Santa Cruz), and anti-beta-tubulin (SIGMA) were incubated overnight at 4^{o}C in 0.5% NFDM in TBS-T. After washing, the filters were incubated with the appropriate secondary peroxidase-conjugated antibody for 1 hour at RT in 0.5% NFDM in TBS-T. The immunoblot was then washed in TBS-T and visualized using the SuperSignal West Pico chemiluminescence reagent (Pierce, Rockford, IL, USA) and autoradiography with Kodak X-OMAT film (Rochester, NY, USA).

Four antibodies to IL1B were used during the course of these studies: R&D Systems MAB-201, MAB-601, AF-201-NA and Santa Cruz Biotechnology SC-52012. MAB-201 is directed to amino acids 117-268 of IL1B while the others are raised against the complete IL1B protein.

Enzyme Linked Immunosorbent Assay (ELISA)

The levels of IL1B in conditioned media from cultured cells was determined by using the Quantikine HS IL-1B Immunoassay ELISA (R&D Systems, Minneapolis, MN, USA) according the manufacturer's directions. The lower detection limits for this ELISA are < 0.1 pg/ml. Fresh growth media was placed on 40-50% confluent cell cultures and conditioned for 24 hours. At this point cell cultures were 80-90% confluent. The conditioned media was centrifuged briefly to remove floating cells and then media was immediately assayed.

Indirect Immunostaining

All cell cultures were grown in 35 mm dishes fitted with polylysine-coated glass coverslips (Matek Corp, Ashland MA, USA). Indirect immunostaining was then performed as previously described (Bundy, Wells et al. 2005) with the following modifications. Anti-IL1B antibody (R&D Systems MAB-201) was used as the primary at a dilution of 1:500 and Hoechst solution (bisBenzimide) was used at 1µg/ml to fluorescently label the nuclear compartment of the cells. The cells were then mounted in Aqua Poly/Mount and visualized on a Leica DM IRB Inverted Microscope equipped with a Nikon DXM1200C camera.

Chromatin Immunoprecipitation and promoter array

MCF10A cells overexpressing C/EBPbeta2 were fixed in 1% formaldehyde for 10 minutes, snap-frozen and shipped to Genpathway to ascertain the binding of C/EBPbeta2 to the promoter of key target genes. At Genpathway, sonication was used to fragment the

DNA into pieces of approximately 300–500 bp. Then C/EBPbeta2 binding to promoter regions or IL1B association with human promoters was assayed.

To determine C/EBPbeta2 binding to promoter regions, an antibody against the T7 epitope tag (Abcam) was then used to precipitate C/EBPbeta2 bound chromatin. After reversal of crosslinks precipitated DNA was purified. C/EBPbeta2 binding to the promoter elements of the target genes was then determined using Q-PCR with primer pairs specific for each promoter region. Q-PCRs were run in triplicate and the averaged Ct values were transferred into copy numbers of DNA using a standard curve of genomic DNA with known copy numbers. Results are normalized for primer pair amplification efficiency using the Q-PCR value obtained with unprecipitated genomic DNA (Input DNA). Results are presented as Binding Events Per 1000 Cells for the promoter region tested. Error bars correspond to the standard deviations from triplicate Q-PCR reactions.

To determine proIL1B association with human promoters, an antibody against IL1B (R&D systems MAB-201) was used to precipitate proIL1B bound chromatin at Genpathway. After reversal of crosslinks precipitated DNA was purified. ProIL1B binding to human promoters was then assessed using the Human Promoter 1.0R Array (Affymetrix). The Cell Intensity Files were analyzed using Affymetrix' Tiling Analysis Software (TAS). TAS is used to generate signal values for all the probes on the arrays. Ratios are then generated by applying averaging and ranking steps.

Bioinformatic Analysis of ChIP-chip data

The following approach was used to determine whether the apparent clustering of proIL1B in the genome was statistically significant. Given a cluster, we can calculate the

range of the cluster, which is: Range = max(location) – min(location). Based on a CHIP with 35 bp probe spacing, we can set k=35. Assuming there are m significant binding sites within this cluster, we can calculate the probability of m significant binding sites within a range as (where n = as.integer(Range/k):

$$prob = \binom{n}{m}\left(\frac{1}{n}\right)^{m}$$

PANTHER pathway analysis was performed as previously described (Thomas, Campbell et al. 2003). The results from this analysis are shown in Table 2. Next, to determine the statistical probability that proIL1B was associated with tumor initiation and various metastatic steps (as defined in 76) over-representation analysis (ORA) was performed (Table 3). The following approach was used and is a derived from the ORA formula previously published by Backes et al. (Backes 2007). Based on the array description, there are approximately m=4,600,000 probes in the array. From these probes, *n*=204 significant binding sites were selected. For a given pathway, assuming there are *l* genes related to *l* binding sites, then there are *k* genes related to *k* binding sites for that pathway within the n=204 significant binding sites. Therefore, we can calculate the P-values for each of these pathways by the following formula:

$$P_C = \begin{cases} \sum_{i=k}^{K} \dfrac{\binom{l}{i}\binom{m-l}{n-i}}{\binom{m}{n}} & if\ k' < k \\ \sum_{i=0}^{k} \dfrac{\binom{l}{i}\binom{m-l}{n-i}}{\binom{m}{n}} & if\ k' \geqslant k \end{cases}$$

Where K = min (n,l).

The R package R.basic was used to conduct the calculation.

Immunohistochemistry

For immunohistochemistry, breast tissue microarrays were purchased from Cybrdi, INC. The arrays were de-waxed, rehydrated, and subjected to thermal antigen retrieval in Retrievit Target Retrieval pH 4 according to the manufacturers instructions using a microwave pressure cooker (InnoGenex). Sections were incubated with primary anti-IL1B antibody (1:25, R&D Systems AF-201-NA) overnight at 4°C, followed by 1-h room temperature incubation with biotinylated anti-goat antibodies (1:25, R&D Systems). Sections were then incubated with avidin-peroxidase (Vector Laboratories, Burlingame, CA), followed by DAB substrate (Invitrogen) and mounted. Using secondary antibody only resulted in no staining. The samples were then imaged on an Olympus BX 51 using the 40X objective plus 20X oculars. The detection of nuclear IL1B was confirmed by Dr. Melinda Saunders, a medical pathologist who specializes in breast cancer.

shRNA and siRNA approaches targeting IL1B

Five shRNAs targeting IL1B were purchased from the SIGMA Mission Lentiviral collection (SIGMA). A control vector expressing GFP was also purchased from the same collection (SIGMA). Virus production and infection of target cells was performed according to the manufacturer's instructions, however, the viral yield was relatively low so optimization was performed. Briefly, 2 x 10^6 Hek 293T cells were plated 24 hours prior to transfection. Lentiphos (SIGMA) was used to transfect in 3 µg of the lentiviral vector. 24 hours after transfection, the media was replaces with fresh media containing 20 mM HEPES buffer. Virus was collected and concentrated 48 hours later. The

concentrated virus was immediately used to infect MCF10-C/EBPbeta2 cells which were 70% confluent. Selection of infected cells was performed using 2 ng/ml of puromycin. After selection the cells were maintained in the same level of puromycin.

I next utilized the pGIPZ construct targeted at IL1B (Open Biosystems). pGIPZ was also a lentiviral construct but contained GFP in addition to the shRNA targeting IL1B. A pGIPZ vector targeted GAPDH was purchased as a control (Open Biosystems). Virus was prepared and used in the same manner as described above. Puromycin selection (2 ng/ml) was needed to obtain pure populations of shIL1B-GFP and GFP cells.

The doxycycline inducible pTRIPZ vectors targeting IL1B and GAPDH were also purchased from Open Biosystems and virus prepared. MCF10A-C/EBPbeta2 cells were then infected and puromycin selected (at 2 ng/ml puromycin). Once a population of puromycin resistant cells had been selected, doxycycline (1 µg/ml) was used to induce expression of the desired shRNA. Cells were then maintained in the presence of doxycycline.

The Adeno-X ViraTrak Expression System 2 was then purchased (Clontech). Oligos targeting IL1B and a scrambled oligo were designed and purchased (SIGMA) and subsequently cloned into the pSIREN-DNR vector and transferred into the pLP-Adeno-X vector according to the manufacturer's directions. The resulting constructs were then digested with PacI prior to transfection into HEK 293 cells which had been plated at 1 x 10^6 in a 60 mm plate 24 hours prior. The cells were monitored for cytopathic effect (CPE). Virus was harvested 7-8 days after transfection by lysing the cells using a series of three rapid freeze-thaw cycles. If CPE had been evident then the virus was used to

infect target cells. If, however, CPE was not evident the virus was amplified by infecting HEK 293 cells.

Dharmocon Smart Pool Plus oligos targeting IL1B and GAPDH were purchased (Dharmocon). Oligos were then transfected into MCF10A-C/EBPbeta2 cells in a similar manner to previously described for parental MCF10A cells (Parsons, Patel et al. 2009). Briefly, the cells were plated in antibiotic free media 24 hours prior to transfection (2.8 X 10^5 cells for p60 or 7.5 x 10^5 cells for p100). Oligofectamine was then used according to the manufacturer's protocol to transfect in 18 or 50 µl of the desired oligo. 36 hours post-transfection the cells were split for future assays which were carried out 30 hours later.

Results

Genomic profiling reveals C/EBPbeta2 gene regulation in MCF10A cells.

We have previously generated a retroviral LZRS expression vector that selectively expresses epitope-tagged C/EBPbeta2 (Bundy and Sealy 2003). MCF10A cells, which are immortalized but not transformed, are epithelial and require EGF in the culture medium for growth; however, overexpression of C/EBPbeta2 results in multiple cancer phenotypes including EGF independent growth (Bundy and Sealy 2003; Bundy, Wells et al. 2005). To characterize C/EBPbeta2 regulation of gene expression in MCF10A cells, RNA was isolated from MCF10A cells and MCF10A cells overexpressing C/EBPbeta2 and hybridized to Affymetrix human genome U133 Plus 2.0 microarrays. 443 genes were found to be statistically differentially expressed upon

C/EBPbeta2 overexpression in MCF10A cells. Of these differentially expressed genes, 86 were found to be upregulated 2 fold or more, while 121 were found to be downregulated 2 fold or more (Table 2).

Table 2. C/EBPbeta regulation of genes in MCF10A cells.

Transcript Abundance Relative to Control		
Affymetrix number	Fold Increase	Common
205403_at	493.0	IL1R2
211372_s_at	396.9	IL1R2
206569_at	103.8	IL24
207526_s_at	77.4	IL1RL1
209719_x_at	74.5	SERPINB3
205067_at	43.7	IL1B
209720_s_at	39.4	SERPINB3
231867_at	35.7	ODZ2
39402_at	27.9	
227070_at	24.0	LOC83468
206421_s_at	22.9	SERPINB7
207808_s_at	10.8	PROS1
210413_x_at	9.9	SERPINB4
221447_s_at	9.8	LOC83468
209909_s_at	9.2	
212099_at	8.6	ARHB
229764_at	7.8	FLJ41238
205542_at	7.6	STEAP
226847_at	7.6	FST
227140_at	7.6	
235236_at	7.4	
235678_at	7.3	GM2A
1553982_a_at	7.2	MGC9726
228121_at	7.0	TGFB2
200665_s_at	6.9	SPARC
202458_at	6.7	SPUVE
230266_at	6.5	MGC9726
201341_at	6.3	ENC1
201289_at	6.0	CYR61
208792_s_at	5.3	CLU
204948_s_at	5.1	FST
208791_at	5.0	CLU
219511_s_at	4.9	SNCAIP
1555007_s_at	4.9	MGC33630
211126_s_at	4.7	CSRP2
226017_at	4.6	CKLFSF7

Table 2 Continued		
35820_at	4.6	
226279_at	4.5	SPUVE
213988_s_at	4.5	SAT
213274_s_at	4.5	
224209_s_at	4.5	GDA
207030_s_at	4.4	CSRP2
206969_at	4.3	KRTHA4
209969_s_at	4.2	STAT1
212737_at	4.1	
240633_at	3.9	FLJ33718
217764_s_at	3.8	RAB31
213689_x_at	3.7	RPL5
204967_at	3.6	APXL
218656_s_at	3.5	LHFP
217979_at	3.4	TM4SF13
225626_at	3.4	PAG
225847_at	3.2	KIAA1363
206343_s_at	3.1	NRG1
200838_at	2.9	CTSB
205896_at	2.9	SLC22A4
222892_s_at	2.9	FLJ11036
218113_at	2.8	TMEM2
225567_at	2.8	na
226844_at	2.7	
200632_s_at	2.7	NDRG1
217996_at	2.6	PHLDA1
208885_at	2.6	LCP1
211571_s_at	2.5	CSPG2
204790_at	2.5	MADH7
201995_at	2.5	EXT1
222877_at	2.5	NRP2
205767_at	2.5	EREG
202718_at	2.4	IGFBP2
204235_s_at	2.3	CED-6
227080_at	2.3	MGC45731
203143_s_at	2.3	
242873_at	2.3	D12S2489E
203708_at	2.2	PDE4B
229568_at	2.2	MOB3B
204237_at	2.2	CED-6
58916_at	2.2	
228010_at	2.2	PPP2R2C
222258_s_at	2.2	SH3BP4
224791_at	2.2	DDEF1
230183_at	2.1	
220987_s_at	2.1	SNARK
221752_at	2.1	SSH1

Table 2 Continued		
217999_s_at	2.1	PHLDA1
217997_at	2.1	PHLDA1
221753_at	2.0	SSH1
203021_at	-100.0	SLPI
213680_at	-93.6	KRT6B
213796_at	-57.8	SPRR1A
205064_at	-46.8	SPRR1B
212190_at	-44.6	SERPINE2
203691_at	-39.9	PI3
41469_at	-28.2	
209301_at	-26.3	CA2
229638_at	-22.5	IRX3
214549_x_at	-22.2	SPRR1A
208966_x_at	-21.7	IFI16
218559_s_at	-20.2	MAFB
209351_at	-19.3	KRT14
201842_s_at	-18.4	EFEMP1
206332_s_at	-16.5	IFI16
217767_at	-16.4	C3
202917_s_at	-16.1	S100A8
202035_s_at	-11.6	SFRP1
209792_s_at	-11.2	KLK10
214211_at	-10.8	FTH1
218966_at	-10.7	MYO5C
239273_s_at	-10.2	MMP28
232165_at	-10.2	EPPK1
203535_at	-8.7	S100A9
208965_s_at	-8.5	IFI16
209955_s_at	-8.4	FAP
201843_s_at	-7.9	EFEMP1
239272_at	-7.8	MMP28
222670_s_at	-7.7	MAFB
204734_at	-7.7	KRT15
217388_s_at	-7.0	KYNU
213348_at	-6.7	CDKN1C
202036_s_at	-6.5	SFRP1
202790_at	-6.4	CLDN7
204971_at	-6.1	CSTA
213711_at	-6.0	KRTHB1
210663_s_at	-5.9	KYNU
202037_s_at	-5.9	SFRP1
241436_at	-5.8	SCNN1G
201131_s_at	-5.6	CDH1
200748_s_at	-5.5	FTH1
226926_at	-5.4	ZD52F10
226931_at	-5.1	ARG99
228575_at	-5.1	MGC34923

Table 2 Continued		
213992_at	-5.1	COL4A6
202728_s_at	-4.9	LTBP1
232164_s_at	-4.8	EPPK1
206166_s_at	-4.8	CLCA2
219127_at	-4.7	MGC11242
209800_at	-4.7	KRT16
232082_x_at	-4.7	SPRR3
226281_at	-4.7	DNER
206165_s_at	-4.5	CLCA2
217528_at	-4.5	CLCA2
201522_x_at	-4.4	SNRPN
226322_at	-4.4	ARG99
226200_at	-4.3	VARS2L
228256_s_at	-4.3	TIGA1
230076_at	-4.2	FLJ10156
232802_at	-4.2	SYT8
202489_s_at	-4.2	FXYD3
212314_at	-4.1	KIAA0746
228708_at	-3.9	RAB27B
227642_at	-3.9	LBP-9
227862_at	-3.9	
210239_at	-3.8	IRX5
204385_at	-3.8	KYNU
203887_s_at	-3.7	THBD
214370_at	-3.7	S100A8
211628_x_at	-3.6	FTHP1; FTHL5
204885_s_at	-3.6	MSLN
219735_s_at	-3.6	LBP-9
212925_at	-3.3	LOC126353
212657_s_at	-3.3	
212328_at	-3.2	KIAA1102
206042_x_at	-3.2	SNRPN
205623_at	-3.2	ALDH3A1
227741_at	-3.1	PTPLB
227480_at	-2.9	
227510_x_at	-2.9	PRO1073
200732_s_at	-2.8	PTP4A1
1563933_a_at	-2.8	FLJ40773
207826_s_at	-2.8	ID3
218990_s_at	-2.8	SPRR3
229465_s_at	-2.7	PTPRS
218807_at	-2.7	VAV3
223578_x_at	-2.7	PRO1073
202729_s_at	-2.7	LTBP1
220945_x_at	-2.6	FLJ10298
229518_at	-2.6	MGC16491
211473_s_at	-2.5	COL4A6

Table 2 Continued		
206008_at	-2.5	TGM1
210538_s_at	-2.5	BIRC3; AIP1
238013_at	-2.4	PLEKHA2
41660_at	-2.4	
213110_s_at	-2.4	COL4A5
225136_at	-2.3	PLEKHA2
223322_at	-2.3	RASSF5
211208_s_at	-2.3	CASK
225079_at	-2.3	EMP2
223125_s_at	-2.2	C1orf21
207388_s_at	-2.2	PTGES; PGES
225968_at	-2.2	PRICKLE2
226925_at	-2.2	FLJ23751
201061_s_at	-2.2	STOM; BND7
228155_at	-2.2	MGC4248
224435_at	-2.2	MGC4248
200706_s_at	-2.1	LITAF
226834_at	-2.1	ASAM
226189_at	-2.1	
201147_s_at	-2.1	TIMP3
213447_at	-2.1	PWCR1
225078_at	-2.1	EMP2
235371_at	-2.1	na
225615_at	-2.1	LOC126917
210367_s_at	-2.1	PTGES
205157_s_at	-2.1	KRT17
203919_at	-2.0	TCEA2; TFIIS
212236_x_at	-2.0	KRT17
204751_x_at	-2.0	DSC2
201149_s_at	-2.0	TIMP3
227443_at	-2.0	na

Genomic Profiling confirms and deepens understanding of C/EBPbeta2 mediated phenotypes

It has been demonstrated that C/EBPbeta2 overexpression in MCF10A cells results in EMT (Bundy, Wells et al. 2005). Genomic profiling reveals C/EBPbeta2 overexpression regulates multiple genes important in EMT and correlated with poor prognosis in human breast cancer (Table 3). These genes include, but are not limited to: (1) secreted protein, acidic,

cysteine-rich (SPARC, 6.9 fold) which is important for stromal de-adhesion needed for invasion and metastasis; (2) cysteine-rich, angiogenic inducer, 61 (CYR61, 6.0 fold) an angiogenic factor known to enhance breast cancer aggressiveness through interaction with its integrin receptor alpha(v)beta(3) and to increase matrix metalloproteinase-1 production leading to enhanced protease-activated receptor 1-dependent migration of breast cancer cells; (3) cathepsin B (CTSB, 2.9 fold) which leads to increased invasion; and (4) E. cadherin (CDH1, -5.6 fold) which is essential for metastatic behavior (Ren and Sloane 1996; Tsai, Bogart et al. 2002; Bervar, Zajc et al. 2003; Jiang, Watkins et al. 2004; Barth, Moll et al. 2005; Nguyen, Kuliopulos et al. 2006). Loss of E. cadherin is an independent prognostic factor in breast cancer (Gould Rothberg and Bracken 2006). Bioinformatics methods to determine pathway involvement based on gene profiling data, such as PathwayAssist analysis, also detected EMT related pathways involving the regulation of the actin cytoskeleton (p-value 0.035) and focal adhesions (p-value 0.040).

Previous work in our lab had indicated ErbB family members were not responsible for the EGF independence observed in MCF10A-C/EBPbeta2 cells based upon the observations that ErbB1 and ErbB2 are not activated nor is the Ras/Raf/ERK1/2 pathway (Bundy, Wells et al. 2005). Genomic profiling of MCF10A-C/EBPbeta2 cells confirms the ErbB family of receptors and their ligands are not overexpressed upon C/EBPbeta2 overexpression (Table 3). Bioinformatic analysis of the data using PathwayAssist also indicated that known ErbB pathways were not involved but detect EMT related pathways (as detailed above). Taken together these results confirm C/EBPbeta2 does not confer EGF independence through enhanced ErbB signaling.

Table 3. Key results of genomic profiling in C/EBPbeta2 overexpressing MCF10A cells. C/EBPbeta2 regulates key genes known to be involved in EMT but does not alter expression of known ErbB signaling components. Strikingly, C/EBPbeta2 overexpression in MCF10A cells dramatically upregulates three interleukin-1 family members. Changes in expression for bolded genes have been confirmed using real-time PCR. No expression (N.E.) is used as the fold change for genes whose expression was not detected at a significant level.

