C/EBPβ DRIVES MAMMARY TUMORIGENESIS

AN ABSTRACT SUBMITTED ON THE EIGHTEENTH DAY OF OCTOBER 2022 TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF MEDICINE OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Breast tumorigenesis relies on complex interactions between tumor cells and their surrounding environment, orchestrated by tightly regulated transcriptional networks. While much is known about the signaling pathways affecting breast cancer cell growth and apoptosis, the transcriptional programs driving these responses are less understood. C/EBPβ is a transcription factor essential for proper cellular proliferation, differentiation, tissue function, metabolism, and the immune response, and is deregulated in breast cancer. C/EBPβ and its protein isoform LIP have long been postulated to promote breast cancer progression, however, the precise mechanisms and gene regulatory networks induced by C/EBPβ are less understood. Analysis of available human patient gene expression data reveals that increased Cebpb mRNA is associated with poor overall survival amongst all breast cancer subtypes, and analysis of available mass spectrometry data shows that increased C/EBPβ total protein is associated with poor overall survival in triple-negative breast cancer patients. To test the function of C/EBPβ in tumor initiation and progression, we designed studies to address the role C/EBPβ in AXL activation, an RTK associated with breast cancer invasiveness. In addition, we designed two mouse models of different molecular subtypes to investigate the roles of C/EBPβ in mammary tumor initiation and progression outside the scope of AXL activation. We observed that LIP can directly activate Axl transcription by binding directly to the promoter, providing
a novel role of LIP in mammary tumorigenesis. In addition, genetic ablation of tumor C/EBPβ in a mouse luminal (MMTV-Neu) breast cancer model results in a complete block in mammary tumorigenesis. In a basal-like C3(1)-Tag model, Ad.Cre-treated CebpΔ/Δ tumor cells fail to grow when transplanted into immunocompetent mice; however, athymic mice support CebpΔ/Δ tumor growth. These data suggest mature T lymphocyte involvement in clearance of CebpΔ/Δ tumor cells in immunocompetent models. RNAseq of CebpΔ/Δ tumor cells indicates upregulation of IRF (interferon regulatory factor) genes, suggesting a potential mechanism of immune clearance of CebpΔ/Δ tumor cells in vivo. Additionally, genes involved in antigen processing and presentation (MHC class I and class II molecules) are suppressed in CebpΔ/+ tumor cells, suggesting a significant role of C/EBPβ in the downregulation of transcriptional networks involved in anti-tumor immune responses. Ongoing in vitro studies of CebpΔ/Δ tumor cells are designed to identify the functional upregulation in antigen processing induced by ablation of C/EBPβ. These studies will have critical implications for determining whether C/EBPβ manipulation within tumors can be utilized to reprogram the cytotoxic anti-tumor response and lead to tumor clearance.
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DEDICATION

This work is dedicated to the amazing female mentors in my lifetime who have encouraged me, inspired me, held me accountable, educated me, and fostered my growth.

Simply by knowing each of them, I truly stand on the shoulders of giants.

Harriet Neville Limper – my loving late grandmother and Chemist

Annie Limper Chauvin – my mother and biggest supporter

Dr. Anna Bourgeois, DVM – first a teacher, and now a friend.
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The works contained within represent what I consider my greatest accomplishment acquired during some of my most challenging times. Earning a PhD is a difficult as is, but these last years have provided among the most significant obstacles I’ve faced. The loss of an important personal relationship, political unrest delivered by a controversial presidency, the COVID19 pandemic, and a category 5 hurricane directly hitting New Orleans required from me resiliency and perseverance I wouldn’t have been able to summon without immense support. As such, this body of work is dedicated to the long list of individuals I’ve leaned heavily on who have provided me with encouragement, sage counsel, compassion, and comfort.

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Second, I acknowledge my mentor, Dr. Heather LaMarca Machado. Her mentorship has sculpted me not only professionally, but also personally. I’m a better scientist and a better woman because of her guidance. She has challenged me when necessary, been patient and compassionate with me in times of struggle, and been a
phenomenal inspiration to me through the years. I’m immensely proud of her career growth and grateful for her counsel.

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Chapter 1: Introduction

**Breast cancer background and progression**

Breast cancer is the most common malignancy affecting women globally. The lifetime risk of developing invasive breast disease for women in the United States hovers around 13%, but is the most prevalent cause of cancer-related deaths behind lung cancer\(^1\). Fortunately, due to increased screening and more specific therapeutics, breast cancer mortality rates have remained steady in women under 50 and are declining in women over 50. From 2013-2018 alone, breast cancer mortality rates dropped 1% per year, but as the most diagnosed cancer in women worldwide, there remains a significant need to understand the molecular underpinnings that drive breast disease to facilitate the development of more relevant therapeutics and to better predict disease relapse. Two of the most significant barriers to successfully treating breast cancer are the intrinsic heterogeneity of breast tumors and tumor recurrence. Despite the immense diversity across breast tumors, four general subtypes have emerged which help dictate clinical treatment strategies. The prognoses, therapeutic strategies, and recurrence rates across each subtype are distinct\(^2\). Breast cancer clinical subtypes are classified by immunohistological cellular surface and proliferation marker expression driven by variations in transcriptional programs\(^3\). Estrogen receptor (ER), progesterone receptor (PR), Her2, and Ki67 expression status determine where breast tumors fit into the
following subgroups: Luminal A, Luminal B, Her2-endriched, and triple-negative. The Luminal A subtype, defined as ER+, PR+, Her2-, generally has a good prognosis as it proliferates more slowly (Ki67-) compared to other subtypes and expresses receptors responsive to hormone therapy. The Luminal B subtype is ER+, Her2- but strongly expresses Ki67, suggesting increased proliferation compared to the Luminal A subtype. Of all subtypes Luminal B are most likely to metastasize to axillary lymph nodes when highly expressing Ki67, presenting a significant challenge in treatment strategies. Her2+ tumors do not express ER or PR and are generally highly proliferative defined by high Ki67 expression but have a more favorable prognosis due to targeted therapies directed at Her2, like trastuzumab. The most difficult to treat subtype with the worst prognosis is the triple-negative breast cancer (TNBC) subgroup, which expresses no hormone receptors. Due to a lack of druggable surface targets, TNBC requires resection of the primary tumor followed by non-specific chemotherapeutics or radiation, which are often ineffective in complete tumor eradication. In fact, 46% of TNBC patients will develop metastatic disease with an average of 13.3 month survival after metastatic diagnosis. Tumor subtype provides significant insight into which treatment strategies will be successful; however, breast tumors are highly dynamic tissues and further research is needed to provide more accurate prognostic indicators.

Disease recurrence has been estimated based on tumor size, lymph node status, subtype, and response to initial therapeutics. Historically, recurrent tumors and metastases were thought to retain characteristics similar to those of their primary tumors; however, more recent studies demonstrated that recurrent tumors can alter hormone
receptor and Her2 receptor status, which can significantly change course of treatment and prognosis upon disease recurrence\textsuperscript{7}. A loss of hormone receptor status after initial treatment often results in a worse prognosis for patients with recurrent disease\textsuperscript{8,9}. Patients with recurrent breast tumors show changes in hormone receptor expression from their primary tumor subtype – termed receptor discordance – in around 24\% of cases\textsuperscript{10–12}. While a significant percentage of tumor cells may be targeted by initial treatment, selective pressures have been removed from tumor cells that do not respond to therapy, allowing for more access to growth factors and nutrients for which they previously could not compete, resulting in recurrence of therapeutic-resistant tumor cells. A similar relationship between primary tumor and metastatic lesions has recently been demonstrated in patients of multiple breast cancer subtypes. When tumor cells migrated to distant sites, as many as 31\% of patients demonstrated receptor discordance between primary and metastatic sites\textsuperscript{13}. As a result, breast tumors have more recently been regarded as significantly dynamic tissues with multiple selective pressures that adapt and evolve as a result of therapeutic intervention and metastasis to novel tissue niches.