	Gene Symbol	Fold Change	p-value
EMT related genes	**SPARC**	6.9	0.033
	CYR61	6.0	0.027
	CTSB	2.5	0.009
	CDH1	-5.6	0.008
ErbB Signaling	**EGFR**	1	n/a
	ERBB2	N.E.	n/a
	ERBB3	N.E.	n/a
	ERBB4	N.E.	n/a
	EGF	N.E.	n/a
	AR	N.E.	n/a
	TGFA	N.E.	n/a
	BTC	N.E.	n/a
	HBEGF	N.E.	n/a
	EPR	N.E.	n/a
	HRG	N.E.	n/a
	NRG2	N.E.	n/a
	NRG3	N.E.	n/a
	NRG4	N.E.	n/a
Interleukin-1 family	**IL1R2**	315.7	0.002
	IlRL1	50.3	0.048
	IL1B	31.9	0.002

C/EBPbeta2 overexpression in MCF10A cells leads to the dramatic upregulation of three interleukin-1 family members

C/EBPbeta2 overexpression in MCF10A cells dramatically upregulates (30 fold or more) a group of cytokines and cytokine receptors in the interleukin-1 family (Table 3). These interleukin-1 family members are interleukin-1 beta (IL1B), interleukin-1 receptor 2 (IL1R2), and interleukin-1 receptor like 1 (IL1RL1, also called ST2). These results were confirmed using real-time PCR (Figure 4). All three of these interleukin-1 family members have been shown to be upregulated in human breast cancer (Jin, Yuan et al. 1997; Werenskiold, Prechtel et al. 2000; Pantschenko, Pushkar et al. 2003). IL1B is found in over 90% of invasive breast carcinomas (Jin, Yuan et al. 1997). IL1R2 is a decoy receptor for IL1B that lacks the cytoplasmic signaling domain (Roy, Sarkar et al. 2006). The upregulation of IL1R2 may prevent IL1B levels from becoming detrimental to the cells, or may play a yet undiscovered role. IL1RL1 is overexpressed in invasive stages of mouse models of breast cancer and is known to be overexpressed in human breast cancer, although at an earlier stage (Werenskiold, Prechtel et al. 2000). IL1RL1 is not known to interact with IL1B or IL1R2 (Gullick and Srinivasan 1998).

Chromatin immunoprecipitation studies demonstrate C/EBPbeta2 binds directly to the IL1B promoter

To determine if the three highly upregulated interleukin-1 family genes are direct targets of C/EBPbeta2, I used chromatin immunoprecipitation (ChIP) to ascertain if C/EBPbeta2 bound directly to the promoter regions of these genes. To ensure I was looking only at C/EBPbeta2 and not other isoforms of C/EBPbeta, an antibody to the T7 epitope was used to immunoprecipitate C/EBPbeta2 bound regions of the genome.

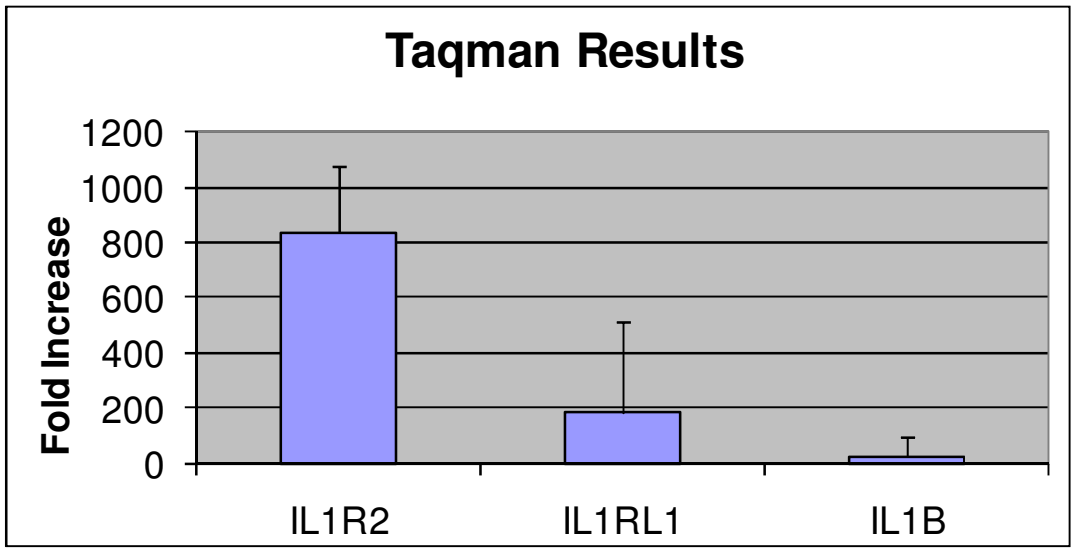


Figure 4. Real-time PCR confirms upregulation of IL1R2, IL1RL1, and IL1B at the mRNA level. Data is shown as fold increase in MCF10A-C/EBPbeta2 cells compared to parental MCF10A cells. The probe for IL1R2 recognizes both the cell bound and secreted mRNA which arise from alternative splicing. IL1R2, IL1RL1, and IL1B are up 835, 183, and 29 fold, respectively. All have a p-value of 0.05 or less.

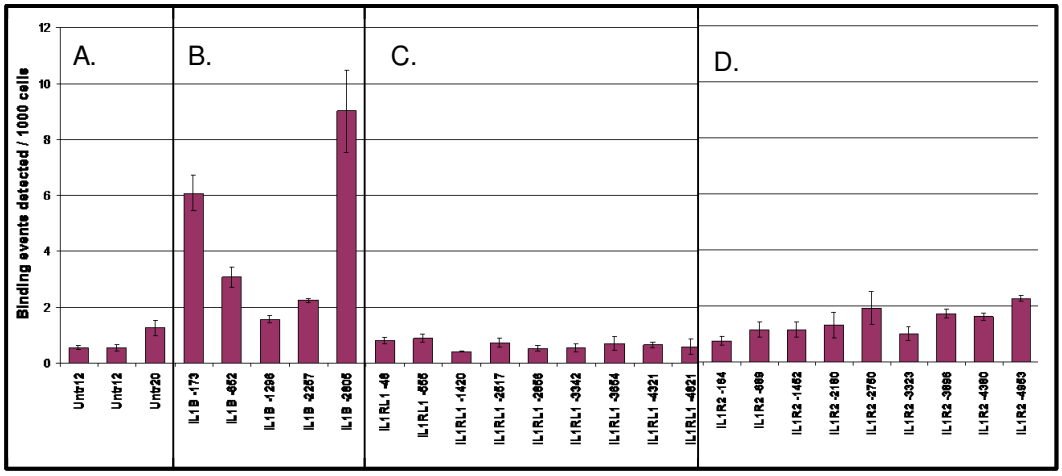


Figure 5. C/EBPbeta2 directly binds the IL1B promoter but not the IL1R2 or IL1RL1 promoters. Chromatin immunoprecipitation (ChIP) was performed using an antibody to the T7 epitope to pull-down C/EBPbeta2 bound regions of the genome. Shown are the quantitative real-time PCR (Q-PCR) analysis for the negative controls (A) and the promoter regions of (B) IL1B, (C) IL1RL1, and (D) IL1R2. For negative controls three untranscribed regions of the genome were used. Q-PCRs were run in triplicate and the averaged Ct values were transferred into copy numbers of DNA using a standard curve of genomic DNA with known copy numbers. Results are normalized for primer pair amplification efficiency using the Q-PCR value obtained with unprecipitated genomic DNA (Input DNA). Results are presented as Binding Events Per 1000 Cells for the promoter region tested. Error bars correspond to the standard deviations from triplicate Q-PCR reactions.

Quantitative real-time PCR analysis of chromatin immunoprecipitated from MCF10A cells overexpressing C/EBPbeta2 was used to examine C/EBPbeta2 binding to IL1B, IL1R2, and IL1RL1 promoters. Two untranscribed regions of the genome were used as negative controls. As seen in Figure 5, C/EBPbeta2 binds directly to the IL1B promoter at two distinct locations. These locations are -1 to -173 and -2258 to – 2805 base pairs upstream of the IL1B transcriptional start site. This agrees with previous studies that have shown C/EBPbeta binds the IL1B between -17 and -107 and also -168 and -258 (Liang, Zhang et al. 2006). It also provides evidence for a new binding site further upstream and demonstrates for the first time that the C/EBPbeta2 isoform can bind to the IL1B promoter. ChIP was also used to investigate 5000 base pairs upstream and downstream of the transcriptional start site of IL1R2 and IL1RL1, but C/EBPbeta2 was not found to bind anywhere within these regions. Nonetheless, IL1R2 and IL1RL1 are dramatically upregulated by C/EBPbeta2 and this upregulation (whether by direct or indirect mechanism) may play an important role in the cancer phenotypes observed.

C/EBPbeta2 overexpression in MCF10A cells regulates IL1B and IL1R2 protein expression

It is known that upregulation of mRNA does not always result in an increase of the cognate protein; therefore I performed immunoblot analysis to determine if the changes seen in mRNA were also seen at the protein levels for IL1B, IL1R2, and IL1RL1. Immunoblot analysis of whole cell lysates from MCF10A-C/EBPbeta2 and control MCF10A cultures was performed (Figure 6). Although, IL1R2 and IL1B were detected at the protein level, IL1RL1 was not. IL1R2 is produced as a secreted form and also as a membrane bound form, both of which are glycosylated (Colotta, Saccani et al.

1996). Both secreted and membrane bound IL1R2 were detected in MCF10A cells overexpressing C/EBPbeta2 but not parental MCF10A cells (Figure 6A). IL1B is synthesized as a 31 kD precursor that is then cleaved by ICE to a 17 kD molecule that is secreted from cells. The17 kD form of IL1B is considered to be the active form, with secretion being the rate limiting step (Song, Voronov et al. 2003). Pro IL1B (31kD) was detected in the C/EBPbeta2 overexpressing MCF10As but not in MCF10A cells (Figure 6B). Interestingly, the active 17kD form of IL1B was not detected by immunoblot analysis in the whole cell lysates or in conditioned media from C/EBPbeta2 overexpressing MCF10A cells (Figure 6B and data not shown). However, low levels (5-50 pg/mL) of IL1B are known to be biologically active and such low levels are below the detection threshold of immunoblot analysis (Streicher, Willmarth et al. 2007). Therefore, an enzyme-linked immunosorbent assay (ELISA) was performed to determine if IL1B was present in the media of the MCF10A cells upon C/EBPbeta2 overexpression. Although conditioned media from MCF10A cells had no IL1B present, conditioned media from MCF10A-C/EBPbeta2 cells had 18.0 ± 0.3 pg/mL (Figure 6C). The ELISA I used is designed to favor the cleaved 17kD form of IL1B and drastically underestimates the amount of uncleaved IL1B present. It is, therefore, impossible to determine with one hundred percent certainty if 17kD "active" IL1B is being secreted or if proIL1B is being detected. The possibility that secreted IL1B was playing a role was assessed multiple ways. First, the active (17 kd) form of IL1B was added to the media of MCF10A cells in the presence and absence of EGF. The addition of 17 kd IL1B did not alter the growth of the MCF10A cells (Figure 7). Next, IL1 signaling was inhibited using

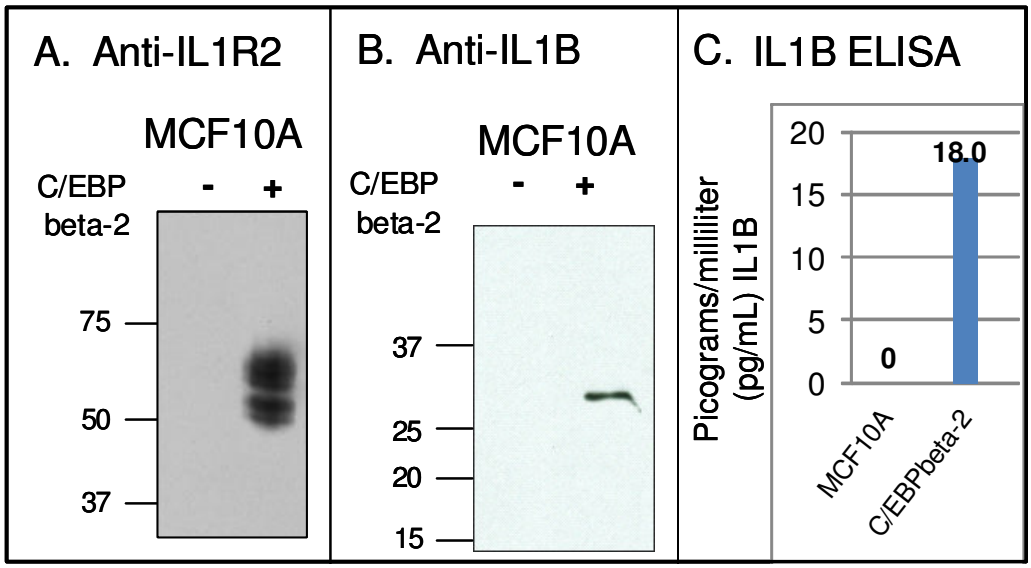
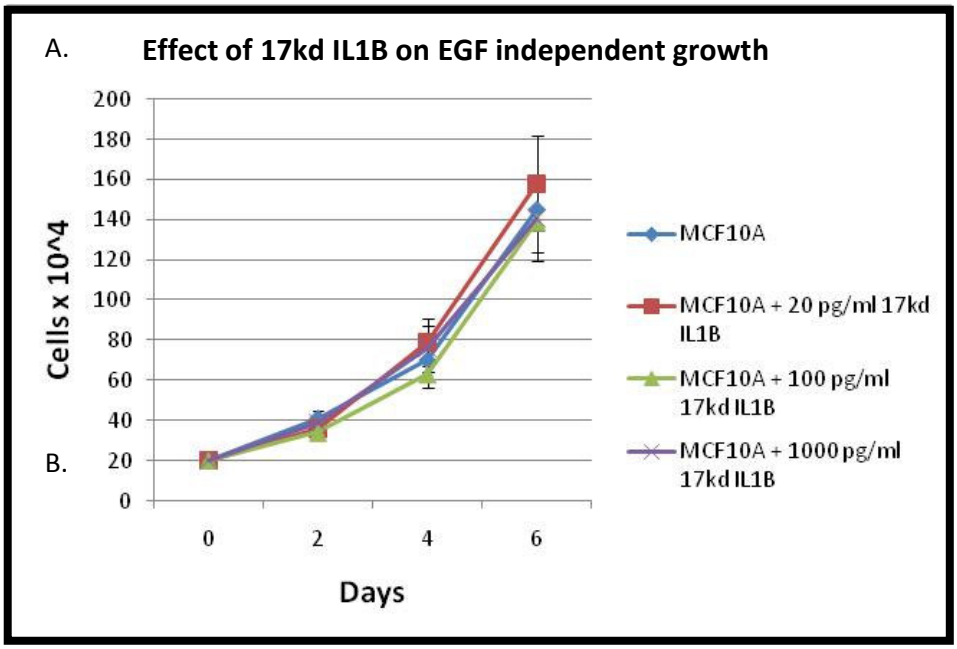


Figure 6. IL1B and IL1R2 are upregulated at the protein level. Equal amounts of protein extracts from MCF10A cells or MCF10A cells overexpressing C/EBPbeta2 were subjected to immunoblot analysis with (A) anti-IL1R2 or (B) anti-IL1B. ELISA was also performed for to determine the concentration of IL1B in the culture supernant of these cells (C). All of these experiments were repeated at least three times with similar results.



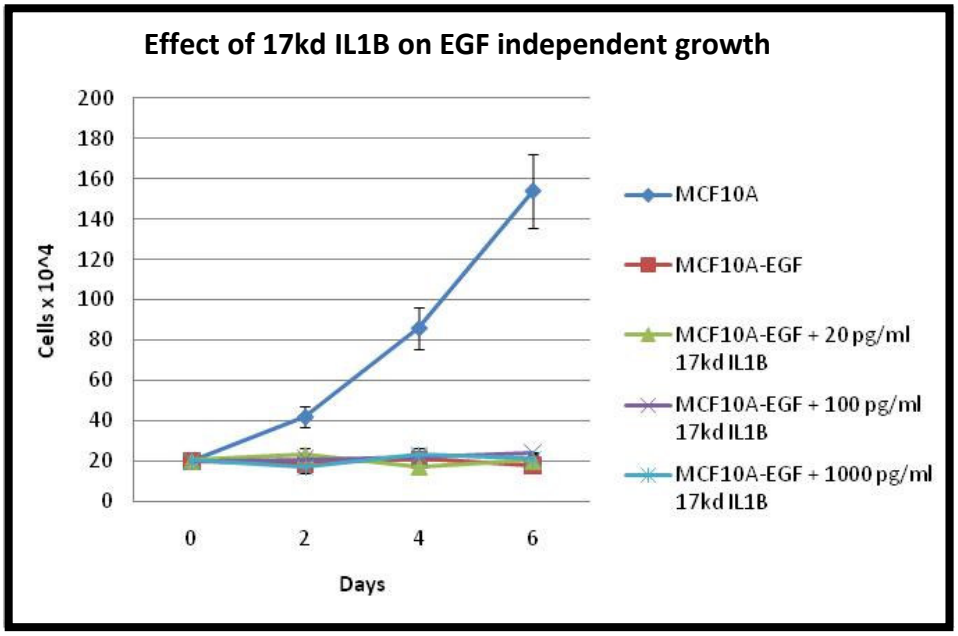
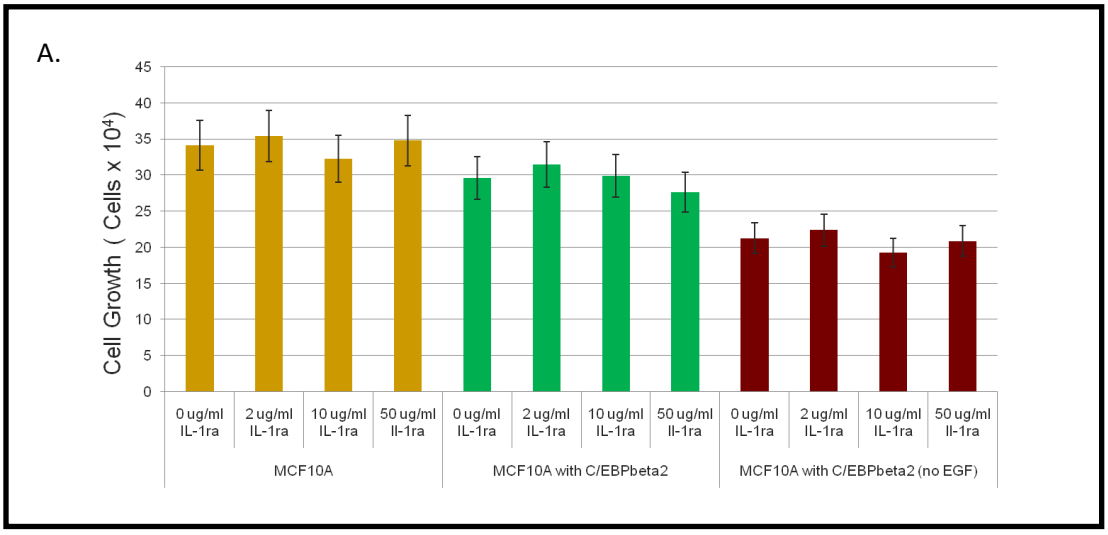


Figure 7. Active IL1B in the media of MCF10A cells does not alter their growth. Addition of 20 -1000 pg/ml of 17 kD, biologically active IL1B did not change the rate of MCF10A growth in the presence (A) or the absence (B) of EGF.