Tumors are heterogeneous and complex; therefore, as selective pressures are applied and removed with surgical and therapeutic intervention, each instance of metastasis and recurrence must be re-characterized, which may require completely different treatment strategies.

Breast tumorigenesis occurs in multi-step genetic and histological process. Tumorigenesis is initiated when luminal cells of the milk duct undergo an oncogenic hit resulting in genetic alterations that confer proliferative advantages. Transformed cells deregulate cell cycle genes required for normal growth and differentiation, allowing for
unchecked proliferation. The most commonly mutated genes in breast cancer are *Tp53*, *Pik3ca*, *Brca1*, and *Brca2* which are related to proliferation, differentiation, apoptosis, and cell motility. Histologically, transformed luminal cells will proliferate to form atypical ductal hyperplasias (ADH) which eventually fill the lumen with atypical hyperplastic cells, resulting in ductal carcinoma in situ (DCIS). While non-invasive, DCIS accounts for around 20% of newly-diagnosed breast cancers in the U.S. DCIS is rarely palpable or clinically symptomatic and is mostly diagnosed through mammography. While DCIS is a non-obligate precursor to invasive ductal carcinoma (IDC), characterized by invasion of hyperplastic cells through the myoepithelial layer into the surrounding stroma, ~43% of DCIS patients will progress to IDC, though the mechanisms driving the transition to invasive disease are poorly understood. Patients diagnosed with IDC will progress to form solid tumors that eventually cause clinical symptoms, often progressing to metastasis at distant sites.

**Transcription factors and their role in tumor progression**

Transcription factors are protein-coding genes that translocate to the nucleus, bind DNA, and activate or repress transcription. They act as genetic switches that modulate the cell cycle, development, response to environmental changes, and oncogenesis. Transcription factors regulate the timing as well as the extent of genetic expression throughout the cellular life cycle in a tightly-coordinated manner. To initiate mRNA transcription, transcription factors serving as activator proteins bind enhancer regions of DNA upstream of the promoter sequence. Once activated, enhancers induce confirmational changes in the DNA to allow other transcription factors access to the
promoter site. Often functioning as multimers, transcription factors complexes recruit RNA polymerase to the promoter site to begin transcription. Inversely, transcription factors can also repress gene expression by binding DNA and physically blocking either the RNA polymerase itself or other essential activators from interacting with a promoter. Strict coordination of transcriptional activation and repression are required for proper homeostasis and division.

Transcriptional activators are precisely regulated through multiple mechanisms. Many transcription factors are excluded from the nucleus in the cytoplasm physically distanced from their target genes until stimulated to translocate to the nucleus. NF-KB, a transcription factor essential to macrophage function, is regulated in this manner. NF-KB is bound by its inhibitor iKB in the cytoplasm until receiving stimuli, like a viral infection. Upon stimulation, iKB is degraded, allowing for NF-KB phosphorylation and translocation to the nucleus to activate transcriptional programs. Interestingly, a target gene of NF-KB transcription is iKB, so inhibition of NF-KB is reestablished shortly after activation. Transcription factors can also amplify their own transcription by binding their own promoter creating a positive feedback loop, resulting in a quick and exponential response to stimuli. These feedback mechanisms allow for precise control over gene expression programs. As another mechanism of transcriptional regulation, transcription factors can alter their DNA binding affinity. In the presence of a bound coactivator, transcription factors undergo conformational changes which increase their DNA binding affinity and subsequently their activation potential. Steroid hormones in the nucleus are an example of this effect – unless coupled to their corresponding steroid hormones from the cytoplasm, they cannot bind their target DNA sequence and activate transcription.
In a similar manner, some transcription factors are inactive by themselves and require dimerization or multimerization to exert their activation effects. Transcription factors can multimerize with the same protein, forming homo-multimers, or they can bind other soluble proteins to form hetero-multimers. In mammalian cells, heat shock factor (HSF) must form a homo-trimer to induce transcription, as the DNA binding affinity for a single HSF molecule is too weak to exert any physiological effect. Regulation of transactivation is another means of transcriptional control in eukaryotic cells. In yeast, HSF is already trimerized and bound to its promoter, but is inactive until an increase in temperature. Upon heat stimulation, the HSF trimer is phosphorylated at multiple sites, inducing a confirmational change that reveals the transactivation domain allowing for transcription initiation. A few molecules improperly translocated to the nucleus can activate cascades of gene expression programs; therefore, cells often utilize multiple means of transcriptional regulation to ensure proper gene expression.

Transcription factors contribute to tumorigenesis in multiple ways. Amplification, point mutations, chromosomal rearrangements, or dysregulation of gene expression can all cause perturbations in cellular programs causing oncogenesis. Transcription factors play a significant role in cancer development due to their ability to induce cascades of expression programs at low cellular concentrations. Additionally, a relatively limited number of transcription factors are overexpressed or overactive in most human cancers, making them an attractive therapeutic target. However, until recently, the protein-protein or DNA-protein interactions central to transcription factor function were deemed “undruggable.” DNA-protein binding sites are generally convex and positively charged, making for a difficult target site for small molecule drug delivery. Additionally, protein-
protein surface interactions tend to be flatter and simpler configurations, lacking the deep pockets found in enzymatic active sites that facilitate binding specificity. However, the discovery of spatially distinct hotspot residues that dictate protein-protein interaction energy has made transcription factor protein-protein interaction inhibitors feasible therapeutic options. Additionally, exploiting allosteric modulation of protein-protein interactions has shown promise in developing small molecule inhibitors for autoimmune disorders and certain cancers.

**CCAAT enhancer-binding protein (C/EBPβ) background**

CCAAT/enhancer binding protein β (C/EBPβ) is a transcription factor that regulates proliferation, differentiation, and apoptosis across multiple tissues and within the mammary gland. The gene encoding both human and mouse C/EBPβ consists of one exon and no introns, giving rise to three protein isoforms by utilization of alternate translation initiation sites (Figure 1). From here forward, we’ll be referring specifically to mouse C/EBPβ, consisting of 38 kDa full length, liver-enriched activation protein (LAP1); 35 kDa LAP2; and the truncated 20 kDa liver-enriched protein (LIP). The N-terminus for the LAP isoforms consist of three activation domains and two regulatory domains. All three isoforms, along with other C/EBP family members, contain a highly-conserved DNA-binding domain at the C-terminus consisting of a basic leucine zipper (bZIP). In the presence of DNA, the basic region of the bZIP domain is stabilized by forming an α-helical structure upon dimerization. All C/EBPs require dimerization to bind DNA. Each C/EBPβ isoform can form homodimers or heterodimers with itself or with other C/EBP family members which then bind DNA with different affinities to
activate transcription\textsuperscript{30}. LIP can dimerize with itself or with LAPs, but it lacks the transactivation domain of the larger isoforms; therefore, LIP can attenuate the transcriptional activation capacity of LAP when functioning as a LIP:LAP heterodimer\textsuperscript{31}. Importantly, LIP is characterized by a higher DNA-binding affinity than the other C/EBPβ isoforms and functions as a dominant negative by occupying C/EBPβ DNA binding sites. The transcriptional inhibitory effect of LIP occurs even at substoichiometric ratios, underscoring the importance of the LIP:LAP ratio in transcriptional networks\textsuperscript{31}. Recent findings have challenged the historical role of LIP as transcriptional repressor and competitive inhibitor of C/EBP transcriptional activation. Genetic knock-in of a single LIP allele in $Cebpb^{-/-}$ mice ($Cebp^{-/-L}$) was shown to coordinate with C/EBPα to restore various physiological functions absent in $Cebpb^{-/-}$ mice. The rescue phenotype of $Cebpb^{-/-L}$ mice included normal adipocyte and keratinocyte differentiation, lipid homeostasis, and partial fertility in females\textsuperscript{32}. These findings challenge the thought of LIP as an on/off transcriptional switch that functions solely as a dominant negative.

Post-translational modifications are responsible for dictating the transcriptional activity, subcellular localization, and protein-protein interactions of C/EBPβ. C/EBPβ is naturally held in a repressed state by its two regulatory domains which sterically hinder its transactivation domain\textsuperscript{33,34}. C/EBPβ can then be phosphorylated, acetylated, methylated, or sumoylated – all resulting in differing transcriptional functions. As is typical of transcription factor phosphorylation, upon phosphorylation of numerous sites, the transactivation domain of C/EBPβ is de-repressed, allowing for transcriptional activation. Phosphorylation of C/EBPβ is regulated by many different pathways.
including, but not limited to the following: Ras-MAPK\textsuperscript{35–37}, protein kinases A and C\textsuperscript{38–42}, ribosomal S6 kinase\textsuperscript{43,44}, Ca\textsuperscript{2+}/calmodulin dependent protein kinase\textsuperscript{45}, growth factors and glycogen synthase kinase 3β (GSK3β)\textsuperscript{46–48}, and the cyclin-dependent kinase pathway CDK1CDK2–CCNA2\textsuperscript{49,50}. C/EBPβ can also be acetylated, but this modification results in differing functions. The Lys39 residue serves as an important regulatory acetylation site that either increases or decreases the transcriptional capability of C/EBPβ. When acetylated, Lys39 can increase C/EBPβ-mediated Fos transcription\textsuperscript{51} but can also decrease transcription of C/EBPβ-mediated adipogenesis target genes when deacetylated by histone deacetylase 1 (HDAC1)\textsuperscript{52}. When C/EBPβ is acetylated at Lys 98, Lys101, and Lys102 by GCN5 and PCAF acetyl transferases during preadipocyte differentiation, HDAC1 and C/EBPβ interactions are reduced, resulting in enhanced transcriptional activity\textsuperscript{44}. Conversely, when C/EBPβ is acetylated at Lys215 or Lys216, transcription of the DNA-binding protein inhibitor ID1 promoter is repressed by decreasing the DNA-binding potential of C/EBPβ; however, this effect is reversed and transcription is activated when C/EBPβ is deacetylated by HDAC1\textsuperscript{54}. As evidenced by the findings from the aforementioned studies, acetylation of C/EBPβ serves as a potent regulatory switch in tightly modulated transcriptional programs. Lys39 not only serves a regulatory role in acetylation of C/EBPβ, but also is involved in methylation and phosphorylation of C/EBPβ. Histone lysine N-methyltransferase H3 lysine-9-specific 3 (G9a) directly methylates Lys39 located in the N-terminal domain of C/EBPβ resulting in transcriptional repression of C/EBPβ; conversely, phosphorylation at this site inhibits methylation, subsequently activating transcriptional activity\textsuperscript{55}. The Lys39 residue of C/EBPβ is conserved across multiple species including rat, chicken, and mouse
indicating the importance of its role in C/EBPβ transcriptional control. Sumoylation is the last post-translational modification that regulates transcriptional functions of C/EBPβ. Sumoylation of transcription factors alters the cellular sub-compartmentalization, nuclear to cytoplasmic trafficking, stability, and interactions within the nucleus and cytoplasm of most cells. Generally, sumoylated transcription factors exhibit repressed transcriptional activity, though enhanced activity has been reported in some cases. Sumoylation of C/EBPβ involves the covalent bonding of either small ubiquitin-like modifier 1 (SUMO1) or SUMO2/3 protein to a lysine residue (Lys132 in mouse or Lys173 in human) in the first inhibitory domain (RD1). SUMO2/3 targets only C/EBPβ-LAP1 and impairs its ability to activate the cyclin D1 promoter\(^5^6\). In mouse T cells, sumoylation of C/EBPβ relieved C/EBPβ-mediated\(\text{Myc}\) repression, encouraging cell cycle progression and T cell proliferation in vivo\(^5^7\). As C/EBPβ is a ubiquitously expressed transcription factor essential for proliferation, differentiation, senescence, and apoptosis, post-translational modifications serve an essential role in the maintenance and strict regulation of C/EBPβ-driven transcriptional programs.

**C/EBPβ and receptor tyrosine kinases in breast cancer**

C/EBPβ is a known downstream effector and regulator of multiple different families of receptor tyrosine kinases (RTKs) in breast cancer. Breast cancer is characterized by aberrant RTK signaling which promotes proliferation and disease progression. The epithelial growth factor (EGF) family includes ErbB1/EGFR, ErbB2 ErbB3, and ErbB4. All are ligand-activated membrane bound RTKs that dimerize and initiate kinase signaling cascades\(^2^6,5^8,5^9\). Both ErbB receptors and ligands play important
roles in normal mammary development and are regularly overexpressed or amplified in breast cancer.\textsuperscript{60–62} In transgenic mouse models and in vitro, ErbB1 signaling stimulates LIP expression through an mRNA-binding protein CUG-binding protein 1 (CUG-BP1).\textsuperscript{63} EGFR signaling has also been shown to regulate C/EBP\(\beta\) isoform expression in a rat model of secondary hyperparathyroidism and in human bronchial epithelial cells\textsuperscript{64,65}. The results of these studies suggest that EGFR signaling can directly increase the LIP:LAP ratio, which then drives proliferation and disease progression. In addition to EGFR signaling, C/EBP\(\beta\) has also been associated with fibroblast growth factor receptor (FGFR) signaling, which is also heavily implicated in human breast cancers.\textsuperscript{66,67} While C/EBP\(\beta\) functions downstream of EGFR signaling, it mediates FGFR signaling in response to EGFR activity by modulating the FGF-binding protein (FGFBP1) promoter. LAP activates and LIP represses FGFBP1 promoter activity in response to EGFR kinase activity, it is unknown whether FGF signaling affects LIP:LAP ratio.\textsuperscript{68} Due to the interconnectedness of growth factor signaling pathways, C/EBP\(\beta\) has been proven to be a critical modulator of RTK signaling in breast cancer.