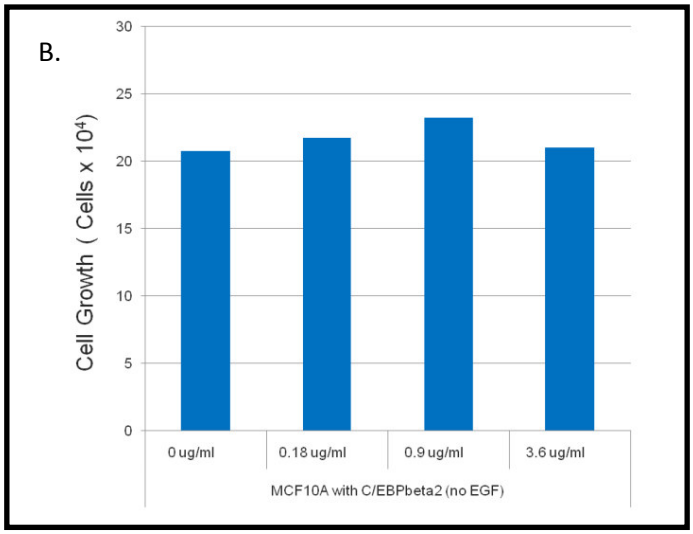


Figure 8. MCF10A-C/EBPbeta2 growth is not altered in the absence of classical IL1B signaling. Inhibiting IL1B signaling using IL1ra (A) or neutralizing antibodies (R&D Systems MAB201, MAB6010) to IL1B (B) does not alter EGF independence growth of MCF10A-C/EBPbeta2 cells.

the recombinant IL1 receptor antagonist (IL1ra). IL1ra binds interleukin-1 receptor 1 (IL1R1), and prevents IL1B mediated signal transduction (Arend, Malyak et al. 1998). Previous studies indicate a 10 to 100 fold molar excess of IL-1ra will effectively block IL1 signaling (Arend, Malyak et al. 1998). IL1ra, even at 1000 fold molar excess, had no effect on EGF independence or the other cancer phenotypes displayed by MCF10A-C/EBPbeta2 cells, nor did addition of a neutralizing antibody against secreted IL1B (Figure 8). Thus, the 'classical' IL1B signaling was demonstrated not to play a role in the cancer phenotypes present in MCF10A cells overexpressing C/EBPbeta2. The role of proIL1B in tumorigenesis has not been previously addressed since pro1ILB is thought to be completely inactive.

ProIL1B present in MCF10A-C/EBPbeta2 cells is localized to the nucleus and is tightly associated with the chromatin

Although IL1B is thought to be inactive in the proform, a growing number of cytokines have been shown to play nuclear roles in their proform. To ascertain the localization of IL1B in MCF10A-C/EBPbeta2 cells, indirect immunofluorescence was performed (Figure 9A). IL1B was detected in the nucleus of C/EBPbeta2 overexpressing MCF10A cells. Visual inspection of Figure 9A indicates some cells express higher levels of IL1B and that there is also some cytoplasmic IL1B in some of the cells. This may be due to the variable expression of C/EBPbeta2 within the population, given a mix of retroviral integration sites within the population. The C/EBPbeta2 overexpressing MCF10A cells are selected as previously described by their anchorage independence and are not sorted for C/EBPbeta2 expression levels (Bundy, Wells et al. 2005). Performing the same indirect immunofluorescence in MCF10A cells results in no signal, as expected.

Also, staining using the secondary antibody alone on the MCF10A-C/EBPbeta2 cells gives no background (Figure 9B and data not shown). Nuclear localization of IL1B was confirmed in C/EBPbeta2 overexpressing MCF10A cells using cell fractionation and immunoblot analysis (Figure 9C). As seen in Figure 9C, the proform of IL1B is detected in both the cytoplasm and nucleus of the MCF10A cells overexpressing C/EBPbeta2. Taken together, these results demonstrate a significant percentage of proIL1B is present in the nucleus of MCF10A cells overexpressing C/EBPbeta2.

Nuclear extraction in buffers of increasing ionic strength can be used to determine how tightly a protein is associated with the chromatin. The first extraction (0.1 M NaCl) removes only those proteins that are very loosely associated with the chromatin. Extraction in 0.3 M NaCl or 0.53 M NaCl releases a large number of nuclear proteins, including a variety of transcription factors. ProIL1B is tightly associated with the chromatin, resisting extraction with 0.53 M NaCl (Figure 10). This data was confirmed and extended by Rachel Jerrell and Linda Sealy (Figure 11). For example, the well-characterized sequence-specific basic leucine zipper transcription factor, CREB (cAMP response element binding protein), is completely extracted from nuclei by 0.6 M NaCl (Figure 11, lane 5). The core histones are among the most tightly bound proteins in chromatin. A1 M NaCl extraction removes 50-75% of histones H2A and H2B (see H2A, Figure 11, lane 6) whereas histones H3 and H4 require 2 M NaCl for complete extraction. Importantly, although some proIL1βeta appears loosely associated and is extracted from nuclei by 0.1 M to 0.3 M NaCl, the majority of nuclear proIL1βeta is tightly bound to the chromatin, resisting extraction by 1.0 M NaCl (Figure 11, lane 7). These results

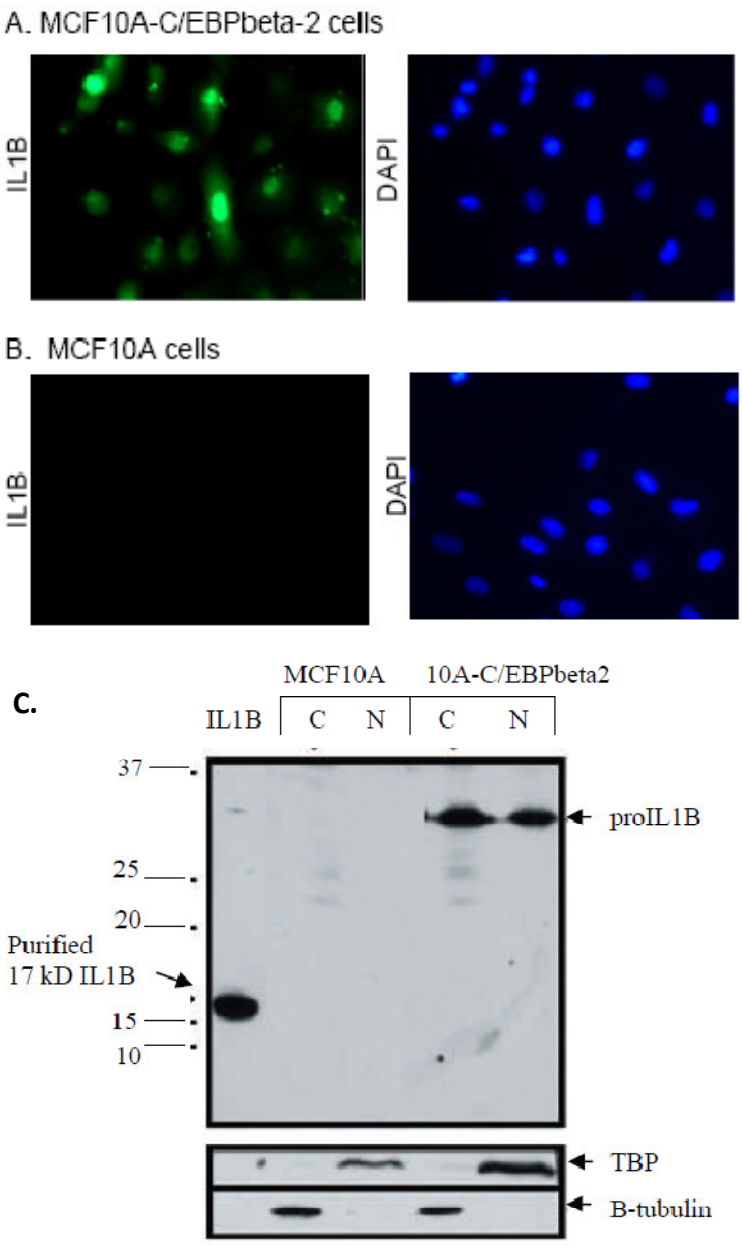


Figure 9. ProIL1B is localized to the nucleus. Indirect immunofluorescence using anti-IL1B was performed on MCF10A-C/EBPbeta1cells (A, left) and parental MCF10A cells (left B). DAPI was used to stain the nuclei (A and B, right). Nuclear localization of proIL1B was also assessed by nuclear fractionation followed by immunoblot analysis (C, top). Efficient cellular fractionation was confirmed using Anti-TFIID as a nuclear specific control (C, middle) and anti-Beta Tubulin as a cytoplasmic marker (C, bottom).

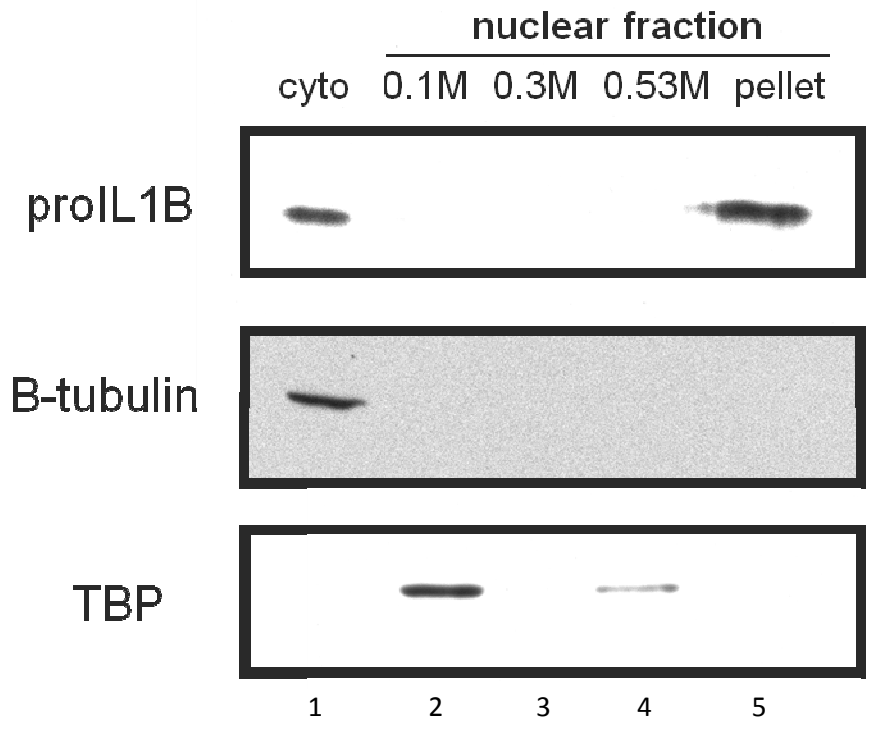


Figure 10. ProIL1B is tightly associated with the chromatin. Nuclei from MCF10A cells overexpressing C/EBPbeta2 were extracted into five fractions sequentially: buffer B containing 0.1 M NaCl (lane 2); or 0.3 M NaCl (lane 3); or 0.53 M NaCl (lane 4); and the nuclear pellet (lane 5). Lane 1 is the cytoplasmic fraction. Cellular fractionation was monitored using anti-beta tubulin as a cytoplasmic marker and anti-TBP as a nuclear marker.

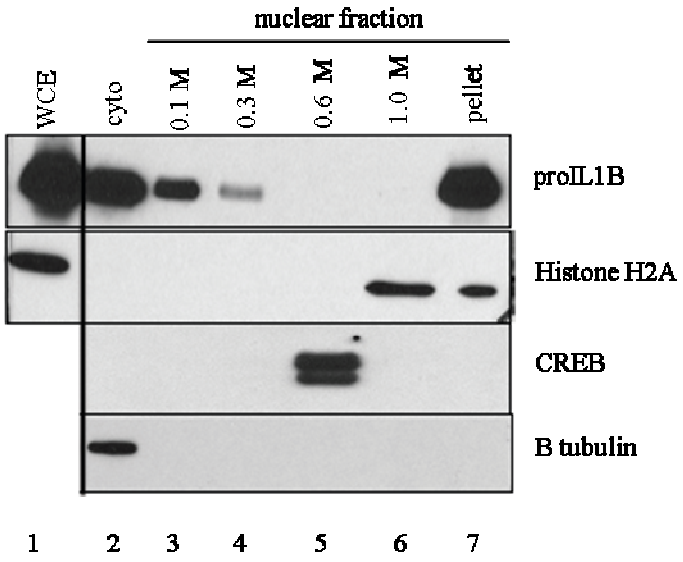


Figure 11. ProIL1B is more tightly associated with the chromatin than Histone H2A. Nuclei from MCF10A cells overexpressing C/EBPbeta2 were extracted into five fractions sequentially: buffer B containing 0.1 M NaCl (lane 3); or 0.3 M NaCl (lane 4); or 0.6 M NaCl (lane 5); or 1.0 M NaCl (lane 6) and the nuclear pellet (lane 7). Lane 2 is the cytoplasmic fraction and lane 1 contains the whole cell extract. Cellular fractionation was monitored using anti-CREB or anti-H2A as nuclear markers and anti-beta tubulin as a cytoplasmic marker (fractionation and immunoblot performed by Jerrell and Sealy).

demonstrate proIL1B is not only present in the nucleus but also interacts with the chromatin even more tightly than the core histone H2A.

ChIP-chip demonstrates nuclear proform IL1B binds specific regions of the chromosome

To gain further insight into a potential nuclear function for proIL1B, we performed chromatin immunoprecipitation followed by hybridization to the Human Promoter 1.0R array (Affymetrix) to identify where proIL1B was bound to chromatin across the genome. The Human Promoter 1.0R array covers over 25,500 promoter regions with probe resolution of 35 bp. Chromatin bound by proIL1B was collected by immunoprecipitation with the IL1B monoclonal antibody MAB201 (R&D Systems). ProIL1B was found to bind at 204 locations in the genome. As shown in Figure 12, all chromosomes contained at least one proIL1B binding site, but these sites did not associate randomly across the chromosomes. Indeed, nearly two thirds of the sites were found in clusters of two or more closely spaced sites with several of the highly statistically significant clusters containing five to seven proIL1B sites (seen visually in Figure 12, and listed with p-values in Table 4). We are currently uncertain as to the functional significance of these multiple site clusters except to note that one of them (7 sites clustered on chromosome 6) coincides with a locus highly enriched for MHC class II genes. Combining the ChIP-chip analysis of proIL1B with the Affymetrix expression profiling of MCF10A-C/EBPbeta2 cells, I can conclude that the sites of proIL1B binding are not closely associated with the promoters of the 443 genes found to be

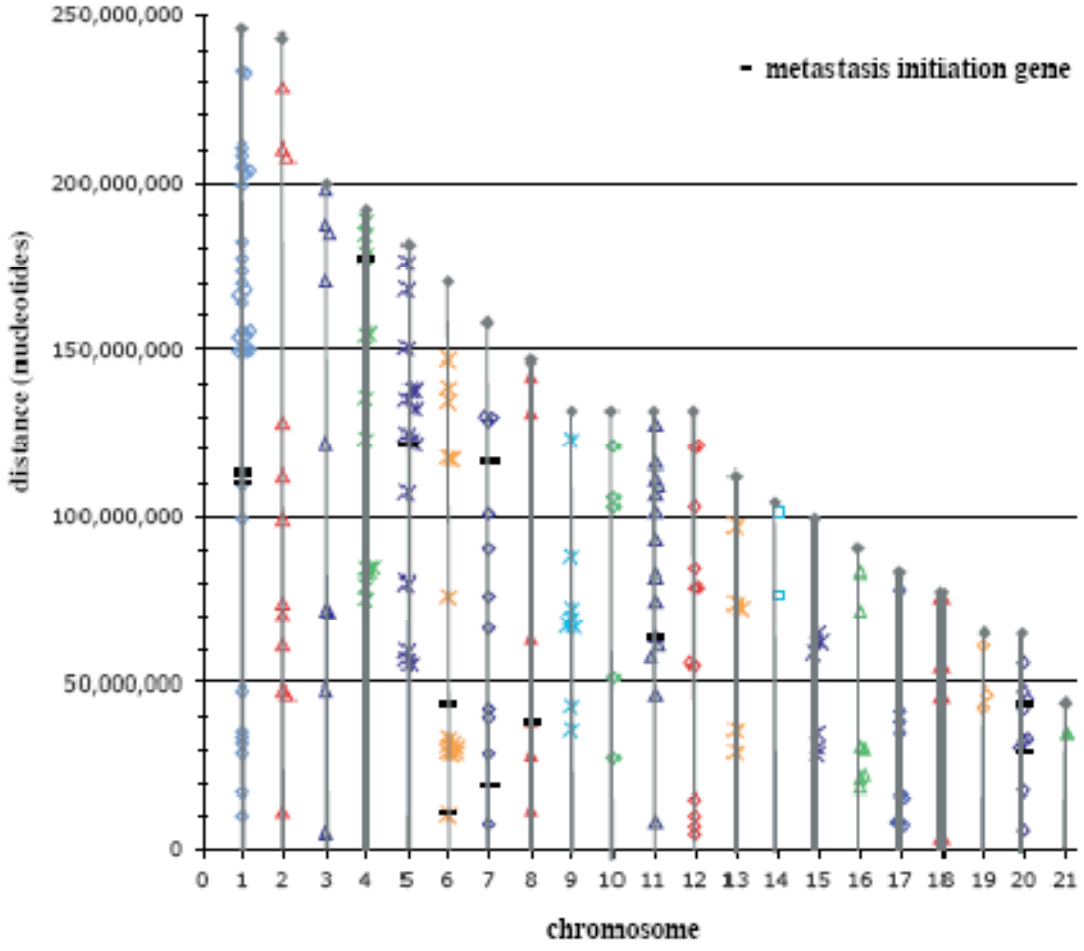


Figure 12. ProIL1B binds at distinct chromosomal locations. Chromatin immunoprecipitation (ChIP) was performed using an antibody to IL1B to pull-down proIL1B bound regions of the genome. After purification, precipitated DNA was then hybridized to the Human Promoter 1.0R array (Affymetrix). Shown are the 204 sites (multicolor) detected to be 2 fold or more enriched when IL1B ChIP DNA was hybridized to the array compared non ChIP DNA. Approximately two thirds of these sites bound in clusters. ProIL1B binding was highly correlated with metastasis initiation genes shown here by black bars.

differentially expressed upon C/EBPbeta2 overexpression. Therefore it seems unlikely that pro-IL1B is functioning as a typical trans-acting transcription factor.

Table 4. ProIL1B associates with the chromatin in a non-random manner as exhibited by statistically significant clustering. See Figure 12 for visual representation and below for chromosome and p-values.

	Chromosome	Probability	Sites in cluster
Clus1	6	0.0002	6
Clus2	1	0.001	6
Clus3	1	0.008	5
Clus4	1	0.04	4
Clus5	1	0.04	4
Clus6	9	0.04	4
Clus7	5	0.04	4
Clus8	1	0.04	4

In the event that proIL1B may regulate the chromatin landscape like the previously described dual function cytokines, I used bioinformatics tools to categorize the genes in the "neighborhood" of proIL1B binding whose expression could be influenced by any proIL1B-dependent changes in chromatin conformation. Pathway analysis using PANTHER for the set of genes within 500KB of proIL1B binding sites identified multiple pathways known to be affected in cancer (Table 5).

In a similar approach, I performed over-representation analysis to determine if pro1L1B binding was correlated with genes involved in tumor initiation, metastasis initiation, metastasis progression, and/or metastasis virulence using gene sets defined in a recent review by Chiang and Massague (Chaing 2008). Interestingly, proIL1B binding is highly significantly correlated with all of these pathways (Table 6). Most highly significant (1.00E-28) were eleven genes (RhoC, LOX, VEGF1,2, CSF-1, ID1, TWIST,

MET, FGFR, MMP-2, NEDD9) associated with metastasis initiation. As shown in Figure 12, six of the eleven genes were located in the neighborhood (<750kB) of proIL1B binding sites, with one gene (VEGFB) in close proximity (<32kB) and another (VEGFC) harboring a site within the 3' end of the gene. These data indicate that future studies to define the mechanism of proIL1B binding to chromatin and its precise functional consequences are warranted.

Table 5. Bioinformatic analysis connects chromatin associated ProIL1B with multiple pathways known to be altered in cancer. Over-representation analysis performed and statistical significance determined using PANTHER Analysis.

Pathway	Total	Found	Expected	p-value
Integrin signaling	227	38	19.94	0.031
PDGF signaling	189	33	16.6	0.039
EGF receptor signaling	150	27	13.18	0.087
Ras signaling	91	19	7.99	0.104
Apoptosis signaling	131	24	11.51	0.137

Table 6. ProIL1B is significantly correlated with genes known to be involved in tumor initiation and metastasis. Over-representation analysis performed and statistical significance determined using modified formula from Backes *et al*. (Backes *et al., 2007*). See methods for detailed description.

	Total Genes	ProIL1B associated	p-value
Tumor initiation	9	3	7.22E-12
Metastatic initiation	11	7	1.00E-28
Metastatic progression	4	2	1.17E-08
Metastatic virulence	5	1	0.000222

Immunohistochemical analysis demonstrates the presence of nuclear IL1B in human breast cancer samples

In order to determine if nuclear IL1B was present in the tumors of breast cancer patients I performed immunohistochemical analysis of human samples. Nuclear IL1B was detected in a subset of these samples, in both the cancer cells themselves and also in infiltrating leukocytes (Figure 13 panels A, C, and E, magnifications in B, D, and F). Most of the samples analyzed were invasive ductal carcinomas. IL1B was detected at varying levels in roughly 80% of these samples, which is similar with previous studies which detected IL1B in 90% of invasive ductal carcinoma via ELISA (Jin, Yuan et al. 1997). In addition, nuclear IL1B was prominent in almost 20% (12 of the 63) of samples analyzed. The representative tumors displaying nuclear IL1B shown in Figure 13 are A) Stage II-III, T2N0M0, ER -, PR -, and ErbB2+++; B) Stage II-III, T2N0M0, ER -, PR ++, and ErbB2-; C) Stage III, T4N3MX, ER -, PR -, and ErbB2++. Incubation with secondary antibody only resulted in no staining. Therefore, these data serve to demonstrate nuclear IL1B occurs in the tumors of breast cancer patients and suggests that a larger study to determine if nuclear IL1B correlates with tumor subtype, stage, and/or grade could be informative.