**Functions of Gas6/Axl signaling axis in cancer**

As previously mentioned, RTK signaling strongly influences tumor progression though multiple mechanisms and can influence disease severity. The same holds true for the Axl signaling axis. Axl is a membrane bound RTK with a canonical C-terminal intracellular kinase domain, a transmembrane domain, and a protease cleavage site near the proximal extracellular surface. The surface receptor consists of two fibronectin type III regions capped by two immunoglobulin-like (Ig-like) domains that contain the ligand
binding site. The ligands for AXL consist of Pros1 and, more commonly, Gas6, a secreted extracellular protein (Figure 3). Upon ligand binding, transmembrane signal transduction occurs resulting in phosphorylation of the intracellular kinase domain which activates downstream signaling. AXL requires dimerization upon ligand binding for proper signal transduction and can be activated in multiple ways. It can homodimerize with itself or AXL monomers on adjacent cells can interact. Additionally, it can heterodimerize with EGF, making it an attractive target for breast cancer therapeutics. It is commonly expressed across multiple cell types including epithelium, endothelium, innate immune cells, and hormone-responsive tissues like the breast and testes. AXL activation has been implicated in many cellular processes like adhesion, migration, invasion, proliferation, and survival. As Axl activation results in downstream activation of PI3K/AKT and MAPK/ERK signaling, it serves as a potent modulator of downstream transcriptional programs essential to normal mammary development and ductal morphogenesis.

Axl has been shown to alter cancer progression and influence patient outcomes. In clinical cancer patients, the degree of Axl expression correlates with disease severity. In acute myeloid leukemia (AML), Axl signaling promotes tumorigenesis by suppressing AKT and MAPK pathways, contributing to proliferation. In prostate cancer, Axl expression is closely associated with metastasis frequency. Specifically within the context of breast cancer, AXL can be used as a prognostic indicator – high expression indicates worse survival outcomes. It has also been shown to regulate epithelial-to-mesenchymal in invasive breast disease. When inhibited in invasive breast cancers, Axl reduces invasion and migration in vitro, suggesting that Axl inhibition may serve as a
potential therapeutic strategy. Interestingly, much of the Axl literature focuses on already invasive tumors, and little is known about how Axl expression regulates early disease states like DCIS. Further investigation is warranted to understand how Axl modulates the switch from pre-invasive to invasive breast disease.

**C/EBPβ functions in the mammary gland**

Before C/EBPβ was investigated within the context of breast cancer, it was shown to be a critical modulator of mammary epithelial cell function and differentiation during pregnancy, lactation, and pubertal development. In vivo, Cebpβ−/− virgin mice exhibit disrupted ductal morphogenesis characterized by distended ducts, reduced side branching, and defective budding. Doppler et al. demonstrated in 1995 that C/EBPβ was not only expressed in HC11 cells, an immortalized mammary epithelial cell line, but also bound and activated expression at the rat β-casein promoter, suggesting a potential regulatory role in lactogenesis during pregnancy. Cebpβ−/− mammary glands transplanted into wild-type stroma displayed a defect in proliferation and terminal differentiation, as determined by a lack of milk production at parturition; conversely, wild-type epithelium transplanted into Cebpβ−/− females showed no lactation defects, suggesting the stunted phenotype was cell-intrinsic rather than influenced by systemic hormones. Importantly, when similar transplantation experiments were performed with mammary epithelia lacking C/EBPα, the other predominant C/EBP expressed in mammary tissue, no phenotype developed, cementing C/EBPβ the more important driver of proliferation and differentiation in mammary development.
C/EBPβ isoforms within the mammary gland play individual and often conflicting roles in development (Figure 2). Raught et al. showed in vivo that expression of each C/EBPβ isoform increased considerably during pregnancy, but LIP expression alone increased over 100-fold, suggesting that a high LIP:LAP ratio supports epithelial proliferation required for adequate milk production. In contrast, upon lactation and involution, the LIP:LAP ratio decreased to expression levels similar to that of the virgin gland and it was established that glucocorticoids relieve LIP repression on the β-casein promoter to support milk production in vitro. These data were the first to indicate lactogenic hormone signaling influences C/EBPβ isoform expression, which modulates downstream milk production genes in mammary tissues. Taken together, these studies were seminal in establishing the importance of epithelial-derived C/EBPβ as a regulator of hormone-driven transcriptional programs during multiple phases of mammary gland development and differentiation.

**C/EBPβ and breast cancer**

The gene encoding C/EBPβ is not mutated in breast cancer but has been shown to be amplified in a small subset of mammary neoplasms. In 2021, Wang et al. discovered that triple negative breast cancer patients that express high C/EBPβ mRNA transcript levels had a worse overall survival compared to low-expressing patients suggesting that C/EBPβ transcription drives invasive disease. The Kaplan-Meier survival data from the study excluded C/EBPβ protein expression analysis, leaving unanswered questions about individual C/EBPβ isoform expression within the cohort. The first correlation between C/EBPβ - specifically, the truncated third isoform, LIP – and invasive breast disease was
established in a small subset of clinical patient samples in 1997. In this study, C/EBPβ expression was assessed in infiltrating ductal carcinoma samples with varying hormone receptor status compared to adjacent normal tissue. LIP was not expressed in adjacent normal tissue but highly upregulated in several invasive tumors in the cohort. A majority (88.9%) of samples with the greatest LIP expression lacked estrogen and progesterone receptor expression (ER−/PR−) and were highly proliferative. Additionally, all LIPhi tumors were aneuploid and poorly differentiated84 – significant indicators of aggressive breast disease. Interestingly, Raught et al. discovered a similar correlation between LIP expression and aggressive disease in mouse mammary tissues. While both transplantable hyperplastic outgrowths and invasive TM6 tumor cells expressed similar LAP1 levels, only the more invasive TM6 cells upregulated LIP. Interestingly, p53−/− and p53+/+ Wnt tumors expressed a high LIP:LAP ratio, suggesting LIP potentially drives the molecular switch from pre-invasive to invasive mammary lesions in mice independent of p53 status85. Although the cohorts for the above studies were small and the results were correlative, they provided rationale to perform functional assays and further in vivo studies to determine the role of LIP in mammary tumorigenesis.

Among the first in vivo investigations into LIP as a potential oncogene in the mammary gland was a study performed by Zahnow et al. in 2001. They generated a mouse model containing a construct driving constitutive LIP expression under the whey acidic protein (WAP) promoter beginning around days 7-10 in pregnancy extending throughout lactation86. WAP-LIP mice lactated and nursed pups with no difficulty and showed no gross histological abnormalities through lactation day 18 (L18). However, after complete involution, when mammary ductal structures normally return to their
virgin-like architecture after mass epithelial apoptotic programs, 30-40% of 6-32 month-aged WAP-LIP mice developed focal and diffuse hyperplasias compared to age-matched wild type controls. These findings suggested that LIP over-expression for the duration of early pregnancy to the onset of involution can foster neoplastic growth within the mammary gland\textsuperscript{87}. To further investigate the function of LIP in metastatic breast disease, Gomis et al. performed a xenograft study utilizing breast cancer cells isolated from pleural effusions of TNBC patients. Their findings indicated that samples with a high LIP:LAP ratio were desensitized to the growth-inhibitory functions of TGF\beta. Upon reduction of the LIP:LAP ratio through constitutive expression of LAP2, TGF\beta-dependent cytostatic functions were restored in the tumor cells and metastasis was overall reduced through LAP2:SMAD-mediated repression of c-MYC\textsuperscript{88}. Taken together, the aforementioned findings emphasized that LIP expression in the mammary gland confers susceptibility to oncogenesis, promotes proliferation, and supports resistance to TGF\beta-mediated growth arrest in metastatic lesions in both human xenograft and transgenic mouse models.

The limitations of the aforementioned studies included a small sample size\textsuperscript{88} and LIP expression from artificial, constitutively-active promoters\textsuperscript{87,88} rather than the endogenous locus; therefore, the oncogenic potential of LIP could be overestimated when expressed without the context of its intrinsic regulatory mechanisms. To address the oncogenic potential of LIP from its endogenous promoter with more biologically relevant transcriptional regulation, Bégay et al. developed a LIP only-expressing (C/EBP\beta\textsuperscript{L/+} or C/EBP\beta\textsuperscript{L/L}) mouse model eliminating the sequence between the LAP1 and LAP2 translation initiation sites, leaving only the endogenous LIP locus intact\textsuperscript{89}. While most
tumor-bearing C/EBPβL/+ mice developed either T- or B-cell lymphoma, some developed mammary carcinomas while the C/EBPβL/+ group developed none – establishing that LIP expression from its native locus can initiate mammary carcinogenesis\textsuperscript{90}. The inverse genetic experiment yielded similar results further validating the oncogenic potential of LIP across multiple tissue types. A genetic mouse model was generated that ablated endogenous LIP by elimination of the upstream open reading frame (uORF) necessary for LIP translation. LAP only-expressing female mice (CebpbΔORF) exhibited 20.6% longer median survival rates and developed fewer tumors compared to wild type females. CebpbΔORF females that did develop tumors had a reduced tumor burden and displayed a delay in tumorigenesis as determined by a significantly higher age at necropsy compared to wild type female controls\textsuperscript{91}. These animal studies conclusively demonstrated that LIP predisposes mice to a multitude of cancers and its ablation serves a protective effect in vivo.

**Mechanisms of immune evasion**

An intact immune system is essential for the surveillance, identification, and elimination of nascent neoplasias\textsuperscript{92,93}. Within the context of cancer, the immune system functions to prevent transformed cells from establishing solid tumors by resolving inflammatory conditions favorable for tumor growth and clearing transformed cells recognized as “non-self”. Anti-tumor immunity identifies and eliminates neoplastic cells mainly by recognition of tumor-specific antigens or by aberrant expression of cellular stress surface molecules\textsuperscript{92}. The two arms of the immune system – innate and adaptive – work in a tightly-coordinated manner to mount an effective anti-tumor response.
A cancer cell under immunosurveillance encounters one of three fates – elimination, equilibrium, or escape. Elimination occurs when the innate immune system – natural killer (NK) cells, dendritic cells (DC), and/or macrophages – responds to pro-inflammatory signaling like interferon-12 (IL-12) or interferon-γ (IFN-γ) and facilitates tumor cell killing. Subsequently, tumor antigen-presenting DCs migrate to the peripheral lymph nodes and prime adaptive immunity by activating tumor-specific CD4+ and CD8+ T lymphocytes. The tumor-specific T cells then infiltrate the nearby tumor tissues and eradicate the remaining tumor cells. However, tumor clonal variants can develop that escape immunosurveillance. This stage of tumorigenesis refers to equilibrium – where the tumor cells are neither eliminated, nor have they faced the final wave of tumor-specific immune responses. Should the emergent tumor cells evade a second immune response, escape occurs, and clinical symptoms inevitably develop.

According to the aforementioned logic that transformed cells are under constant surveillance by the immune system, the successful establishment of tumors implies that cancer cells have escaped immunodetection. The prevalence of certain cancers in immunocompromised patients serves as evidence that defective immunological monitoring confers tumor susceptibility, but this effect was mostly observed in viral-induced cancers not affecting breast tissue. Defective or incomplete anti-tumor immunosurveillance, therefore, is not the sole cause of tumor escape, particularly in epithelial-derived cancers. In fact, tumors are enriched with immune cells. Pathologists have long characterized that many solid tumors of different origins were heavily infiltrated by both innate and adaptive immune cell types resembling infiltrations seen in chronically inflamed, non-cancerous tissues undergoing wound-healing. It is now
well established that tumor-infiltrating immune cells work in conflicting ways. Multiple studies have demonstrated that immune infiltrates exacerbate and promote tumorigenesis in epithelial cancers and even serve as negative prognostic indicators\textsuperscript{99–101}. These reports showed that tumor cells reprogram their microenvironments by suppressing anti-tumor immunity and recruiting pro-tumor immune cells. Among the most important innate immune cells that promote a hospitable environment for tumor growth are tumor-associated macrophages (TAMs). TAMs promote tumor progression by secreting IL-1, tumor necrosis factor, CCL2, and CXCL8 which facilitate angiogenesis and tissue-remodeling\textsuperscript{94}. Additionally, they produce free radicals which induce DNA damage, further promoting oncogenesis. Another immunosuppressive cell type heavily responsible for facilitating tumor growth is myeloid-derived suppressor cells (MDSCs). Tumor-derived granulocyte macrophage colony-stimulating factor (GM-CSF) drives production of MDSCs, which acquire cancer stem-like properties and inhibit T cell cytotoxicity\textsuperscript{102,103}. TAMs, MDSCs, and other granulocytes work in concerted effort to generate an immunosuppressive, chronically inflamed local microenvironment conducive to tumor growth.

Within the context of breast cancer, elevated systemic inflammatory biomarkers like C-reactive protein (CRP) and serum amyloid A (SAA) were shown to be indicators of reduced overall and disease-free survival in patients, demonstrating that systemic inflammation directly worsens clinical outcomes\textsuperscript{104}. In support, the Women’s Health Initiative revealed that twice-weekly NSAID usage protected women from breast cancer risk by 21\%, though the results have difficult to replicate\textsuperscript{105}. Liu et al. were among the first to identify a mechanism in vivo demonstrating how chronic inflammation drives
mammary tumorigenesis. Utilizing transgenic mice, they established that NF-Kβ-driven inflammation is fundamental in promoting mammary tumorigenesis and the expansion of mammary tumor stem cells\textsuperscript{106}. These findings highlighted that within the context of mammary tumors, inflammation and immune infiltration is more likely to promote tumorigenesis than to inhibit it. In addition to recruitment of pro-tumor immune cells, tumor cells evade cytotoxic effectors by decreasing expression of receptor-bound recognition proteins. Transformed cells actively downregulate immunogenic surface receptors like MHCI to avoid direct cell killing by cytotoxic CD8\textsuperscript{+} lymphocytes\textsuperscript{92}; however, this effect isn’t recapitulated by all solid tumor types, suggesting that immune composition of the intratumoral landscape, among other factors, determines tumor elimination or escape.

Taken together, these data demonstrate that immune escape is likely driven by multiple mechanisms working in concert to evade recognition and promote growth. Recent developments of potent immunotherapies have strengthened the arsenal of clinical weapons to treat invasive cancers, but many cancer types remain resistant to therapy – as such, understanding and characterizing the diverse mechanisms driving tumor growth and immune escape remains imperative to develop more effective treatment strategies.