Attempted knock-down of IL1B

Knocking-down IL1B expression would be very informative. Thus, I have made every attempt to do so. Unfortunately, knock-down of IL1B has not been achieved; however, I have successfully been able to knock-down GAPDH in parallel samples. During these attempts I have utilized 5 different shRNAs targeting IL1B from the SIGMA Mission Lentiviral collection; these vectors contain a puromycin resistance

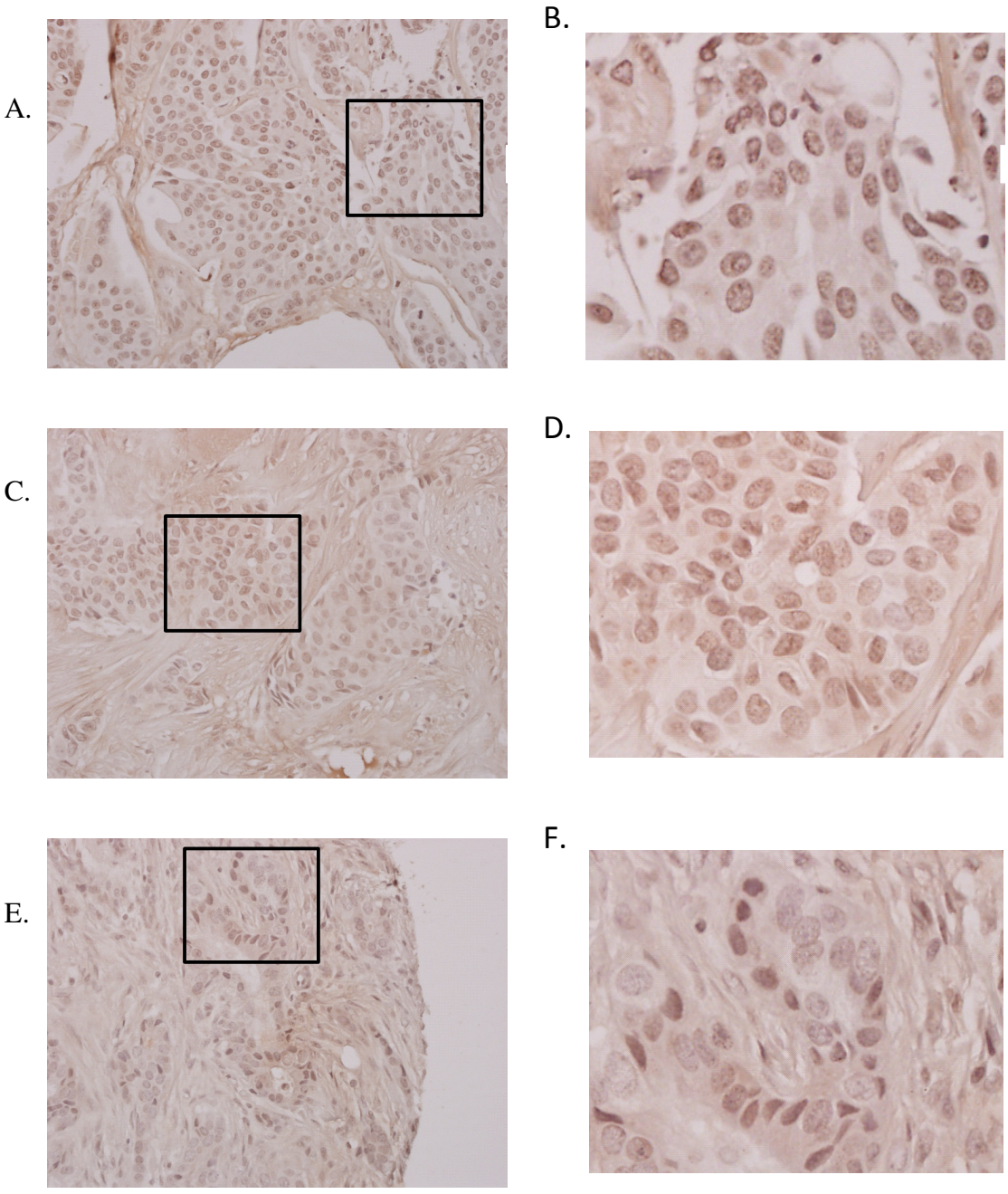


Figure 13. Immunohistochemical analysis of human breast cancer samples reveals nuclear IL1B in invasive ductal carcinoma. The tumors shown have the following characteristics: A) Stage II-III, T2N0M0, ER -, PR -, and ErbB2+++; C) Stage II-III, T2N0M0, ER -, PR ++, and ErbB2-; E) Stage III, T4N3MX, ER -, PR -, and ErbB2++. Magnification of each of the tumors is shown in panels B, D, and E.

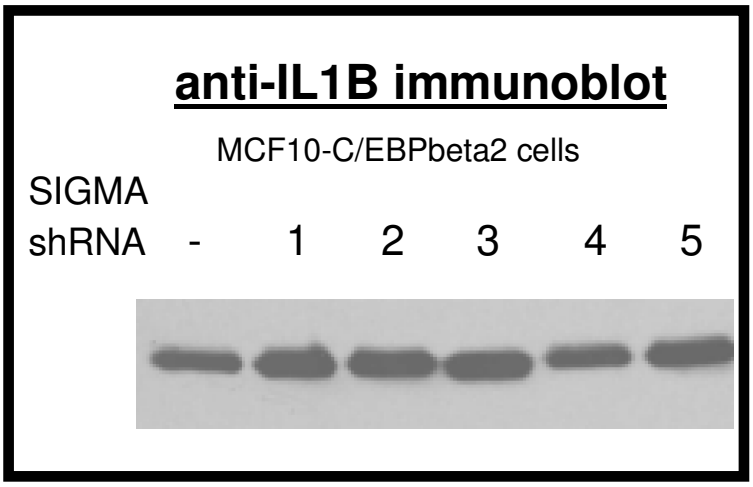


Figure 14. SIGMA Mission lentiviral shRNA against IL1B has no effect on IL1B protein levels. Equal amounts of protein extracts from MCF10A -C/EBPbeta2 cells and MCF10A-C/EBPbeta2 cells engineered to express one of the five shRNA constructs against IL1B were subjected to immunoblot analysis with anti-IL1B.

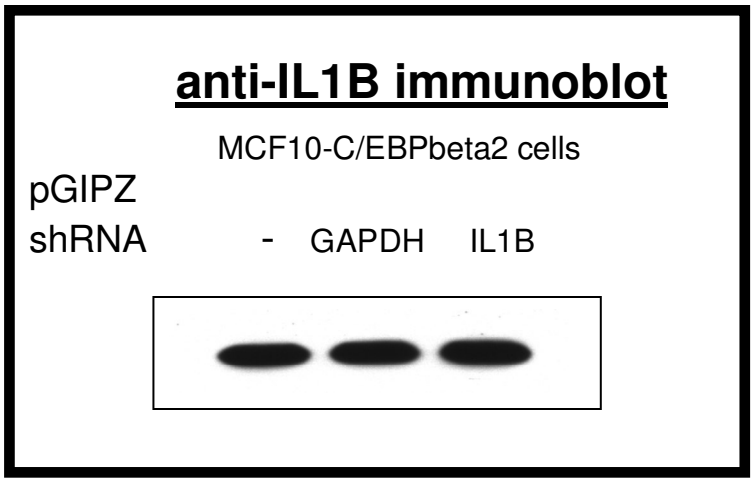


Figure 15. Use of the pGIPZ shRNA against IL1B does not result in diminished protein expression. Equal amounts of protein extracts from MCF10A -C/EBPbeta2 cells and MCF10A-C/EBPbeta2 cells engineered to express shRNA against GAPDH or IL1B were subjected to immunoblot analysis with anti-IL1B.

cassette. Despite selection of puromycin resistant populations, none of these five constructs resulted in the down-regulation of IL1B mRNA or protein (Figure 14). I then tried the Open Biosystems pGIPZ shRNA construct targeted at IL1B. The pGIPZ constructs are also lentiviral based constructs but contain a GFP reporter. Despite the selection of pure GFP positive cell populations IL1B was not affected (Figure 15).

I next attempted to make adenovirus targeting IL1B, knowing this would allow us to target 99.9% of the cells with a suitable MOI of adenovirus-shRNA against IL1B, alleviating the need for selection. I utilized the Adeno-X ViraTrak Promoterless Expression System 2 (Clontech). However, despite the successful production of control adenovirus no titer was ever achieved for the adenovirus containing shRNA against Prior approaches did not result in a pure population of shRNA expressing cells. Adenovirus is known to generate an inflammatory response resulting in induction of IL1B, so perhaps IL1B is necessary for the production of adenovirus (Bowen et al., 2002).

I then decided that it would be advantageous to use an inducible shRNA directed at IL1B, selecting for cells harboring the shRNA-IL1B prior to activating its expression. This way if cells expressing shRNA against IL1B died upon IL1B knock-down I would see the effect in the whole population upon shRNA-IL1B expression.
Prior approaches did not result in a pure population of shRNA expressing cells immediately and so it is possible that cells capable of knocking-down IL1B were lost prior to analysis. To circumvent this problem I utilized the Open Biosystems pTRIPZ construct. This construct constitutively expresses the reverse Tet activator and a

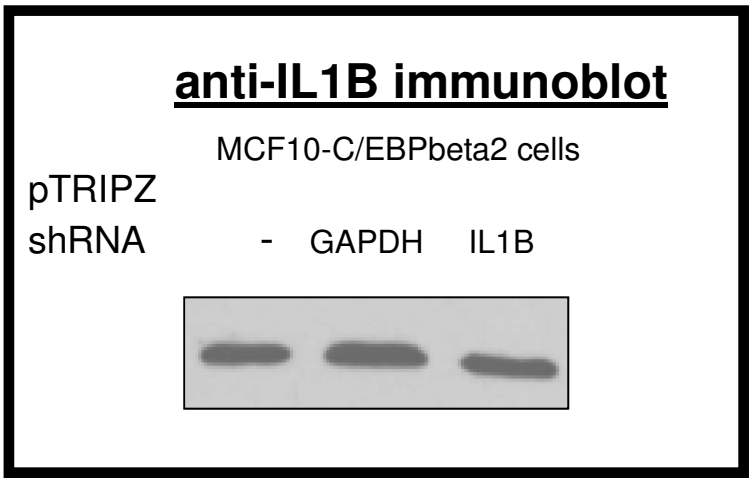


Figure 16. The inducible pTRIPZ vector targeting IL1B does not diminish IL1B protein expression. Equal amounts of protein extracts from MCF10A -C/EBPbeta2 cells and MCF10A-C/EBPbeta2 cells engineered to express inducible shRNA against GAPDH or IL1B were subjected to immunoblot analysis with anti-IL1B.

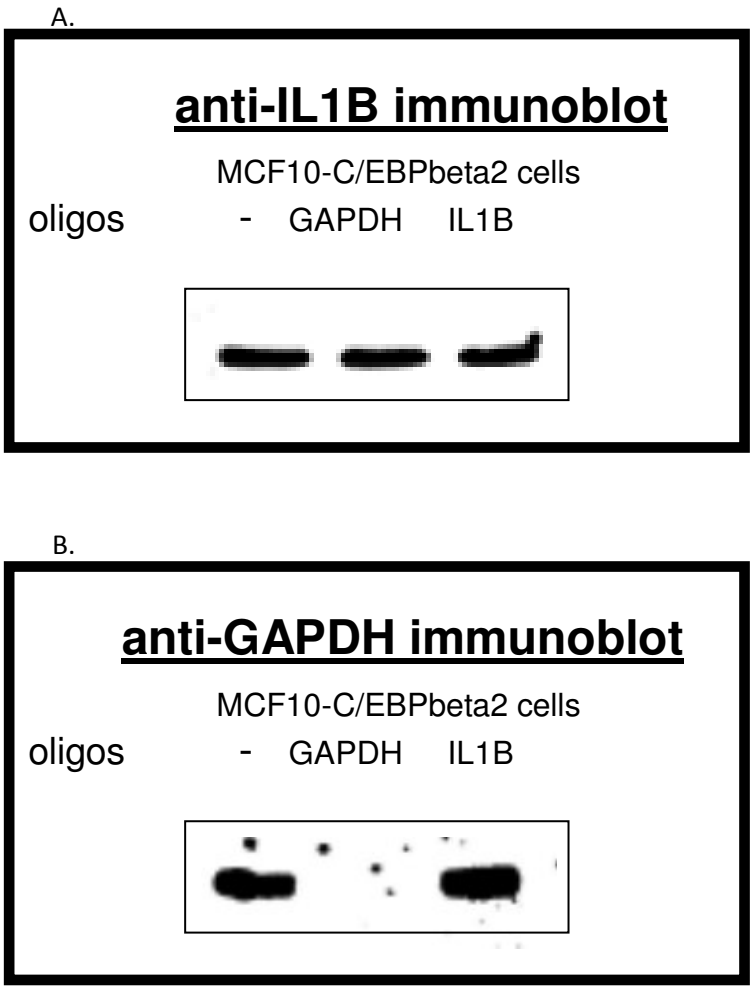


Figure 17. siRNA mediated knock-down of GAPDH but not IL1B is achieved. Equal amounts of protein extracts from MCF10A -C/EBPbeta2 cells and MCF10A-C/EBPbeta2 cells transfected with siRNA against GAPDH or IL1B were subjected to immunoblot analysis with (A) anti-IL1B and (B) anti-GAPDH.

puromycin resistant cassette. Thus, the cells can be infected and selected without shRNA against IL1B being actively produced. Induction using doxycycline induces the shRNA against IL1B and also RFP. Using this method, RFP was induced, but no knock-down of IL1B was detected (Figure 16). No major cell death was seen and the expression of IL1B has been monitored for up to two weeks to ensure the absence of knock-down was not due to the high stability of IL1B protein. Only one shRNA targeting IL1B was available in this vector, so it is possible that the shRNA used is not capable of knocking-down IL1B. To this end, the lab will continue to pursue knock-down of IL1B using the pTRIPZ system but expressing shRNA targeted at multiple different regions of IL1B.

Finally, I had avoided using siRNA technology due to the fact that MCF10A cells are notoriously difficult to transfect, however, recent publications (including (Parsons, Patel et al. 2009)) have demonstrated the ability to knock-down targets in MCF10A cells using DHARMACON Smart Pool Plus oligos. However, IL1B knock-down was not detected using this methodology either, despite the successful knockdown of GAPDH in parallel experiments, thereby validating the methodology (Figure 17).

Discussion

C/EBPbeta2 overexpression in MCF10A cells results in the upregulation of three interleukin-1 family members: IL1B, IL1R2, and IL1RL1. These interleukin-1 family members are all known to be upregulated in human breast cancer (Jin, Yuan et al. 1997; Pantschenko, Pushkar et al. 2003; Nguyen, Kuliopulos et al. 2006). Furthermore, I demonstrated by chromatin immunoprecipitation that IL1B is a direct target of C/EBPbeta2 transactvation in MCF10A cells, showing for the first time that this isoform

of C/EBPbeta can bind the promoter. One of the C/EBPbeta binding sites identified is consistent with a previously observed C/EBPbeta binding site in different cell types (Yang, Wara-Aswapati et al. 2000). Our data also indicates the presence of a novel C/EBPbeta binding site further upstream of the IL1B promoter.

As detailed in the introduction, it is well established that secreted IL1B plays an important role in breast cancer. Although MCF10A-C/EBPbeta2 cells have a small amount of IL1B in the culture medium, the IL1R2 protein is also concomitantly elevated in these cells. As a decoy receptor devoid of any cytoplasmic signaling domain, IL1R2 would be expected to block mature IL1B from classically signaling though IL1R1. Indeed I found that addition of interleukin-1 receptor antagonist or IL1B neutralizing antibodies did not alter the EGF independent growth of MCF10A-C/EBPbeta2 cells. Thus in contrast to IL1A and SUM149 cells, secreted mature IL1B is not likely contributing to ErbB independent growth of MCF10A-C/EBPbeta2 cells. While functional studies of IL1B have always focused on the mature, secreted protein because proIL1B is considered biologically inactive, our results presented here clearly call this notion into question. The majority of IL1B protein present in MCF10A-C/EBPbeta2 cells is found in the 31 kD proform and a substantial fraction of proIL1B is actually tightly bound to chromatin in the nucleus of these cells. Therefore, it appears likely that IL1B, like IL1A, IL33, and HMGB1, is also a dual function protein, with roles as both a secreted cytokine and an intracellular nuclear factor. IL1B has a putative NLS (PKKKMEK) and it is known that mature (cleaved) IL1B can translocate to the nucleus with the receptor after IL1R1 mediated endocytosis. This translocation is believed to be receptor dependent as mutation of the NLS did not affect IL1B nuclear localization in this

system (Grenfell, Smithers et al. 1991). It is very unlikely that interaction with IL1R1 is responsible for the localization of proIL1B to the nucleus in our studies, since the proform of IL1B does not bind to IL1R1 (Mosley, Urdal et al. 1987). Very recently, while investigating the possibility of nuclear localization of proIL1A in microglia, Lusheshi et al. detected nuclear proIL1B as well (Luheshi, Rothwell et al. 2009). They found that proIL1A nuclear translocalization was NLS mediated and involves Ran-dependent active transport. In contrast, proIL1B nuclear translocalization was passive and NLS independent (Luheshi, Rothwell et al. 2009).

I used genomic and bioinformatics approaches to begin to address the possibility that nuclear proIL1B in MCF10A-C/EBPbeta2 cells was positioned in such a way as to contribute to the cancer phenotype by chromatin remodeling. ChIP-chip data indicate proIL1B is bound at distinct, non-random locations along the chromosome, and bioinformatic approaches demonstrate proIL1B is positioned in such a way to contribute to multiple cancer connected pathways. ProIL1B is tightly bound to chromatin (resisting extraction by 1.0M NaCl), but the details of how this cytokine binds to chromatin are at this point still obscure. The IL-1 like cytokine IL33 contains a short (12 aa) chromatin-binding motif (CBM) that docks into an acidic pocket formed by the histone H2A-H2B dimer at the nucleosome surface (Roussel, Erard et al. 2008). By interacting with nucleosomes, IL33 was shown to regulate chromatin compaction. However, the IL33 CBM, $IL33_{40\text{-}58}$, is not well conserved (4 out of 9 aa) in IL1B (or IL1A whose mechanism of chromatin interaction is also not well established). Thus, future experiments will be required to identify the IL1B CBM and to determine whether IL1B also interacts with histones and/or some other chromatin-associated proteins.

As proIL1B may be altering chromatin architecture, via a yet to be elucidated mechanism, it is of interest that cancer associated pathways and certain genes known to be involved in metastasis initiation, including the processes of invasion, angiogenesis, and epithelial-to-mesenchymal transition, were correlated with proIL1B sites (Table 2 and Table 3). In this setting, C/EBPbeta2 upregulation of proIL1B may result in association of proIL1B with the chromatin and/or chromatin remodeling complexes facilitating changes in gene expression related to initiating EMT and promoting metastatic initiation, and to a lesser degree, metastatic progression and metastatic virulence. A direct role for nuclear proIL1B in the changes in EMT pathway genes seen in MCF10A-C/EBPbeta2 cells could be directly addressed by IL1B knock-down. Unfortunately, multiple attempts using both shRNA and siRNA technologies to knock-down IL1B have been unsuccessful to date, although GAPDH knock-down was achieved.

The accumulation of the proIL1B in MCF10A-C/EBPbeta2 cells raises the question of why the majority of this protein is not processed. IL1B mRNA is typically absent in cells and is induced by inflammatory signals such as TLR ligands, which lead to the activation of NFkappaB and C/EBPbeta. Once IL1B is synthesized, the inflammasome must be assembled and activated for proIL1B to be cleaved. Multiple negative regulators of the inflammasome activation are now known (Martinon, Mayor et al. 2009); however, based on our genomic profiling data none of these negative regulators are expressed in MCF10A or MCF10A-C/EBPbeta2 cells at a significant level. These include: CARD17, CASP12, COP, ICEBURG, and SERPINB9. Although ICE is expressed, other known components of inflammasome complexes are not expressed at a significant level. These include: NALP1, NALP3, CARDINAL ,caspase-5, and

PYCARD. The very low steady state mRNA level and/or lack of these components may explain the absence of the 17kD protein in MCF10A-C/EBPbeta2 cells.

It is notable that PYCARD, an important component of both the NALP1 and NALP3 inflammasomes, is silenced due to extensive promoter methylation in nearly half of breast cancer cell lines and over a third of human breast tumors (Conway, McConnell et al. 2000; Levine, Stimson-Crider et al. 2003). Since IL1B is present in over 90% of invasive breast carcinomas by ELISA, it is reasonable to expect that a subset of these breast cancers lack PYCARD expression due to promoter methylation, and therefore IL1B may be present in the proform (Jin, Yuan et al. 1997). In fact, immunohistochemical analysis detected nuclear IL1B in human breast cancer samples. Further studies need to be done to determine if lack of PYCARD occurs in any or all of these tumors. As listed in Table 4, PYCARD promoter methylation has been observed in many other cancer types. When combined with the known expression of IL1B in many of these same cancer types, it is possible proIL1B may be present in these cancers as well (Table 7). A subset of the following tumor types have been documented to express IL1B or to have PYCARD promoter methylation. Studies have not been done looking at IL1B expression and PYCARD expression in the same tumors**.** Although this study began with a model "normal" mammary epithelial cell line in which C/EBPbeta2 overexpression upregulates IL1B, it is important to note the upregulation of IL1B in many cancers, including breast cancer, may not necessarily require C/EBPbeta2 overexpression.

Table 7. ProIL1B may be present in multiple tumor types which express IL1B but lack PYCARD.

Cancer	IL1B	PYCARD Promoter	References
breast	Present	Methylated	Jin *et al.*, 1997; Conway *et al.*, 2000
glioblastoma	Present	Methylated	Saidi *et al.*, 2008 ;Stone *et al.*, 2004
ovarian	Present	Methylated	Hefler *et al.*, 2002; Terasawa *et al.*, 2004
melanoma	Present	Methylated	Patel *et al.*, 2002; Guan *et al.*, 2003
small cell lung	??	Methylated	Virmani *et al.*, 2003
non-small cell lung	Present	Methylated	Zienolodiny *et al.*, 2004;;Virmani *et al.*, 2003
colorectal	Present	Methylated	Csiszar *et al.*, 2004;Yokoyama *et al.*, 2003
hepatocellular	Present	Methylated	Bortolami *et al.*, 2008; Zhang *et al.*, 2007

As with most genes, the expression of IL1B is regulated by multiple transcriptional regulatory factors. For example, activated NF-kappaB is known to synergize with C/EBPbeta and also to lead to IL1B expression (Kunsch, Lang et al. 1994). NF-kappaB has been shown to be overactive in a wide variety of cancers and to play a role in the progression of these cancers (Colotta, Saccani et al. 1996). Regardless of how the IL1B gene is transcriptionally activated, future studies to define the role of proIL1B as an intracellular nuclear factor capable of modulating chromatin architecture may provide insight into understanding the metastatic and ErbB-independent phenotypes

of breast and other cancers. It will also be important to determine if nuclear IL1B in breast cancer samples correlates with tumor subtype, stage, and/grade.

It is interesting that nuclear IL1B was detected in roughly half of the ErbB2+++ samples on the tissue microarray although no trend with tumor subtype, stage, and/grade was readily detected. The cancer phenotypes mediated by C/EBPbeta2 overexpression in MCF10A cells are remarkably similar to those that result when ErbB2 is overexpressed/activated in MCF10A cells or immortalized human mammary epithelial (HME) cells. Activation of ERBB2 in MCF10A and HME cells results in anchorage independence, an invasive phenotype, epidermal growth factor independence, and altered architecture in 3D-culture models (Ignatoski, Lapointe et al. 1999; Ignatoski, Maehama et al. 2000; Muthuswamy, Li et al. 2001).

C/EBPbeta2 is likely a key downstream effector of ErbB signaling in MECs. C/EBPbeta2 is known to be downstream of multiple signaling pathways, including those that activate the ERK and RSK kinases (Wegner, Cao et al. 1992; Nakajima, Kinoshita et al. 1993; Buck, Poli et al. 1999; Hanlon, Sturgill et al. 2001; Shuman, Sebastian et al. 2004). The ErbB family of receptor tyrosine kinases in breast cancer often activate ERK and RSK kinases via the Shc- and/or Grb2-activated Ras-Raf-MAPK pathways and phosphatidylinositol-3-kinase (PI-3 K) pathways (Prenzel, Fischer et al. 2001; Yarden and Sliwkowski 2001). C/EBPbeta2 is essential for Ras transformation in multiple cell types (Zhu, Yoon et al. 2002; Wessells, Yakar et al. 2004). For example, C/EBPbeta null mice are completely resistant to carcinogen-induced skin tumors involving mutant Ras (Zhu, Yoon et al. 2002). Taken together these data suggest C/EBPbeta2 is a key

downstream effector of ErbB signaling in MEC. Thus, if C/EBPbeta2 is downstream of ERBB2 the presence of nuclear IL1B in ERBB2 positive tumors may be a reflection of this.

CHAPTER III

C/EBPbeta2 MEDIATED RESISTANCE TO TRASTUZUMAB

Introduction

Although there is a significant improvement in relapse-free survival when patients with ErbB2 overexpressing breast cancer receive trastuzumab, intrinsic and acquired resistance to trastuzumab continue to be a major clinical concern (Piccart-Gebhart, Procter et al. 2005; Romond, Perez et al. 2005; Dahabreh, Linardou et al. 2008). Recent preclinical studies indicate overexpression of ErbB-1 (EGFR) may confer resistance to trastuzumab (Ritter, Perez-Torres et al. 2007; Wang, Xiang et al. 2008; Narayan, Wilken et al. 2009). This is supported by data demonstrating tykerb, which inhibits both ErbB-1 and ErbB-2, synergizes with trastuzumab and has clinical activity in trastuzumab-resistant tumors (Medina and Goodin 2008). This is despite the fact that no significant correlation between ErbB1 expression in ErbB2 positive human breast tumors and resistance to trastuzumab has been detected even though large amounts of clinical data have been analyzed (Gori, Sidoni et al. 2009). This may suggest that ErbB1 can only confer trastuzumab resistance in a context dependent manner and so only a subset of tumors that overexpress ErbB1 are resistant to trastuzumab. This is supported by the fact that tykerb works only in a subset of patients whose tumors co-express ErbB2 and ErbB1 (Paul, Trovato et al. 2008).

Evidence suggests C/EBPbeta2 may be a key downstream effector of ErbB signaling in MECs. Aberrant ErbB signaling in breast cancer results in the sustained

activation of the Ras-Raf-MAPK and PI3K pathways (Prenzel, Fischer et al. 2001; Yarden and Sliwkowski 2001). C/EBPbeta2 is known to be downstream of both of these pathways (Wegner, Cao et al. 1992; Nakajima, Kinoshita et al. 1993; Buck, Poli et al. 1999; Hanlon, Sturgill et al. 2001; Shuman, Sebastian et al. 2004). It has also been demonstrated that C/EBPbeta is necessary for Ras transformation in multiple cell types (Zhu, Yoon et al. 2002; Wessells, Yakar et al. 2004). Importantly for our studies, C/EBPbeta2 confers EGF independence in MCF10A cells which is independent of ErbB signaling (Chapter 2 and (Bundy, Wells et al. 2005)).

Given that MCF10A cells overexpressing C/EBPbeta2 are no longer dependent on ErbB signaling for survival, I sought to investigate whether aberrant C/EBPbeta-2 expression could contribute to the resistance of some breast cancers to ErbB targeted therapies, such as trastuzumab. The data presented in this chapter provides evidence that C/EBPbeta2 can mediate resistance to trastuzumab, albeit via distinct mechanisms in basal and luminal MECs.

Materials and Methods

Cell culture

HCC1954, HCC1569, BT474, SKBR3, and ZR7530 human mammary epithelial cell lines which overexpress ErbB2 due to genomic amplification were already in hand or were obtained from the American Type Culture Collection (ATCC) in Manassas, VA. Cells were grown according to the ATCC recommendations. Briefly, cells were grown in RPMI 1640 with 2.0 nM glutamine (Invitrogen Corp., Grand Island NY) supplemented

with 10% fetal bovine serum (FBS) (Hyclone, Lakewood NJ) and 50 U/ml penicillin, and 50 micrograms/ml of streptomycin.

HCC1954, BT474, SKBR3, and ZR7530 cells overexpressing T7 epitope tagged C/EBPbeta2 were established via a similar method to C/EBPbeta2 overexpressing MCF10A cells. Cells were retrovirally infected with LZRS-his-C/EBPbeta2-GFP or LZRS-GFP in the presense of 8 µg/ml polybrene. At 3-5 hours after infection, growth media was added bringing the polybrene concentration to 4 µg/ml. After 18-20 hours of incubation, the cells were placed in fresh growth media and maintained as detailed above. Fluorescence activated cell sorting (FACS) was then used to generate pure GFP-expressing LZRS-his-C/EBPbeta2-GFP or LZRS-GFP populations. Infected populations were trypsinized and pelleted in media containing 20% FBS at 500 × g for 5 minutes. The cells were then resuspended in RPMI 1640 media containing 0.5% FBS and filtered through a sterile 0.95 micron nylon mesh (Small Parts Inc., Miami Lakes, FL) prior to sorting with a BD FACSAria equipped with FACSDiva software (Becton Dickinson, San Jose, CA). GFP-expressing cells were collected under sterile conditions in FBS media containing 20% FBS, 200 U/ml penicillin and 200 mg/ml streptomycin after which the sorted populations were expanded in cell culture.

Growth Assays

Growth assays on HCC1954 cells were performed in the presence and absence of trastuzumab as previously described (Wang, Chan et al. 2007). Briefly 2 x 10^5 cells were plated in duplicate in 60 mm plates. 24 hours after plating the media was replaced with fresh media with or without 50 µg /mL of trastuzumab. Cells were photographed and

counted 36 hours after treatment. Growth assays on BT474, SKBR3, and ZR7530 cells were performed in a similar manner. 2 x 10^5 cells were plated in duplicate in 60 mm plates. 24 hours after plating the media was replaced with fresh media with or without 50 µg /mL of trastuzumab. The cells were then counted every 2 days. Replating was done as needed.

Whole cell lysates, cell fractionation, and immunoblot analysis

Whole cell extracts were prepared from 100 mm dishes which were 80-90% confluent by scraping into chilled phosphate-buffered saline with 100uM NaVandadate and collected by centrifugation as described previously (Bundy and Sealy 2003). The pellet is then resuspended in saline tris EDTA (STE; 100 mM NaCl, 10 mM Tris pH 7.5, and 1 mM EDTA) with protease and phosphatase inhibitors, as described previously (Bundy and Sealy 2003). Nuclear extracts were also prepared from 80-90% confluent cultures but were scraped into a chilled phosphate-buffered saline containing 1 mM EDTA. After they were collected by centrifugation, the cells were resuspended in 10 volumes 10 mM HEPES pH 8, 0.5M sucrose, 50 mM NaCl, 1mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 7 mM beta-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 µg of leupeptin per liter, 0.1µM pepstatin, 1 ng/ml aprotinin, and phosphatase inhibitors (Buffer A). After vigorous vortexing, nuclei were collected by centrifugation at 1000 X *g* for 5 min. The supernatant (cytoplasmic fraction) was removed from the tube and the nuclei resuspended in an equal amount of buffer A used in the previous step. An equal volume of 2X Laemmli sample butter was added to whole cell lystates, cytoplasmic fractions, and

nuclear fractions and they were boiled for 5 minutes. Protein Assay Reagent (BioRad Laboratores, Hercules, CA, USA) was used as per the manufacturer's instructions to ensure equal amounts of protein were loaded onto a 10% SDS-PAGE. After separation by electrophoresis the proteins were transferred to an Immobilon P filter. The filters were blocked in 5% nonfat dry milk (NFDM) in TBS-T (TBS with 0.1% Tween 20). Primary antibodies anti-ErbB2 (Abcam), anti-IL1B (R&D Systems), anti-TFIID (Santa Cruz), and anti-beta-tubulin (SIGMA) were incubated overnight at 4^{o}C in 0.5% NFDM in TBS-T. After washing, the filters were incubated with the appropriate secondary peroxidase-conjugated antibody for 1 hour at RT in 0.5% NFDM in TBS-T. The immunoblot was then washed in TBS-T and visualized using the SuperSignal West Pico chemiluminescence reagent (Pierce, Rockford, IL, USA) and autoradiography with Kodak X-OMAT film (Rochester, NY, USA).

Genomic Profiling

RNA was submitted to the Vanderbilt Microarray Shared Resource for quality assurance and microarray analysis. In short, after confirming RNA quality, biotinylated complementary RNA was prepared, fragmented, and hybridized to Affymetrix GeneChip U133 PLUS 2.0 arrays. Total RNA was isolated from MCF10A cells stably overexpressing C/EBPbeta2 and parental MCF10A cells using the RNeasy Mini kit and RNase-Free DNase kit (Qiagen). Streptavidin coupled with phycoerythrin was used to detect and visualize hybridized complementary RNA using a GeneChip Scanner 3000 7G Plus 2. GeneChip Operating System (GCOS, Affymetrix, Santa Clara, CA) was used to

grid images and generate .CEL and .CHP files for further analysis. CEL files were imported in GeneSpring 7.0 (Agilent Technologies).

Results

Rationale for selection of cell lines

It has been well documented that breast cancer tumors and cell lines exhibit distinct gene expression profiles which allow for the molecular classification of breast cancer (Perou, Sorlie et al. 2000; van 't Veer, Dai et al. 2002; Sotiriou, Neo et al. 2003; Bertucci, Finetti et al. 2005; Charafe-Jauffret, Ginestier et al. 2006; Hu, Fan et al. 2006). Although several studies have proposed ErbB2 overexpressing tumors form their own molecular subtype, these studies often include a subset of ErbB2 positive tumors that do not cluster with the ErbB2+ subtype (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001; Charafe-Jauffret, Ginestier et al. 2006). In fact, unsupervised clustering can also result in the ErbB2+ cell lines and tumors being scattered throughout the luminal and basal-like subtypes (Figure18) (Neve, Chin et al. 2006). Our initial data demonstrating that C/EBPbeta2 overexpression results in ErbB independence was performed in MCF10A cells. Although MFC10A cells are considered a model of normal MECs they were included in this genomic profiling study performed on breast cancer cells lines and found to cluster with the basal subtype. I hypothesized the molecular division between the basal and luminal subtypes might represent, in addition to other things, the chromatin

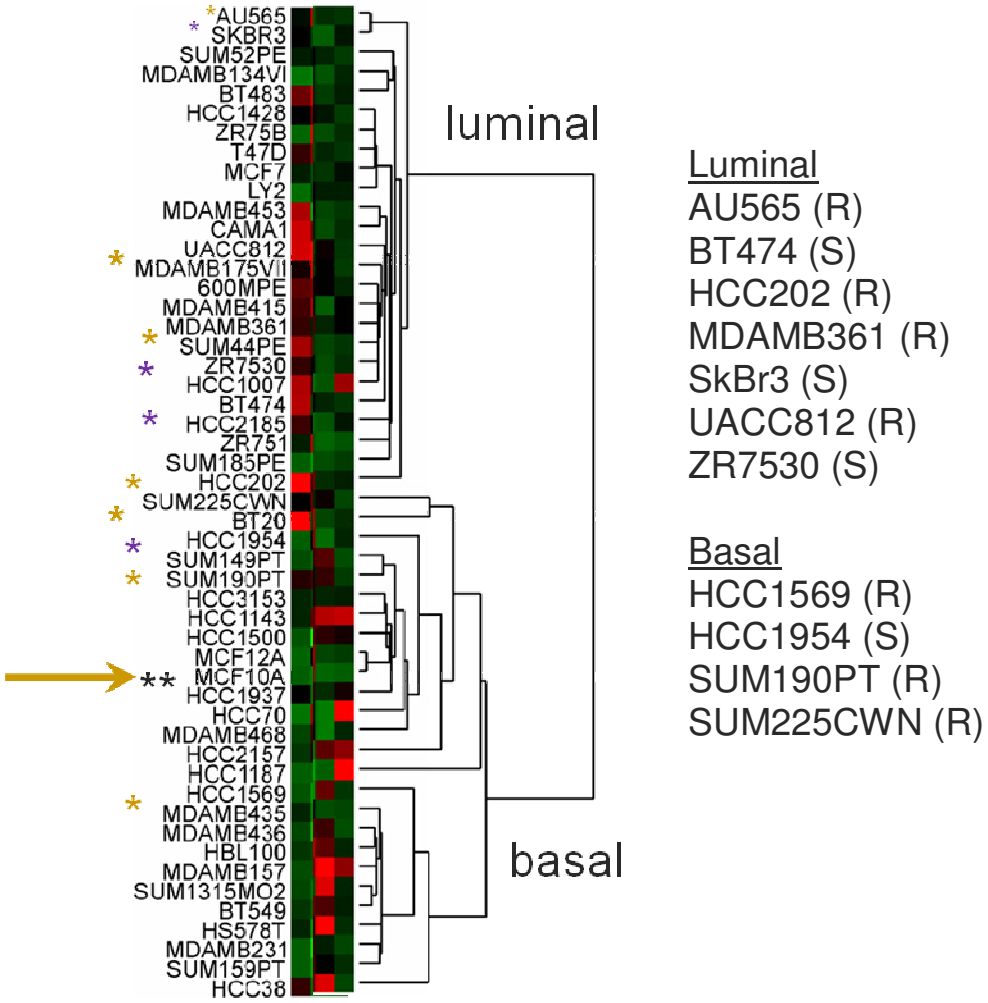


Figure 18. ErbB2+ cell lines are found within both the luminal and basal subtypes. Both trastuzumab resistant (R) and sensitive (S) lines are found with in both subtypes. MCF10A cells are basal (see arrow). Used with permission from Neve, Chin et al. 2006.

Variable	AU565	BT474	HCC202	HCC1569	HCC1954	MCF10A	MDAMB36	SKBR3	SUM190PT	SUM225CV	UACC812	ZR7530
AU565	?	0.929965	0.901094	0.914029	0.922025	0.89403	0.929051	0.968936	0.92979	0.92651	0.931912	0.932033
BT474	0.929965	?	0.920134	0.912901	0.91721	0.884793	0.952995	0.938016	0.928809	0.918115	0.935036	0.943625
HCC202	0.901094	0.920134	?	0.898127	0.899139	0.83438	0.905143	0.919622	0.903388	0.87535	0.885862	0.916043
HCC1569	0.914029	0.912901	0.898127	?	0.929738	0.9083	0.907155	0.922061	0.928217	0.898221	0.904436	0.914922
HCC1954	0.922025	0.91721	0.899139	0.929738	?	0.929759	0.911796	0.926804	0.947424	0.912833	0.907744	0.918281
MCF10A	0.89403	0.884793	0.83438	0.9083	0.929759	?	0.887257	0.890861	0.92422	0.892243	0.888749	0.88535
MDAMB36	0.929051	0.952995	0.905143	0.907155	0.911796	0.887257	?	0.927187	0.928713	0.928335	0.936772	0.94331
SKBR3	0.968936	0.938016	0.919622	0.922061	0.926804	0.890861	0.927187	?	0.927649	0.903248	0.921777	0.936787
SUM190PT	0.92979	0.928809	0.903388	0.928217	0.947424	0.92422	0.928713	0.927649	?	0.93839	0.923617	0.931599
SUM225CV	0.92651	0.918115	0.87535	0.898221	0.912833	0.892243	0.928335	0.903248	0.93839	?	0.928972	0.922276
UACC812	0.931912	0.935036	0.885862	0.904436	0.907744	0.888749	0.936772	0.921777	0.923617	0.928972	?	0.933812
ZR7530	0.932033	0.943625	0.916043	0.914922	0.918281	0.88535	0.94331	0.936787	0.931599	0.922276	0.933812	?

Figure 19. Correlation between ErbB2 overexpressing lines and MCF10A cells. Shown here are the Pearson correlation results calculated using Partek Genomics Suites and the entire genomic profile of each cell line. This chart is formatted so that the highest similarities are shown in blue which fades to yellow as similarity decreases and finally to red for the least similar.

landscape of the cell and play a role in determining the effect of introducing C/EBPbeta2 (or any other transcription factor) into the cells. Partek Genomic Suites was utilized to perform a correlation similarity analysis using the default Pearson (linear) correlation (Figure 19). This analysis compared each cell line to all of the others based on their entire genomic profile, whereas the classification shown in Figure 18 separated the cell lines based on only 305 genes proposed to distinguish between luminal (and its subtypes) and basal. Thus, the Pearson correlations shown in Figure 19 demonstrate a more thorough comparison of the cell lines. Interestingly, the relationships between the lines were very similar, if not identical, to those determined by the 305 proposed molecular subtype classification genes.

It was determined that HCC1954 and HCC1569 cells were similar to MCF10A and would be utilized to determine the effect of exogenous expression of C/EBPbeta2 in a trastuzumab sensitive line (HCC1954) and to compare the levels of endogenous C/EBPbeta2 in basal trastuzumab sensitive (HCC1954) and resistance (HCC1569) lines. BT474 are luminal in nature and are highly correlated with the luminal SKBR3 and ZR530 lines. Thus, these three lines would be used to investigate the effect of exogenous C/EBPbeta2 expression in trastuzumab sensitive luminal breast cancer lines.