**C/EBPβ and immune implications in cancer**

Recent literature has indicated that C/EBPβ may contribute to immune evasion within the intratumoral landscape. As discussed previously, the ability of a tumor cell to avoid clearance by cytotoxic effectors depends on multiple mechanisms involving multiple cell types. Tumor cells can downregulate immunogenic surface receptors and
recruit immunosuppressive TAMs and MDSCs through altered chemokine signaling and metabolic pathways.\textsuperscript{107} As C/EBP\textbeta\ regulates cytokine and chemokine signaling, cell cycle progression, and metabolism in multiple tissue types, the potential involvement of C/EBP\textbeta\ in tumor cell immune escape seems plausible. To determine shifts in genetic expression upon C/EBP\textbeta\ deletion, mice were treated with DMBA to initiate squamous cell papilloma tumors. When the tumors reached an appreciable size, C/EBP\textbeta\ recombination was induced with tamoxifen administration. Mice with tumors lacking C/EBP\textbeta\ showed a reduction in tumor volume and tumor burden. Gene set enrichment analysis was performed on the regressing C/EBP\textbeta^\textDelta^\textDelta\ tumors and showed that IFN-\alpha and IFN-\gamma gene signatures were the most differentially upregulated genes, suggesting potential changes in immune recruitment, though these observations were not tested functionally in this study.\textsuperscript{108} Evidence from CRISPR deletion of C/EBP\textbeta\ in vitro supports that tumor-derived C/EBP\textbeta\ may promote “immuno-masking” and tumor escape. Gene set enrichment analysis of the human BT-20 TNBC cell line indicated that antigen processing and presentation genes as well as MHC class I and II were the most significantly upregulated genes in the C/EBP\textbeta\ deleted group; these data suggest that tumors rely on C/EBP\textbeta\ to suppress antigen presentation processes essential for clearance by cytotoxic CD8+ T cells\textsuperscript{109}. LAP over-expression in TNBC cells was shown to induce both G-CSF and GM-CSF expression, suggesting that tumor-derived C/EBP\textbeta\ can potentially induce MDSC expansion in vivo. Conversely, when LAP was knocked out in 4T1 transplanted TNBC tumors, tumor-protecting MDSC populations decreased and cytotoxic CD8+ T cells increased within the tumors, implicating a potential promotional role for C/EBP\textbeta\ in immunosuppression.\textsuperscript{107}
**Statement of the problem**

As breast cancer is the second-most common cause of death in women in the United States\(^1\), understanding the molecular drivers at all stages of disease – from pre-invasive lesions to metastasis – is essential to identifying predictive biomarkers and developing effective therapeutic interventions. Breast cancer is a complex disease involving tumor cell-stromal interactions, altered transcriptional programs that influence immune function, and restructuring of surrounding stroma to support invasion and angiogenesis. A thorough understanding of the transcriptional networks driving the tumor-promoting functions within the epithelium that influence the microenvironment is essential to combat and treat invasive breast disease.

The studies performed herein investigate the functional roles of epithelial-derived C/EBP\(\beta\) in Axl activation, a known driver of breast cancer invasion, and its overall contributions to mammary tumorigenesis. **We hypothesize that C/EBP\(\beta\) contributes to mammary tumorigenesis and will influence immune recruitment to the tumor microenvironment.** The following three specific aims have been developed to test these hypotheses.

**Specific Aim 1** is to determine if C/EBP\(\beta\) functions as a direct transcriptional activator of the Axl promoter. These studies will utilize cell lines lacking C/EBP\(\beta\) expression to address promoter activation.

**Specific Aim 2** determines the role of C/EBP\(\beta\) in tumor initiation and progression.

**Specific Aim 3** investigates whether tumor-derived C/EBP\(\beta\) regulates immune clearance.
The final two aims utilize two transgenic mouse models generated in our lab. The first model utilizes a transgenic Cre recombinase to conditionally delete C/EBPβ from the epithelium in a spontaneous mammary tumor model. With this model, we will address tumor formation and tumor burden. The second method involves a transplant model of mammary tumorigenesis where C/EBPβ is deleted utilizing an exogenous adenoviral Cre recombinase. After recombination, cells are transplanted into recipient animals to assess tumor formation and the immune implications of C/EBPβ deletion.
Figure 1 *Cebpb* gene structure and protein isoforms

*Cebpb* belongs to the C/EBP bZIP family of transcription factors due to its conserved DNA binding domain which consists of a leucine zipper with a 5’ basic region. It contains three transactivation domains and two regulatory domains when translated into its full-length isoform. The third truncated isoform, LIP, lacks the transactivation domain and a significant portion of the first regulatory domain, causing it to be canonically considered a transcriptional repressor.
The LIP:LAP ratio plays a fundamental role in maintaining cell states in healthy tissues and in tumorigenesis. If the LIP:LAP ratio is low, normal processes of differentiation, lineage commitment, and properly timed progression through cell cycle are maintained. When the LIP:LAP ratio increases to stochiometric levels > 1, excessive LIP drives proliferation and encourages oncogenesis.

Figure 2 The LIP:LAP ratio influences multiple cell states in homeostasis and oncogenesis
Gas6, the extracellular soluble ligand for Axl, binds the extracellular domain and initiates homodimerization. Upon dimerization, the C-terminal intracellular domains are phosphorylated at specific tyrosine residues, initiating downstream phosphorylation cascades which results in cellular processes of migration, invasion, EMT, and survival.

**Figure 3 Axl signaling regulates multiple cell processes during oncogenesis**
Chapter 2: C/EBPβ regulation of Axl during early breast cancer progression

Specific Aim 1 is to determine if C/EBPβ functions as a direct transcriptional activator of the Axl promoter. These studies will utilize cell lines with minimal C/EBPβ expression to address promoter activation.

Introduction

Axl/Gas6 signaling has been heavily implicated in carcinogenesis. Axl activation has been linked to multiple hallmarks of cancer including migration, invasion, and apoptotic resistance. Additionally, constitutive Axl expression has been shown to confer resistance to targeted anticancer therapeutics. As Axl signaling in cancer is reliant on overexpression, uncovering the transcriptional mechanisms that activate Axl expression remains imperative to understanding its function in malignant disease.

Axl signaling has been implicated as a driver of invasion in many different forms of cancer; however, the role of Axl in the switch from pre-invasive DCIS to invasive tumors is poorly characterized. Axl is overexpressed in multiple cancer types, including breast cancers, though little is known about the transcriptional mechanisms regulating Axl expression. In clear cell renal carcinoma, hypoxia-induced transcription factors (HIF) HIF-1 and HIF-2 have been shown to activate Axl expression by binding hypoxia-response element in the Axl promoter, driving an invasive and mesenchymal
phenotype. Similarly, Dang et al. demonstrated that the transcription factor ΔNp63α induces Axl expression and drives cell migration in basal-like breast cancer, further cementing that transcriptional activation of Axl promotes invasive disease, especially in the breast. Prior experiments performed by a former Machado lab member, Sheng Zheng, indicated that the truncated isoform C/EBPβ-LIP isoform can induce proliferation, migration, and invasion in the pre-invasive cell line DCIS.com; interestingly, LIP over-expression increased Axl transcripts, while overexpression of LAP had the opposite effect. Interestingly, when Axl was knocked down in LIP-overexpressing DCIS.com cells, the phenotype reversed, suggesting LIP may play a potential role in the transcriptional regulation of Axl in preinvasive cells.