Exogenous C/EBPbeta2 results in trastuzumab resistance in basal MECs

To investigate the effect of C/EBPbeta2 on trastuzumab sensitivity in basal breast cancer cells overexpressing ErbB2, a population of C/EBPbeta2 overexpressing

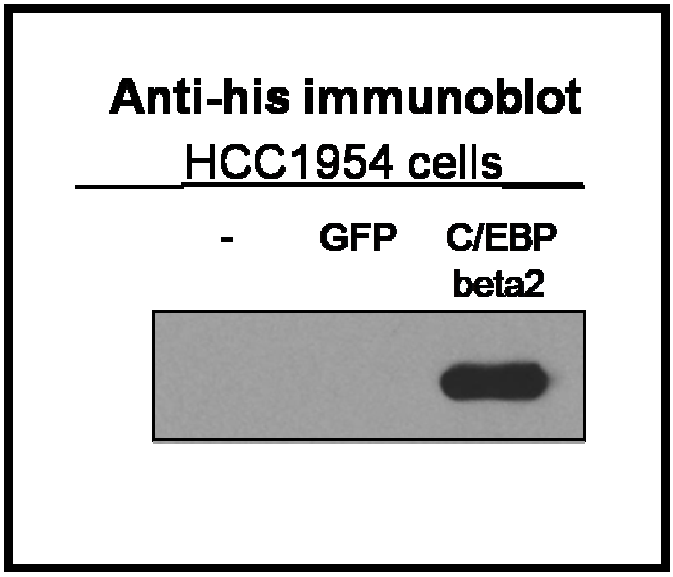


Figure 20. Exogenous expression of C/EBPbeta2 in HCC1954 cells. Equal amounts of protein extracts from HCC1954 cells and HCC1954 cells engineered to express GFP only or C/EBPbeta2-GFP were subjected to immunoblot analysis with anti-T7 (Novagen).

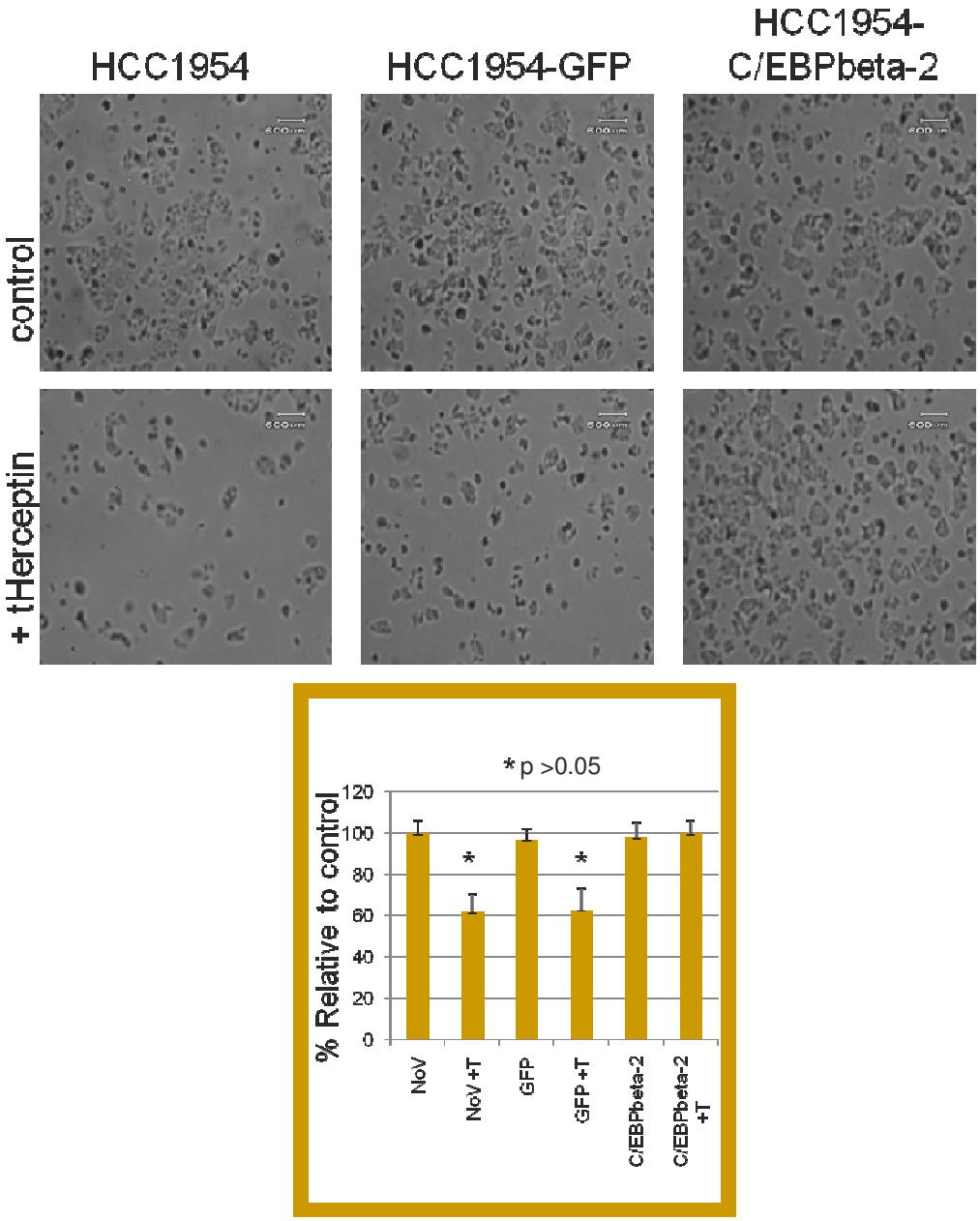


Figure 21. Expression of C/EBPbeta2 in HCC1954 cells results in trastuzumab (Herceptin) resistance. C/EBPbeta2 mediated trastuzumab resistance is demonstrated by photomicrograph (top) and cell number (bottom) 36 hours after treatment.

HCC1954 cells was generated by infection with LZRS-C/EBPbeta2-IRES-GFP (Figure 20). These cells expresses a his-tagged C/EBPbeta2 along with GFP. GFP only HCC1954 cells were also generated and used as a control. Sensitivity to trastuzumab was assayed as previously described (Wang, Chan et al. 2007). Briefly, 2 x 10^5 cells were plated in duplicate in 60 mm plates. 24 hours after plating the media was replaced with fresh media with or without 50 µg /mL of trastuzumab. Cells were photographed and counted 36 hours after treatment (Figure 21). Parental HCC1954 cells and HCC1954 cells engineered to express GFP only are sensitive to trastuzumab as shown by decreased cell number upon trastuzumab treatment. HCC1954 cells engineered to overexpress C/EBPbeta2 are resistant to trastuzumab as evidenced by normal growth in the presence of trastuzumab.

I performed immunoblot analysis to determine if IL1B and IL1R2 were upregulated in the HCC1954 cells upon C/EBPbeta2 overexpression as they were in MCF10A cells. IL1R2 was not altered (Figure 22 and also by real-time PCR data not shown), however, IL1B was upregulated at both the mRNA and the protein level (Figure 23). I observed an absence of the 17kd "active" form of IL1B but the 31 kd proform of IL1B was readily detected. ELISA was performed to determine if IL1B was present in the media of the HCC1954-C/EBPbeta2 cells. No IL1B was detected in the media of these cells. The nuclear localization of proIL1B in HCC1954-C/EBPbeta2 cells was demonstrated using cell fractionation and immunoblot analysis (Figure 24).

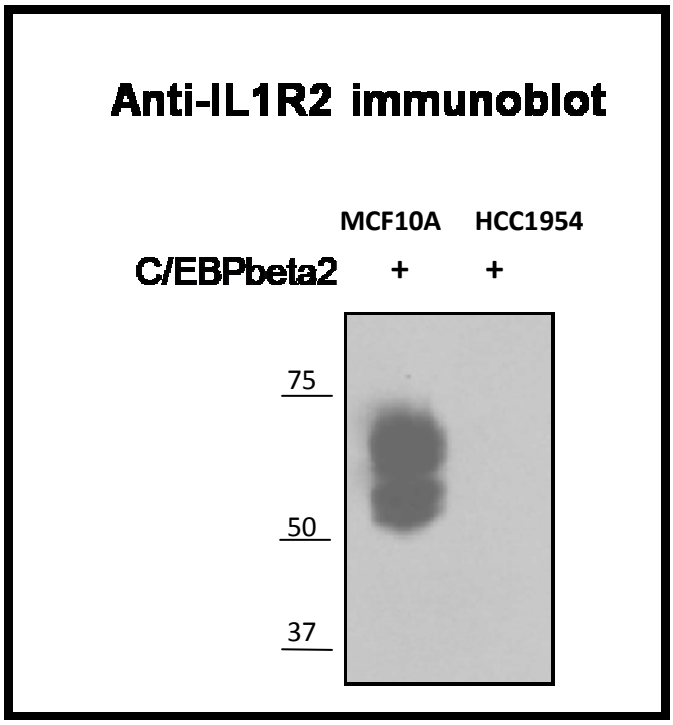


Figure 22. IL1R2 is not upregulated upon C/EBPbeta2 overexpression in HCC1954 cells. Equal amounts of protein extracts from MCF10A-C/EBPbeta2 and HCC1954-C/EBPbeta2 were run on a 10% SDS-PAGE gel and imunoblot analysis performed using anti-IL1R2 (R&D Systems).

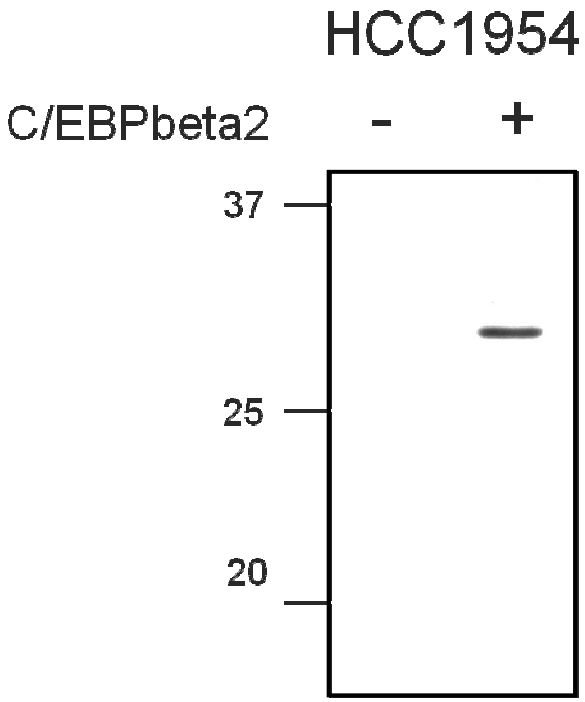


Figure 23. ProIL1B is upregulated at the protein level upon C/EBPbeta2 overexpression in HCC1954 cells. Equal amounts of protein extracts from HCC1954 cells or HCC1954 cells overexpressing C/EBPbeta2 were subjected to immunoblot analysis with anti-IL1B (R&D Systems MAB-201). Note the absence of the 17 kd form of IL1B (which was confirmed using ELISA).

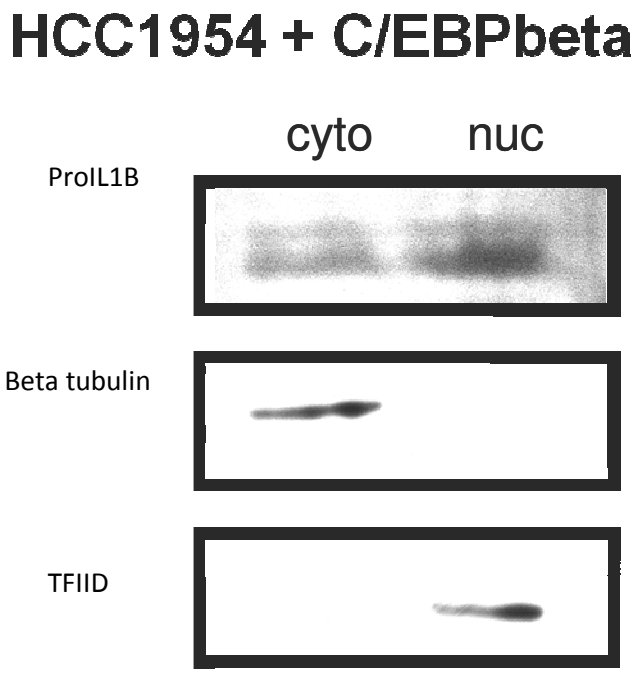


Figure 24. ProIL1B is localized to the nucleus upon C/EBPbeta2 overexpression. Nuclear localization of proIL1B was also assessed by nuclear fractionation followed by immunoblot analysis (top). Efficient cellular fractionation was confirmed using anti-beta tubulin as a cytoplasmic marker (middle) and anti-TFIID as a nuclear specific control (bottom).

Characterization of endogenous C/EBPbeta2 in basal MECs.

The demonstration that exogenous expression of C/EBPbeta in HCC1954 cells results in trastuzumab resistance begs the question: Does endogenous C/EBPbeta2 contribute to trastuzumab resistance in basal ErbB2 overexpressing MECs? In order to address this question, HCC1569 cells were purchased from the ATCC. HCC1569 cells are basal ErbB2 overexpressing cells that are highly resistant to trastuzumab. Their resistance to trastuzumab has recently been shown to be caused by ErbB2/ ErbB3 heterodimerization in the absence of ligand resulting in aberrant activation of downstream signaling, specifically the PI3K pathway (Junttila, Akita et al. 2009). C/EBPbeta2 is activated via phosphorylation on threonine 235 in response to the PI3K and MAPK pathways (Nakajima, Kinoshita et al. 1993; Hanlon, Sturgill et al. 2001). Thus, I hypothesized high levels of activated C/EBPbeta may be present in the trastuzumab resistant HCC1569 cells as compared to the trastuzumab sensitive HCC1954 cells. Previous research had demonstrated that C/EBPbeta2 can be sequestered to the cytoplasm (Eaton, Hanlon et al. 2001). Therefore, I performed nuclear/ cytoplasmic fractionation prior to immunoblotting so that I could look specifically at the active (phosphorylated) C/EBPbeta2 in the nucleus (Figure 25). It is important to note that when phosphorylated on T235, C/EBPbeta2 and the threonine 235 to aspartate phosphomic mutation both run at approximately 60 kd, while unphosphorylated C/EBPbeta2 runs around 44 kd. To our surprise, pT235 C/EBPbeta2 was more abundant in the trastuzumab sensitive line, HCC1954. Therefore, I went on to look at the levels of unphosphorylated C/EBPbeta in the nucleus of these cell lines (Figure 26). The

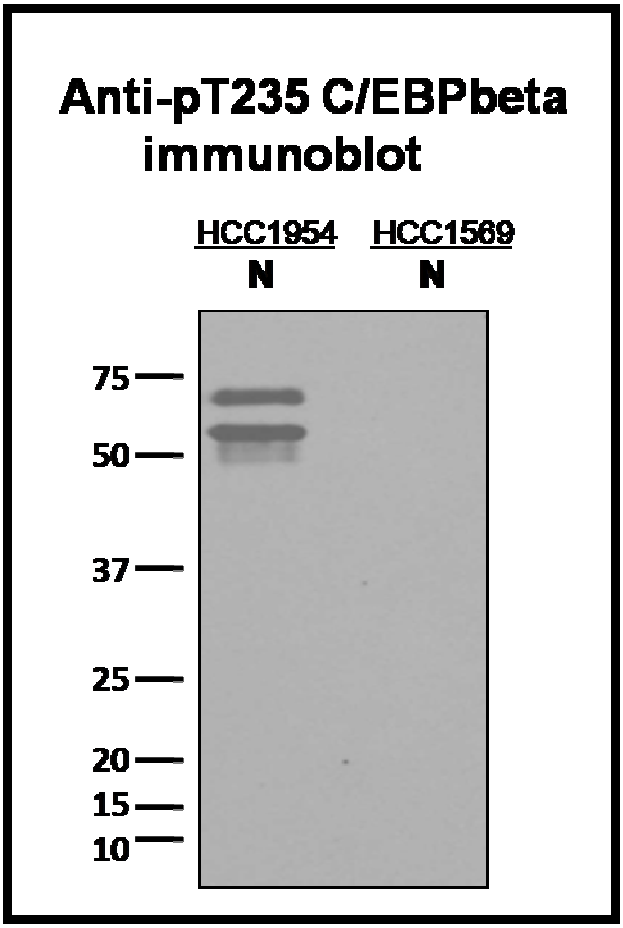


Figure 25. Nuclear phosphorylated C/EBPbeta2 in HCC1954 and HCC1569 cells. Levels of nuclear C/EBPbeta2 phosphorylated on threonine 235 in HCC1954 (trastuzumab sensitive) and HCC1569 (trastuzumab resistant) cells was assessed by cellular fractionation followed by immunoblot analysis.

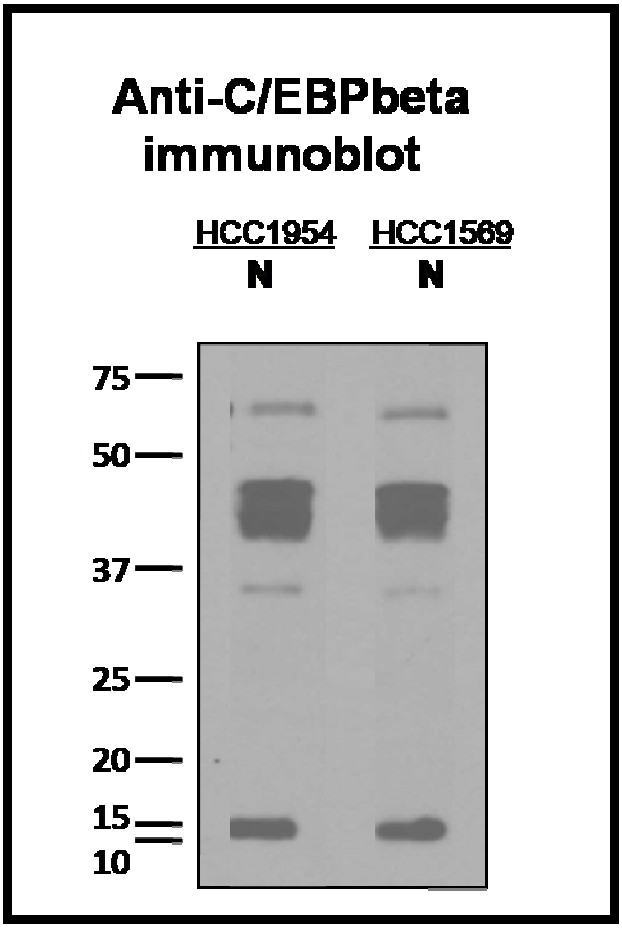


Figure 26. Total nuclear C/EBPbeta2 in HCC1954 and HCC1569 cells. Levels of nuclear C/EBPbeta2 phosphorylated on threonine 235 in HCC1954 and HCC1569 cells was assessed by cellular fractionation followed by immunoblot analysis.

antibody used to do this is a C-terminal antibody that detects C/EBPbeta1, C/EBPbeta2, and C/EBPbeta3 and is purchased from Santa Cruz Biotechnology. This antibody does not detect the C/EBPbeta2 that is phosphorylated on threonine 235. The immunoblot shown in Figure 26 demonstrates that HCC1954 and HCC1569 have roughly equal amounts of unphosphorylated C/EBPbeta2 and both contain C/EBPbeta3 as well.

Exogenous C/EBPbeta2 and trastuzumab resistance in luminal MECs.

To investigate the effect of C/EBPbeta2 on trastuzumab sensitivity in luminal breast cancer cells overexpressing ErbB2, a population of C/EBPbeta2 overexpressing BT474 cells was generated using the LZRS-C/EBPbeta2-IRES-GFP retrovirus (Figure 27). These cells expresses a his tagged C/EBPbeta2 along with GFP. GFP only BT474 cells were also generated and used as a control. BT474 cells were initially chosen because they were luminal, and our correlation studies had confirmed they were dissimilar to MCF10A cells. 2×10^5 cells were plated in duplicate in 60 mm plates. 24 hours after plating the media was replaced with fresh media with or without 50 µg /mL of trastuzumab and the cells were counted every two days. The results demonstrate C/EBPbeta2 results in resistance to trastuzumab in BT474 cells (Figure 28). Unexpectedly, these results also show that C/EBPbeta2 results in the dramatically increased proliferation of BT474 cells. C/EBPbeta2 did not confer resistance to trastuzumab to the ZR7530 of SKBR3 cell lines.

Since the introduction of C/EBPbeta2 into BT474 cells did not result in the upregulation of IL1B at the mRNA or protein level, genomic profiling was performed to investigate the mechanism of C/EBPbeta2 mediated resistance in BT474 cells. This

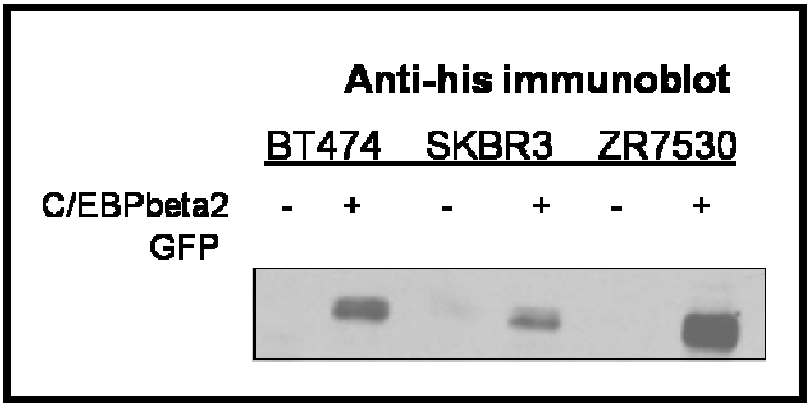


Figure 27. Exogenous expression of C/EBPbeta2 in luminal breast cancer lines. Equal amounts of protein extracts from BT474, SKBR3, and ZR7530 cells with and without C/EBPbeta2-GFP were subjected to immunoblot analysis with anti-T7 (Novagen). All three luminal cell lines express C/EBPbeta2 although to varying levels.

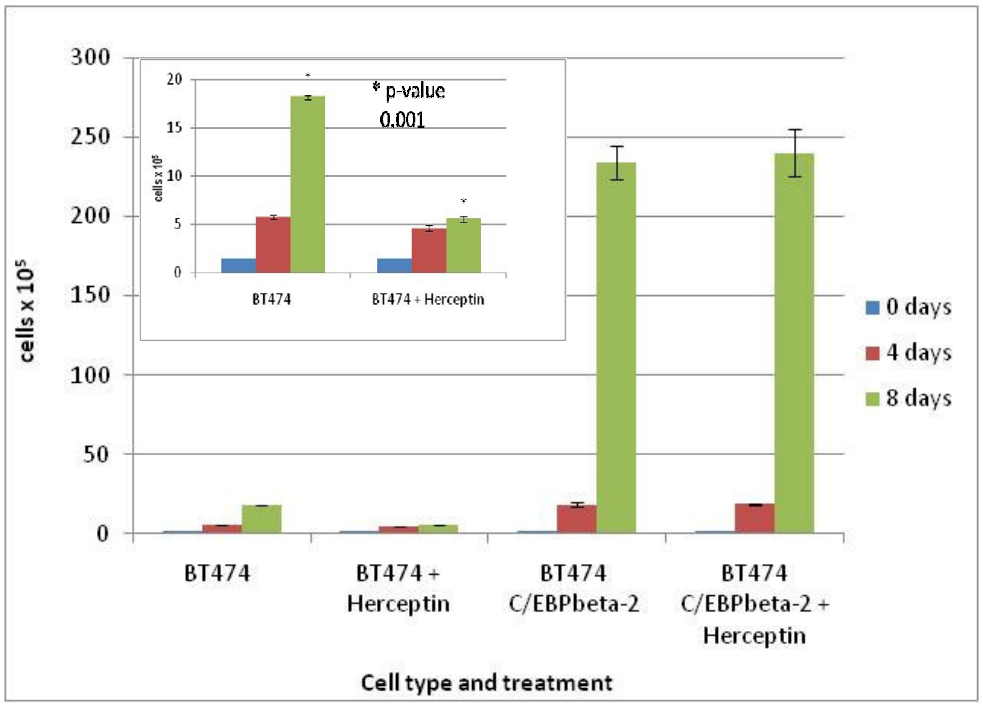


Figure 28. Expression of C/EBPbeta2 in BT474 cells results in trastuzumab resistance and altered growth. C/EBPbeta2 mediated trastuzumab resistance is demonstrated by lack of growth inhibition of BT474 cells when C/EBPbeta2 is exogenously expressed. The insert shows the growth inhibition of parental BT474 cells more clearly since the scale of the larger chart is much greater due to the increased rate of growth of BT474 cells when C/EBPbeta2 is introduced.

confirmed that IL1B expression was not altered upon C/EBPbeta2 introduction into BT474 cells. Once the genomic profiling was completed, it was very clear why the BT474-C/EBPbeta2 cells were resistant to trastuzumab – they had greatly reduced expression of ErbB2. The ErbB2 transcript, which was detected by the Affymetrix probe 216836_s_at, went from a value of 27,189 to 538. The loss of ErbB2 was confirmed at the protein level (Figure 29). Interestingly, genomic profiling also indicated the loss of ErbB1 and ErbB3 which are expressed at low levels in BT474 cells. Thus, I again observed ErbB independent growth mediated by C/EBPbeta2.

Genomic profiling of BT474-C/EBPbeta2 cells indicated ER and PR were also lost. ER/PR/ErbB2 negative tumors are most often categorized by their gene expression profile as basal and patients with this type of tumor have a poor prognosis (Sorlie, Perou et al. 2001; Charafe-Jauffret, Ginestier et al. 2006). As a consequence of this observation I investigated whether C/EBPbeta2 had caused the luminal BT474 line to become basal in nature. Basal markers were not upregulated in the BT474 cells (Table 8).

Table 8. Basal markers in BT474 and BT474-C/EBPbeta2 cells.

Basal Marker	**BT474**	**BT474 + C/EBPbeta-2**
Cytokeratin 5	Absent	Absent
Cytokeratin 14	Absent	Absent
Cytokeratin 17	Absent	Absent
Cytokeratin 4	Absent	Absent
Cytokeratin 16	Absent	Absent
p63	Present	Absent
ErbB1	Present	Absent

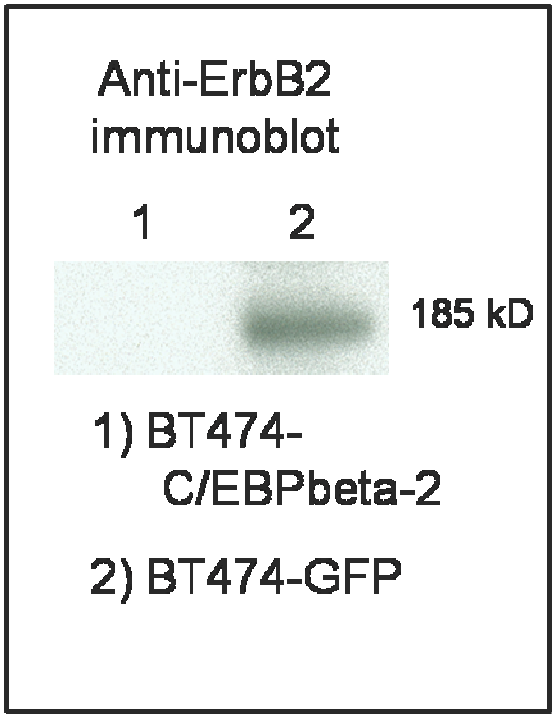


Figure 29. ErbB2 is lost in BT474 cells upon C/EBPbeta2 expression. Equal amounts of protein extracts from BT474-C/EBPbeta2 cells or BT474-GFP cells were subjected to immunoblot analysis with anti-ErbB2.

While studying the genomic profiling data, I noticed that lin-28 homolog B (LIN28B) was upregulated in the C/EBPbeta2 overexpressing BT474 cells. LIN28B is found in stem cells but is lost as differentiation occurs (Yang and Moss 2003; Darr and Benvenisty 2009). LIN28B has recently been found in a subset of breast, lung, colon, cervical, and hepatocellular cancers (Guo, Chen et al. 2006; Viswanathan, Powers et al. 2009). Consequently, I set out to determine if C/EBPbeta2 caused the BT474 cells to develop a more stem-cell like gene profile. In order to do this I looked at 21 previously described mammary stem cell markers (Dontu, Abdallah et al. 2003). As shown in Figure 30, BT474-C/EBPbeta2 cells have a more stem cell-like genotype than the parental BT474 cells.

This trastuzumab resistance and induction of a stem cell-like genotype was demonstrated twice in BT474 cells upon C/EBPbeta2 expression, but was not observed in later populations of BT474-C/EBPbeta2 cells nor SKBR3-C/EBPbeta2 or ZR7530-C/EBPbeta2 cells. To the best of our knowledge all variables, other than the FBS lot, remained the same. Thus, the phenotypes described above may be dependent on a yet to be elucidated component that is present in some batches of FBS. Efficient expression of C/EBPbeta2 in the luminal MECs listed is shown in Figure 27.

Discussion

C/EBPbeta2 overexpression may result in trastuzumab resistance in both basal and luminal MECs. The presence of nuclear proIL1B in HCC1954 (basal) cells upon overexpression of C/EBPbeta2 indicates C/EBPbeta2 mediated trastuzumab resistance

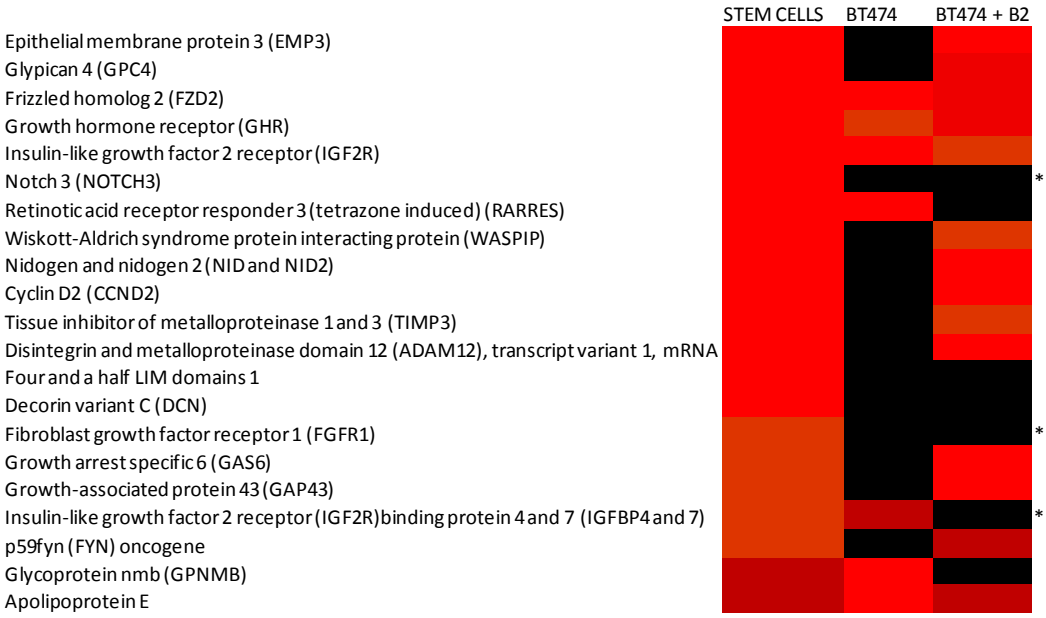


Figure 30. C/EBPbeta2 results in a more stem cell-like genotype in BT474 cells. Parental BT474 cells express 7 of 21 previously described mammary stem cell markers. Upon introduction of C/EBPbeta2 this number rises to 14/21 or 17/21 if induction of close family members is included.

may occur via the same mechanism seen (but not fully understood) in MCF10A cells. This mechanism likely involves nuclear proIL1B. Multiple attempts to knock-down IL1B in HCC1954 cells have been conducted, however, none have been successful to date. IL1B is not detected in the HCC1569 cells (basal ErbB2 overexpressing cells resistant to trastuzumab) by real-time PCR or immunoblot analysis. This may indicate that the HCC1569 cells did not acquire trastuzumab resistance via C/EBPbeta2 or that C/EBPbeta2 may mediate resistance via multiple pathways some of which do not require nuclear proIL1B.

The involvement of C/EBPbeta2 in luminal MECs may be more complex. Under some situations C/EBPbeta2 may actually lead to reduced expression of ErbB2 and a stem cell-like genotype in luminal MECs. This is supported by recent data demonstrating that C/EBPbeta regulates stem cell maintenance and differentiation (LaMarca, et al., 2010). Thus, overexpression of C/EBPbeta2 in a subset of cells within an ErbB2 positive breast tumor could give rise to a resistant population that was able to survive trastuzumab treatment and result in relapse. The ability of C/EBPbeta2 to generate a stem cell-like genotype is dependent on some yet to be elucidated factor. This factor may or may not be present in the tumor microenviroment. As more becomes known about the regulation of stem and cancer stem cells, the factor(s) important for C/EBPbeta2's ability to cause a stem cell-like genotype may become apparent. At that point, further studies regarding C/EBPbeta2 involvement in trastuzumab resistance via reduction of ErbB2 and acquisition of a stem cell-like genotype should be performed.

Due to a variety of factors it is very difficult to determine if endogenous C/EBPbeta2 correlates with trastuzumab resistance. One problem is that there are

relatively few ErbB2 positive cell lines available for study and ErbB resistance in the available cell lines may arise from multiple different alterations. However, even if there was only one pathway to resistance and it was through C/EBPbeta2 this would still be difficult to determine. Our studies looking at endogenous C/EBPbeta2 and trastuzumab resistance relied on the premise that nuclear C/EBPbeta2 phosphorylated on threonine 235 is the active form of C/EBPbeta2. Based on previous work from our lab and others this was a reasonable hypothesis at the time (Nakajima, Kinoshita et al. 1993; Hanlon and Sealy 1999). Recent work in the lab carried out by Rachel Jerrell and Linda Sealy, however, now indicates that pT235 C/EBPbeta2 detected via immunoblot analysis may not be the active form of C/EBPbeta2. This is based on data generated using a threonine 235 to aspartate phosphomimic (T-D mutant). This mutant, like pT235 C/EBPbeta2, is shifted to a mobility of around 60 kd (whereas unphosphorylated runs around 44 kd). Recent data has demonstrated the shift of the T-D mutant is due to the modification of lysine 286, and that this shifted form of C/BEPbeta2 is transcriptionally inactive. The exact modification is currently unknown. Thus, if the endogenous C/EBPbeta2 detected by the pT235 antibody is similarly modified on lysine 286, it is likely transcriptionally inactive. The active form of pT235 C/EBPbeta2 may not be detected by immunoblot analysis due to isomerization by peptidyl-prolyl isomerase, NIMA-interacting 1 (pin1), and subsequent degradation. Pin1 is a peptidylprolyl cis-trans isomerase which specifically isomerizes proline if it is directly after a phosphorylated threonine or serine (Zhou, Lu et al. 1999). Rachel Jerrell and Linda Sealy have also demonstrated pin1 isomerization of C/EBPbeta2 results in high transcriptional activity but also in its loss at

the protein level. Hence, maximal activation for C/EBPbeta2 may be dependent on pin1 and linked to its rapid degradation.

This exciting line of research needs further investigation and validation. One approach would be to inhibit pin1 activity and then monitor C/EBPbeta2 expression using the pT235 antibody. Inhibition of pin1 by 5-hydroxy-1,4-naphthoquinone, also called juglone, has been demonstrated (Hennig, Christner et al. 1998). The problem is that inhibition of pin1 results in changes to the function and stability of a large number of proteins making the results hard to interpret. This is especially true in our case since pin1 has been shown to be important for the stability of both ErbB2 and AKT (Lam, Burga et al. 2008; Liao, Wei et al. 2009). In fact, siRNA mediated knock-down of pin1 resulted in reduced levels of ErbB2 due to degradation leading to growth inhibition in BT474 and SKBR3 cells (Lam, Burga et al. 2008). Loss of ErbB2 and AKT, one of its downstream signaling components, could greatly alter the level of C/EBPbeta2 phosphorylation. This loss of C/EBPbeta2 phosphorylation could in turn obscure the accumulation of pT235 C/EBPbeta2 that I might expect if pin1-mediated isomerization of C/EBPbeta2 is prevented. To truly understand the effects of pin1 on C/EBPbeta2 it may be necessary to have a mutated C/EBPbeta2 which is resistant to pin1 isomerization. The site of pin1 isomerization in unknown and C/EBPbeta2 has multiple thereonine-proline and serine-proline motifs where pin1 may act. Consequently, determining the site of pin1 isomerization is not a trivial task. In addition, this approach still would not allow us to look directly at the endogenous active C/EBPbeta2 levels in ErbB2 positive breast cancer cell lines.

Since directly assessing the level of active C/EBPbeta2 is a difficult task, I hypothesized it might be interesting to use nuclear proIL1B as a surrogate for C/EBPbeta2 activity. Specifically I was interested in the basal ErbB2 positive cell lines that were resistant to trastuzumab. The HCC1569 cells were the only commercially available cell line that fit this description, and they do not express IL1B at the mRNA or protein level (data not shown). SUM190PT cells were requested from the Ethier lab but the requests were never answered. None of the luminal ErbB2 overexpressing cells studied expressed IL1B at the mRNA or protein level. Thus, looking for nuclear proIL1B in ErbB2 positive breast cancers may be necessary to determine if proIL1B occurs in these cells and if it correlates with trastuzumab resistance. The immunohistochemistry necessary for this experiment is described in chapter 1, along with examples of breast cancer samples containing nuclear IL1B.

CHAPTER IV

SUMMARY AND DISCUSSION

Summary

This work demonstrates for the first time that IL1B is a dual function cytokine and provides insight into the role of proIL1B in breast cancer (Figure 31). While the ErbB family of receptors play an important role in normal mammary gland development, aberrant ErbB signaling is observed in early stages of breast cancer and contributes to cancer progression (Jackson-Fisher, Bellinger et al. 2004; Andrechek, White et al. 2005). Overexpression of ErbB2 is associated with aggressive tumors and poor prognosis (Paik, Hazan et al. 1990; Press, Bernstein et al. 1997). This is likely due in large part to the activation of C/EBPbeta2 which is known to be downstream of ERK and RSK kinases which are activated by the ErbB family of receptor tyrosine kinases via the Shc- and/or Grb2-activated Ras-Raf-MAPK pathways and phosphatidylinositol-3-kinase (PI3 K) pathways (Wegner, Cao et al. 1992; Nakajima, Kinoshita et al. 1993; Buck, Poli et al. 1999; Hanlon, Sturgill et al. 2001; Shuman, Sebastian et al. 2004, Fischer et al. 2001; Yarden and Sliwkowski 2001). C/EBPbeta2 activation leads to the expression of known genes associated with EMT and to the upregulation of IL1B. The proIL1B produced is not cleaved but instead translocates to the nucleus where it tightly associates with the chromatin and likely alters the chromatin landscape allowing for expression of key genes needed for the metastatic cascade and perhaps ErbB independence.

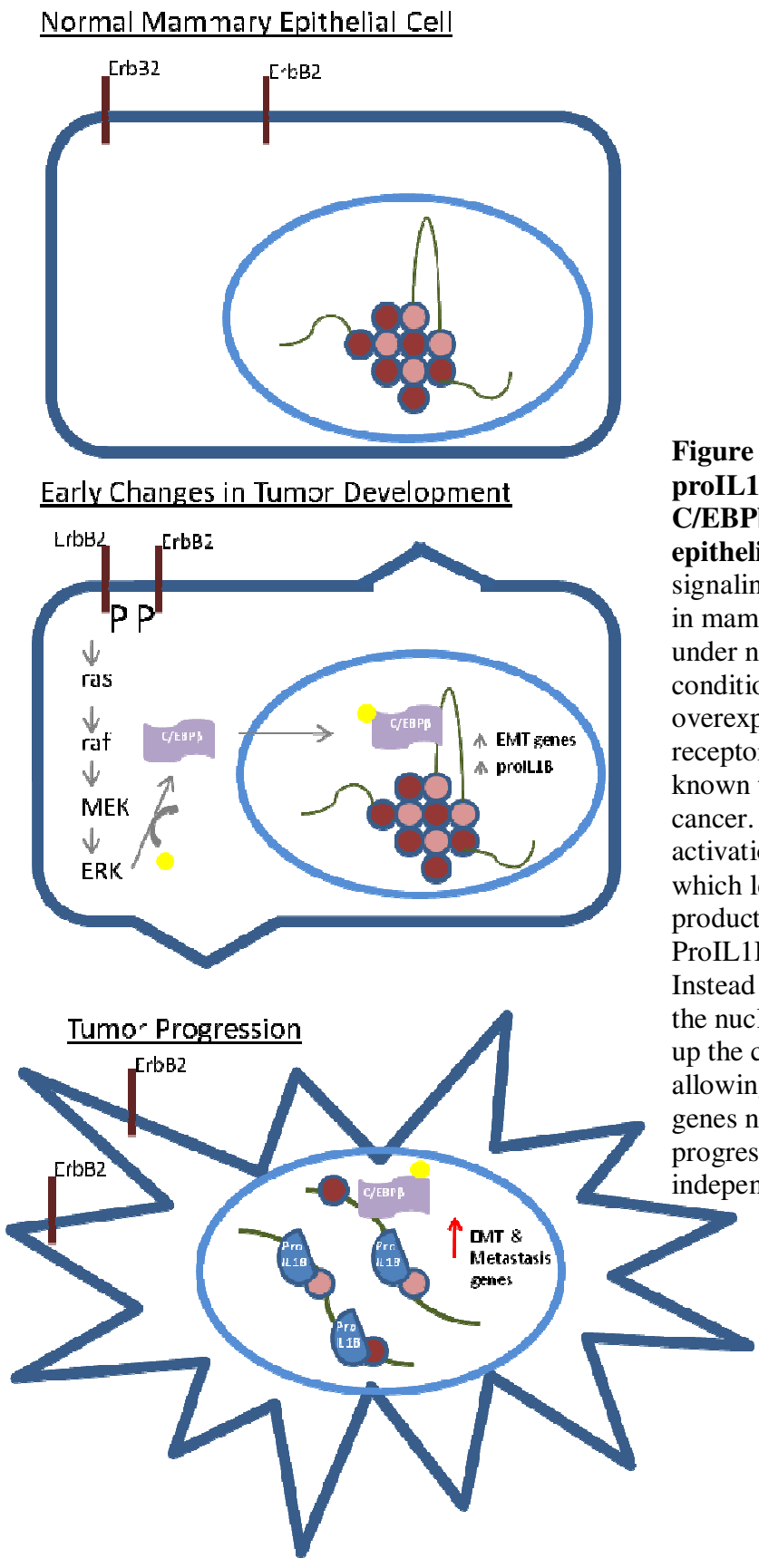


Figure 31. Effect of proIL1B upregulation by C/EBPbeta2 in mammary epithelial cells. ErbB signaling is tightly regulated in mammary epithelial cells under normal growth conditions. However, overexpression of the ErbB receptors or their ligands is known to occur in breast cancer. This results in activation of C/EBPbeta2 which leads to the production of proIL1B. ProIL1B is not cleaved. Instead it is translocated to the nucleus where it opens up the chromatin structure, allowing the expression of genes needed for metastatic progression and ErbB independence.