The above studies provide intriguing results; however, multiple mechanisms may be at play. LIP expression may induce Axl by competing for binding sites on the Axl promoter, relieving potential repressor function by serving as a dominant-negative. Perhaps more interestingly is the idea that LIP, which lacks a transactivation domain, directly binds Axl and functions as transcriptional activator – a function much more rarely observed. Here we investigate the direct transcriptional functions of each C/EBPβ isoform at the Axl promoter site.
Materials and Methods

Cell lines and reagents

Human cell line MCF10DCIS.com was purchased in 2007 from Asterand (Detroit, MI, USA) and maintained in 5% horse serum (Invitrogen #26050-088), 1% Penicillin-Streptomycin (Invitrogen #15140-122), 1.05mm CaCl₂, and 10 mM HEPES (Invitrogen #1563-000) in DMEM/F12 (Invitrogen #11320). HEK293T cells were kindly donated from Jeffrey Rosen in 2013 from Baylor College of Medicine. HEK293Ts were maintained in 10% FBS (Invitrogen #10082-147), 1% Penicillin-Streptomycin (Invitrogen #15140-122) in DMEM (Invitrogen #11995-073). Cell lines were passaged at 75% confluence by rinsing twice with DPBS (Invitrogen #14190-136) following with the addition of either 0.25% or 0.05% trypsin-EDTA (Invitrogen #25200-056) for MCF10DCIS.com and HEK293T cells, respectively. Cells were incubated at 37°C, 5% CO₂ until detached. The trypsin-cell suspension was neutralized by addition of equal volume of complete media and cells were pelleted in an Eppendorf centrifuge (5810R) at 450 g x 5 minutes.

Lentiviral propagation and concentration

HEK293T cells were seeded at 1.25x10⁷ cells per 15 cm dish in complete media and incubated overnight at 37°C, 5% CO₂. The following day, cells were treated with 25 uM chloroquine in complete media for 3 h. before adding plasmids and reagents for transfection. Plasmids and transfection reagent PEI (polyethylenamine, branched; Sigma-Aldrich, #408727) were added to plain DMEM (Invitrogen #11995-073) and incubated for 15 min. at room temperature. Plasmid:PEI complexes were added dropwise to each
plate and left to transfect overnight at 37°C, 5% CO₂. Plasmid and reagent ratios per plate are listed in the table below. After 16 h. of transfection, media was removed and replaced with 40 uM caffeine in complete media. After 24 h., viral media from each plate was collected, stored at 4°C in 50 ml conical tubes, and replaced with complete media. After another 24 h., viral media was collected and combined with the previous day’s media collection. Collected media was spun in an Eppendorf centrifuge (5810R) at 450 g x 5 minutes to pellet unwanted epithelial cells. Viral media was then passed through a 0.22 low protein binding PVDF filter (Millipore-Sigma, SLGV004SL) and aliquoted into sterile 38.5 ml ultracentrifuge tubes (Beckman Coulter, #344058). Media-containing tubes were loaded into SW32 Ti rotor (Beckman Coulter) and ultracentrifuged at 25,000 rpm for 2 h. at 4°C with no brake. Supernatant was discarded and viral pellets were left to dry at room temperature for 10 min. Viral pellets were resuspended in complete media totaling 240 ul per condition resulting in a 1000x concentration.

<table>
<thead>
<tr>
<th>Plasmid/Reagent</th>
<th>Function</th>
<th>Mass per plate (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-VSVG</td>
<td>Lentiviral packaging</td>
<td>4.98</td>
</tr>
<tr>
<td>RSV-REV</td>
<td>Lentiviral packaging</td>
<td>11.25</td>
</tr>
<tr>
<td>MDLg-RRE</td>
<td>Lentiviral packaging</td>
<td>22.49</td>
</tr>
<tr>
<td>pEIT</td>
<td>Expression vector</td>
<td>22.49</td>
</tr>
<tr>
<td>pEIT-hLIP</td>
<td>Expression vector</td>
<td>22.49</td>
</tr>
<tr>
<td>Optimem</td>
<td>Low serum media</td>
<td>2247 ul</td>
</tr>
</tbody>
</table>

**Lentiviral tittering**

HEK293T cells were seeded at 7.0x10⁵ cells per well in 24 well plates in complete media and incubated overnight at 37°C, 5% CO₂. The following day, one well of cells were trypsinized and counted at time of transduction. Using complete media, serial dilutions ranging from 10⁻² to 10⁻⁷ were made with concentrated viral media in 250 ul volumes. Complete media was removed and 200 ul of each serial dilution was added to
each well. 24 h. post-transduction, viral media was removed and replaced with complete media. 72 h. post-transduction, cells were trypsinized and brought to FACs analysis for quantitation of fluorescence. Titer was then calculated by the formula below.

\[ \text{titer} \left( \frac{\text{TU}}{\text{ml}} \right) = \frac{P \times N}{D \times V} \]

- \( P \) = percent of fluorescent cells (i.e. 20% equals 0.2)
- \( N \) = number of cells at time of transduction
- \( D \) = dilution factor (i.e. \( 1 \times 10^{-3} \))
- \( V \) = viral inoculum (i.e. 0.2 ml)

**Lentiviral transduction**

MCF10DCIS.com cells were seeded at 500,000 cells per well in complete media and incubated overnight at 37°C, 5% CO\(_2\). 24 h. after plating, cells were treated overnight with lentivirus at MOI 3 (calculation shown below) and 8 ug/ul polybrene (Millipore Sigma, #28728-55-4) in a total volume of 1 ml of complete media. The next morning, viral media was removed and replaced with complete media. Cells were analyzed by FACS 72 h. post-transduction.

**Protein extraction and quantitation**

Adherent cells were washed 2x with ice cold DBPS and treated with 1X RIPA at a concentration of 100 ul per 1.0x10\(^6\) cells. Cells were manually scraped to remove from the plate then the mixture was transferred to 1.5 ml Eppendorf tubes and placed on ice. Cells were passed 10 times through a 26 G needle to shear cell membrane and nucleocomplexes. Samples were rocked at 4°C for 15 min. to homogenize, then centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were then transferred to new
tubes and protein concentrations were quantitated using the Pierce™ BCA Protein Assay Kit (ThermoFisher #23225) according to manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Components</th>
<th>Manufacturer/Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA extraction buffer</td>
<td>8.1 ml DI water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05g sodium deoxycholate</td>
<td>DOC Sigma #D6750-25g</td>
</tr>
<tr>
<td></td>
<td>300 ul 5M NaCl</td>
<td>Fisher #AC424290010</td>
</tr>
<tr>
<td></td>
<td>1 ml of 10% NP-40 (igapel)</td>
<td>Sigma #I8896-100ml</td>
</tr>
<tr>
<td></td>
<td>300 ul 1M Tris, pH 7.5</td>
<td>Fisher #BP1521</td>
</tr>
<tr>
<td></td>
<td>1 tablet PhosSTOP</td>
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<tr>
<td></td>
<td>1 tablet Complete Ultra</td>
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</tr>
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</table>

**Immunoblotting**

50 ug of protein lysates were mixed with 12.5 ul of 4X loading dye, 1M DTT to 0.4 uM final concentration, and DI H₂O to a total volume of 50 ul per sample. Samples were boiled for 5 min. at 100°C then transferred to ice. Samples were spun at 4°C for 1 min. at 14,000 rpm to collect condensation from tube lid, then loaded into the 10% tris-gly gels (Thermofisher XP00100BOX) contained in a Novex Mini-Cell XCell SureLock™ electrophoresis chamber filled with 1X running buffer (Novex LC2675-5, diluted 10x with DI H₂O). 10 ul of MagicMarkXP™ chemiluminescent protein ladder was added to a single well to estimate protein band size. Samples were run for 25 min. at 70V until samples lined up in the stacking gel, then voltage was increased to 115V for 1.5 h. Transfer buffer was made while gels ran and placed at -20°C to cool before gel transfer to membrane. Invitrolon PVDF filter paper membranes (ThermoFisherLC 2005, 0.45 uM pore size) were activated in methanol for 30 s., then soaked in transfer buffer for 5 min. After electrophoresis, the gels were removed from cassettes and wet transfers were performed in BioRad Mini Trans-Blot® cell PROTEAN® Tetra Systems in 1L of transfer buffer according to manufacturer’s instructions. Transfer occurred at 100V for 1 h. at
4°C. Membranes were treated with 5 ml Ponceau stain (5% acetic acid [Fisher UN789], 0.01% Ponceau S stain [Sigma P3504-10G]) while rocking and destained with 1% acetic acid to visualize protein bands before blotting. Membranes were rinsed to remove Ponceau stain, then blocked with 5% milk (Nestle Carnation NFDM) in 1X Tris-buffered saline (TBS [Alfa Aesar #62662-K3]) with 0.1% Tween (Sigma #P7949-500ml) (TBST) for 1 hr. with rocking at room temperature. Primary antibodies were then applied to the membranes according to the table below and rocked overnight at 4°C.