Prior to this work it was known that C/EBPbeta2 is not detected in normal mammary tissue but is highly expressed in 70% of invasive mammary carcinomas (Eaton, Hanlon et al. 2001). It was also known that C/EBPbeta2 overexpression in MCF10A cells, an immortalized but not transformed mammary epithelial cell line, results in anchorage independence, an invasive phenotype, epidermal growth factor (EGF) independence, altered acinar architecture in 3D-culture models, and epithelial to mesenchymal transition (EMT) (Bundy and Sealy 2003; Bundy, Wells et al. 2005).

Here I demonstrate that overexpression of C/EBPbeta2 in MCF10A cells results in the upregulation of IL1B mRNA and expression of proIL1B at the protein level (Figure 4 and Figure 6). Interestingly, indirect immunofluorescent staining and cellular fractionation showed the proIL1B is present in both the cytoplasm and nucleus (Figure 7). I went on to demonstrate, using nuclear extraction in buffers of increasing ionic strength, that proIL1B is tightly associated with the chromatin (Figure 8). Impressively, proIL1B is not extracted even at 1 M NaCl but remains associated with the chromatin (Figure 9). Genomic and bioinformatic approaches were then used to begin to address the role of nuclear proIL1B in MCF10A-C/EBPbeta2 cells. ChIP-chip data indicated proIL1B binds 204 distinct, non-random locations along the chromosome (Figure 9 and Table 4). Bioinformatic analysis revealed proIL1B is positioned in such a way as to contribute to multiple cancer connected pathways (Table 5). In fact, overexpression of C/EBPbeta2 in HCC1954 cells resulted in nuclear proIL1B and resistance to Herceptin (Figure 21 and Figure 24). A pilot study using immunohistochemistry to look at IL1B in a tissue array of breast cancers, presented in Chapter 2, was performed and nuclear IL1B was demonstrated in a subset of breast cancers. These studies show that nuclear IL1B is

present in human mammary carcinoma. Taken together our findings demonstrate IL1B has a nuclear role in its proform and that nuclear IL1B is found in human breast cancer.

Discussion and Future Directions

Mechanism of proIL1B chromatin interaction

Previous studies of IL1B have always focused on the mature, secreted protein due to the belief that proIL1B is biologically inactive (Mosley, Urdal et al. 1987). For example, mature IL1B increases tumor invasiveness and metastasis by modulating cell adhesion and angiogenesis (Suswam, Nabors et al. 2005; Apte and Voronov 2008). Although IL1B is considered to be inactive in the proform, the highly related protein interleukin 1alpha (IL1A) is active in both the mature form and the proform. ProIL1A can be found in the nucleus and has been shown to modulate chromatin structure and interact with several transcription factors (Maier, Statuto et al. 1994; McMahon, Garfinkel et al. 1997; Hu, Wang et al. 2003; Pollock, Turck et al. 2003; Werman, Werman-Venkert et al. 2004; Kawaguchi, Nishimagi et al. 2006; Cheng, Shivshankar et al. 2008). Another member of the interleukin 1 family, IL33, has been shown to localize to the nucleus where it also modifies chromatin structure by interacting with the H2A-H2B dimer; this is in addition to its role as a secreted cytokine (Roussel, Erard et al. 2008). Nuclear HMGB1, the first recognized dual cytokine, binds in a non sequence dependent manner to DNA inducing a ninety degree bend and allowing access to a variety of transcription factors (Agresti and Bianchi 2003). Thus, all of these dual function cytokines mediate changes in the chromatin landscape resulting in increased or

decreased transcription. ProIL1B is very tightly associated with the chromatin but the details of how this cytokine binds to the chromatin and exerts its effects are still obscure and warrant further research.

Comparison of proIL1B structure with these known dual function cytokines provides insight into the possible mechanism of proIL1B chromatin association. In the mature, cleaved form both IL1A and IL1B bind to IL1R1 with similar affinities and lead to similar biological outcomes (Rupp, Cameron et al., 1986). In addition, both are translated as precursor molecules which lack a classical peptide secretion sequence (271 and 269 amino acids respectively). ProIL1B is processed by the IL1B-converting enzyme (ICE, also called caspase-1), while proIL1A is cleaved by calpain (Apte and Voronov 2008). At the protein level there is less than 30% sequence homology between IL1A and IL1B, with most of this homology residing in the carboxy-terminal half of the precursor (or IL1R1 binding region) which contains a six-stranded barrel with three hairpins that cap one end of the barrel (Finzel, Clancy et al., 1989). This beta-trefoil structure is a defining characteristic of IL1 family cytokines and is also seen in IL33 (Schmitz, Owayang et al, 2005). However, the literature indicates that proIL1A intranuclear actions are dependent on the pro-piece not the conserved beta-trefoil region ((Maier, Statuto et al. 1994; McMahon, Garfinkel et al. 1997; Hu, Wang et al. 2003; Pollock, Turck et al. 2003; Werman, Werman-Venkert et al. 2004; Kawaguchi, Nishimagi et al. 2006; Cheng, Shivshankar et al. 2008). This indicates that although the pro-piece of IL1B may play a role in chromatin association that it likely interacts with distinct proteins from nuclear proIL1A. In fact, I believe that proIL1B chromatin association is likely dependent on the pro-piece. The IL1 family member, IL33,

associates with the chromatin via the H2A-H2B acidic pocket resulting in increased chromatin compaction and transcriptional repression (Carriere, Roussel et al. 2007; Roussel, Erard et al. 2008). This interaction was shown to be dependent on amino acids 40-58 which is in the pro-piece of IL33 (Roussel, Erard et al. 2008). However, the IL33 CBM, $IL33_{40-58}$, is not well conserved (4 out of 9 aa) in IL1B (or IL1A whose mechanism of chromatin interaction is also not well established). In addition, IL1B is not related to HMGB1. HMGB1 has been shown to bind DNA using side by side DNA-binding regions called HMG box domains and acidic tails. These bind to the minor grove of DNA in a sequence independent manner (Agresti and Bianchi 2003). In support of this, recent studies investigating the structure of IL1B confirm that there are no HMB boxes present in proIL1B (Hailey, Li et al., 2009).

Although the proIL1B is unlikely to interact with the same proteins as proIL1A, it is possible that the same approaches used to identify proIL1A interactions can be employed to elucidate proIL1B binding partners. Immunoprecipitation has been used by three separate groups to identify nuclear proIL1A binding partners (Hu, Wang et al., 2003; Buryskova, Pospisek et al., 2004; Kawaguchi, Nishimagi et al., 2006). Hu et al. demonstrated that proIL1A interacts with necdin in fibroblasts from systemic sclerosis patients (Hu, Wang et al., 2003). When not bound to proIL1A, necedin inhibits cell growth via interactions with DNA. ProIL1A likely prevents the direct binding of necedin to DNA or other transcription factors (Hu, Wang et al., 2003). Buryskova et al. showed in HEK293 cells that nuclear proIL1A interacts with histone acetyltransferase complexes resulting in increased transcriptional activity and proinflammatory excitability (Buryskova, Pospisek et al., 2004). Finally, Kawaguchi et al. found that proIL1A in

systemic sclerosis interacts with HS1-associated protein X-1 (HAX1) and IL1R2 and that disrupting the interaction with HAX1 resulted in loss of nuclear localization and the loss of IL6 and procollagen type I production (which are characteristic of the disease) (Kawaguchi, Nishimagi et al., 2006).

In order to determine potential proIL1B binding proteins, one could use nuclear extracts from MCF10A-C/EBPbeta2 cells and polyclonal anti-IL1B (R&D Systems AF-201-NA) to perform co-immunoprecipitation. Co-immunoprecipitation using nuclear extracts from MCF10A cells will be performed to control for non-specific binding. Since only proIL1B is found in the nucleus of the cells all immunoprecipitated IL1B will be proIL1B. The resulting protein mix would be run on a SDS-PAGE which will then be stained with colloidal commassie. Matrix assisted Laser Desorption Ionisation - Mass Spectrometry – Mass Spectrometry (MALDI-MS-MS) would then be performed in collaboration with the Vanderbilt Mass Spectrometry Core. Ten serial bands will be cut from the polyacrylamide gel to cover all proteins present. Prior to tandem mass spectrometry tryptic digestion will be carried out directly on the resulting polyacrylamide gel slices. After MALDI-MS-MS bioinformatic analysis will be utilized to identify the proteins present from MS-MS spectra databases using Mascot. Immunoblot analysis will confirm the identity of key proteins identified using this method.

I anticipate the combination of co-immunoprecipitation (co-IP) and mass spectrophotometry will lead to the identification of core histones or other chromatin associated proteins. It is likely that this will confirm that proIL1B, like proIL1A, is able to interaction with proteins in such a way as to open up the chromatin and allow for increased expression of specific genes. This is based on the fact that proIL1B is bound to

the chromatin in locations statistically related to genes whose expression is important for known cancer pathways and metastatic progression (Tables 5 and 6). Thus, proIL1B may enable cancer cells to express the needed genes for continued tumor progression. Co-IP may also reveal specific transcription factors that interact with proIL1B. Combining ChIP-chip analysis of proIL1B binding sites with Affymetrix expression profiling of MCF10A-C/EBPbeta2 cells, I can conclude that the sites of proIL1Beta binding are not closely associated with the promoters of the 443 genes found to be differentially expressed upon C/EBPbeta2 overexpression. In addition, when IP of C/EBPbeta2 was performed by Rachel Jerrell no proIL1B was detected. Taken together these data indicate that proIL1B and C/EBPbeta2 do not directly interact. TFSEARCH 1.3 and MatInspector 8.0, software designed to identify transcription factor binding sites, were used to interrogate the DNA sequences associated with proIL1B binding sites. No transcription factor was identified by these programs to bind a significant percent of these sites. However, computer algorithms are notoriously unreliable at correctly identifying weak binding sites. Our lab has seen experimentally that multiple weak sites can result in higher affinity and binding of transcription factors than a single consensus site. So it is possible a single, or small set of, transcription factor(s) is associated with the majority of the proIL1B binding sites and is responsible for the nuclear localization of proIL1B in our system. The identification of these proteins will give insight into the mechanism by which proIL1B contributes to the cancer phenotypes of MCF10A-C/EBPbeta2 cells and human breast cancer.

Function of nuclear proIL1B

Knock-down of IL1B was attempted (detailed in Chapter II) and would have allowed us to investigate the role of nuclear proIL1B. Unfortunately, said knock-down was never achieved despite many shRNA and siRNA mediated attempts. However, a key caveat is that IL1B knockdown would result in the loss of both nuclear and cytoplasmic IL1B, rendering it impossible to determine if the effects were due to nuclear IL1B specifically. One way to investigate the activity of nuclear proIL1B would be to cause its cleavage so that it is no longer found in the nucleus due to secretion from the cells.

Accumulation of proIL1B in our system is likely due to the absence of inflammasome activation. As discussed in the introduction and multiple recent reviews, cleavage of proIL1B requires activation of caspase-1 and the activation of caspase-1 is dependent on the assembly of a macromolecular complex called the inflammasome (Mosley, Urdal et al. 1987; Petrilli, Papin et al. 2005; Ogura, Sutterwala et al. 2006; Petrilli, Dostert et al. 2007; Netea, van de Veerdonk et al. 2008; Franchi, Eigenbrod et al. 2009; Martinon, Mayor et al. 2009). The status of the inflammasome has not been well studied in MECs. Review of our Affymetrix expression data led us to determine that negative regulators of inflammasome activity are not upregulated upon C/EBPbeta2 overexpression in MCF10A cells. Instead I find that key components of the inflammasome itself are not expressed at significant (mRNA) levels. They are NALP1, NALP3, CARDINAL, caspase-5, and PYCARD. Absence of these proteins could prevent the formation of the inflammasome and thus cleavage of proIL1B. PYCARD is of much interest because it plays an important role in all forms of the inflammasome

identified to date and it is known to be silenced in a high percentage of breast cancers (Conway, McConnell et al. 2000; Levine, Stimson-Crider et al. 2003).

It is possible that forced expression of PYCARD in the MCF10A-C/EBPbeta2 cells could result in the processing of proIL1B into the mature form. MCF10A cells and MCF10A-C/EBPbeta2 cells could be retrovirally infected with PYCARD-IRES-GFP or GFP alone. Infected cells will be selected using fluorescence activated cell sorting. Immunoblot analysis will then be utilized to confirm PYCARD expression in the MCF10A-PYCARD and MCF10A-C/EBPbeta2-PYCARD cells. Once PYCARD expression is confirmed, cell fractionation studies in combination with immunoblot analysis will be used to assess the level and localization of proIL1B remaining in the MCF10A-C/EBPbeta2- PYCARD cells. Once decreased nuclear proIL1B has been confirmed, these cells will be used to investigate the role of proIL1B on the cancer phenotypes observed in MCF10A-C/EBPbeta2 cells. Thus, it might be possible to ascertain the role of proIL1B in the EMT and other cancer phenotypes observed in MCF10A cells upon overexpression of C/EBPbeta2.

Alternatively, mutation of proIL1B and/or its binding partners could be used to investigate the not only the molecular mechanism of their interactions but also the function of proIL1B itself. A series of truncation mutations could be generated and the effect on chromatin association and interaction with binding partners could be assessed using nuclear fractionation and co-IP respectively. When used to study proIL1A interactions these approaches demonstrated that the N-terminal pro-piece of proIL1A was necessary for interaction with necdin and that lack of the pro-piece resulted in lack of nuclear localization (Hu, Wang et al., 2003). Loss of the proIL1A binding partners,

HAX1 and/or IL1R2, via shRNA mediated knock-down resulted in loss of nuclear localization of proIL1A and the absence of expression of key genes related to the systemic sclerosis phenotype (Kawaguchi, Nishimagi et al., 2006). Since proIL1B lacks a known nuclear localization signal it is likely its nuclear localization is dependent on a binding partner (Grenfell, Smithers et al. 1991). It is known that mature (cleaved) IL1B can translocate to the nucleus with the receptor after IL1R1 mediated endocytosis. This translocation is believed to be receptor dependent as mutation of the NLS did not affect IL1B nuclear localization in this system (Grenfell, Smithers et al. 1991). It is very unlikely that interaction with IL1R2 is responsible for the localization of proIL1B to the nucleus in our studies, since the proform of IL1B does not bind to IL1R1 or IL1R2 (Mosley, Urdal et al. 1987). In addition, no nuclear IL1R2 was observed in the MCF10A-C/EBPbeta2 cells using indirect immunofluorescence or cellular fractionation techniques. As a key effector of C/EBPbeta2 in MECs it is likely that nuclear proIL1B is responsible for some or all of the cancer phenotypes observed in MCF10A-C/EBPbeta2 cells and that disrupting nuclear location or interaction with binding partners as described above will result in a more normal phenotype. This is supported by the association of proIL1B binding sites in the genome with multiple pathways known to be associated with cancer.

Nuclear IL1B in breast and other cancer samples

Based on the literature it seemed reasonable that nuclear IL1B would be present in a subset of breast cancers. PYCARD, an important component of all studied inflammasome complexes, is silenced due to extensive promoter methylation in nearly

half of breast cancer cell lines and over a third of human breast tumors (Conway, McConnell et al. 2000; Levine, Stimson-Crider et al. 2003). IL1B is present in over 90% of invasive breast carcinomas by ELISA (Jin, Yuan et al. 1997). Thus, it is reasonable to expect that a subset of these breast cancers lack PYCARD expression due to promoter methylation, and therefore IL1B may be present in the proform. In fact, immunohistochemical analysis detected nuclear IL1B in human breast cancer samples.

I performed our initial optimization of IL1B immunohistochemistry on human breast tumor samples already in the lab and then utilized a commercial tissue microarray (Cybridi). Our hope was that by using a tissue microarray containing normal breast tissue and various stages of breast cancer that I would be able to determine if nuclear localization of proIL1B is associated with tumor grade and/ or clinical subtype (ER/PR+, ErbB2+, or triple negative). However technical difficulties including tumor degradation on the array and non specific background staining of some samples prevented us from using the first analysis to answer these questions.

Further optimization of the immunohistochemistry protocol along with larger tumor sample numbers should allow these problems to be overcome. I anticipate that nuclear IL1B will correlate with tumor stage and grade since bioinformatic analysis of proIL1B binding locations indicates proIL1B may be involved tumor initiation and in the metastatic cascade (Table 4). It is more difficult to speculate on the correlation of nuclear proIL1B with clinical subtype. However, one might imagine proIL1B may correlate with ErbB2 positive tumors since C/EBPbeta2 is a key downstream effector of ErbB2 signaling and upregulated proIL1B. On the other hand, C/EBPbeta2, or other transcription factors known to activate IL1B transcription, may be active in all subtypes

of breast cancer. Therefore nuclear IL1B may not correlate with subtype at all. In addition to looking at the relationship between nuclear IL1B and tumor stage and subtype, it would be interesting to look at nuclear IL1B in regard to trastuzamab resistance. To do this immunohistochemistry could be performed on trastuzumab resistant and sensitive ErbB2 overexpressing tumor samples. This is technically a relatively simple experiment to conduct but realistically very hard to accomplish due to the lack of breast tissue samples of known trastuzumab response. I hypothesize nuclear IL1B would correlate with resistance to trastuzumab.

Further studies need to be done to determine if lack of PYCARD occurs in any or all of the tumors containing nuclear IL1B. The relationship between PYCARD and nuclear IL1B in human breast cancer could be investigated using tissue microarrays containing serial sections. It is likely that breast tumors lacking PYCARD would have nuclear IL1B since these cells would not be able to assemble the inflammasome, which is critical for IL1B cleavage and cleaved IL1B is secreted. I expect that tumors with nuclear IL1B will lack PYCARD expression and this correlation will indicate that the nuclear IL1B observed in breast tumor samples is proIL1B.

As listed in Table 7, PYCARD promoter methylation has been observed in many other cancer types which are known to express IL1B. Studies have not been done looking at IL1B and PYCARD expression in the same tumor sample for any of these tumor types**.** It is expected nuclear proIL1B is present in a subset of these cancer types as well. Data indicates proIL1B associates with the chromatin and/or chromatin remodeling complexes facilitating changes in gene expression related to initiating EMT and promoting metastatic initiation, and to a lesser degree, metastatic progression and

metastatic virulence. The location of proIL1beta binding sites relative to genes involved in metastasis is intriguing, given that IL1beta is a well established target gene for C/EBPbeta in multiple cells types and C/EBPbeta has recently emerged, along with the transcription factor, STAT3, as synergistic initiators and master regulators of mesenchymal transformation in malignant glioblastoma (Liang, Zhang et al. 2006; Carro, Lim et al., 2010). In fact, recent research in the lab by Linda Sealy and Rachel Jerrell has demonstrated proIL1B is present in HTB14 glioblastoma cells and that it is tightly associated with the chromatin.

In conclusion, proIL1B is a dual function cytokine which contributes to cancer progression in breast cancer (and likely other tumor types). The work presented within provides the foundation for understanding the function of nuclear proIL1B in cancer and opens many new lines of inquiry. This work along with the proposed future studies may lead to therapeutic interventions in breast cancer and to a validated biomarker for ErbB2 resistance which can be used clinically.

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