Membranes were washed 1X for 15 min., then 2x for 5 min. at room temperature. The appropriate horseradish peroxidase-conjugated secondary antibodies were diluted in 5% milk in TBST and were applied to membranes with rocking for 1 hr. at room temperature. The HRP-conjugated secondary bands were treated with Pierce ECL Western Blot Substrate (ThermoFisher #32106) according to manufacturer’s instructions and imaged on a BioRad ChemidocMP imaging system.

<table>
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<tbody>
<tr>
<td><strong>4x loading dye</strong></td>
<td>5 ml 1M Tris, pH 6.8</td>
<td>Fisher #AC327360010</td>
</tr>
<tr>
<td>(200 mM Tris, pH 6.8, 400 nM DTT, 8% SDS, 0.04% Bromphenol blue, 40% glycerol)</td>
<td>2 g SDS</td>
<td>Sigma #L-3771-100g</td>
</tr>
<tr>
<td></td>
<td>10 ml 1M DTT</td>
<td>Sigma#43815-5G</td>
</tr>
<tr>
<td></td>
<td>0.01 g BPB</td>
<td>Sigma #B0126-25G</td>
</tr>
<tr>
<td></td>
<td>10 ml glycerol</td>
<td>Sigma #G5516-1L</td>
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<tr>
<td><strong>Transfer buffer</strong></td>
<td>28.0 g glycine</td>
<td>Fisher BP381-1</td>
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<tr>
<td>(200 mM glycine, 25 mM Tris base, 40% methanol)</td>
<td>6.0 g Tris base</td>
<td>Fisher #AC327360010</td>
</tr>
<tr>
<td></td>
<td>400 ml methanol</td>
<td>Fisher A412-4</td>
</tr>
<tr>
<td></td>
<td>1.6 L DI H$_2$O</td>
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<table>
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<th>Antibodies</th>
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<tbody>
<tr>
<td>C/EBPβ</td>
<td>1:200</td>
<td>Santa Cruz SC79-62</td>
</tr>
<tr>
<td>β actin</td>
<td>1:1000</td>
<td>Cell Signaling #4967</td>
</tr>
<tr>
<td>Goat anti-mouse IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32230</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32260</td>
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</table>
RNA extraction, cDNA construction, and RT-qPCR

RNA was extracted using Trizol® reagent (Ambion #15596026) according to manufacturer’s instructions. To remove trace genomic DNA, samples were treated with DNA-free™ DNA Removal Kit according to manufacturer’s instructions. RNA purity and concentration was determined by NanoDrop2000 spectrophotometer (ThermoFisher ND2000CLAPTOP). After concentration was determined, cDNA synthesis was performed using 1000 ng of RNA and the iScript cDNA Synthesis kit (BioRad #170-8891). Thermal cycler settings (Applied Biosystems, 2720 thermal cycler) for cDNA synthesis are outlined in the table below. RNA was stored at -80ºC once quantified.

<table>
<thead>
<tr>
<th>cDNA synthesis conditions</th>
<th>25ºC</th>
<th>5 min.</th>
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<tr>
<td>46ºC</td>
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<tr>
<td>95ºC</td>
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<td>4ºC</td>
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Reverse-transcriptase quantitative PCR (RT-qPCR) was performed on cDNA samples to quantitate relative expression of genes of interest. Bio-Rad iQ™ SYBR® Green Supermix (Bio-Rad #1708880) was combined with 10 uM of primers (hAx1 forward 5’-CGTAACCTCCACCTGGTC-3’, hAx1 reverse 5’-TCCCCATCGTCTGACAGCA-3’, 18s forward 5’-GTAACCGTTGAACCCATT-3’, and 18s reverse 5’-CCATCCAAATCGGTAGTAGCG-3’), 1ul of cDNA, and nuclease-free water totaling 20 ul and added to 96 well plates and sealed with plastic film (Bio-Rad 2239441, Bio-Rad MSB1001, respectively). The sealed reaction plates were briefly pulsed in the centrifuge to collect all liquid in the bottom of the well, then placed on thermal cycler (Bio-Rad Real-Time System CFX96) with the following program:
Melt curves were analyzed to ensure specific amplification products and relative expression values were calculated using the $2^{-\Delta\Delta C_T}$ (delta-delta CT) method in the Bio-Rad CFX Manager software program.

**Plasmids and cloning for promoter assays**

Human C/EBP\(\beta\) plasmid constructs in the pHIV-Zsgreen (pEIZ, Addgene #18121) backbone were kindly donated by Brian Welm. Human C/EBP\(\beta\) plasmid constructs were digested from their pEIZ backbone. Similarly, SP1 was digested from the pOTB7 backbone. pOTB7-SP1 plasmid provided by Dr. Gerry Rubin, clone ID HsCD00338893. Each hC/EBP\(\beta\) DNA construct and SP1 were cloned into pHIV-dTomato (pEIT, Addgene #21374) using BamHI-HF and EcoRI-HF restriction sites. Digestion reaction included 1 ug of DNA, 5 ul of 10X CutSmart Buffer, 1 ul of BamHI-HF and EcoRI-HF enzymes incubated at 37°C for 15 min. To inactivate digestion enzymes, samples were incubated at 60°C for 1 hr. Digested plasmids were run on a 1.5% agarose gel for 45 min. at 100V, then gel purified using PureLink Quick Gel Extraction Kit (Invitrogen #K2100-12). Gel-purified hC/EBP\(\beta\) DNA isoforms and SP1 were then
ligated for 15 min. at RT into 25 ng of pEIT at a molar ratio of 1:2 insert:vector using T4 ligase (New England Biolabs, #M0202). Samples were incubated at 65ºC for 10 min. then transformed into New England Biolabs Stable Competent *E. coli* (NEB #3040H) using standard manufacturers’ protocol. Cells were streaked onto selection plates to isolate single colonies which were screened for correct insertion by restriction digest.

**Luciferase promoter assay**

Dual-Luciferase® Reporter Assay System (Promega, E1910) was used to determine promoter binding activity according to manufacturer’s protocols. HEK293T cells were seeded at 9.0x10^4 per well in a 24 well dish and incubated over night at 37ºC, 5% CO₂ in complete media. The following morning, when cells reached 80% confluency, cells were transfected with 25 ng of pRL-SV40, 500 ng of Axl construct plasmid (either pGL3-Axl-556 or pGL3-Axl-2376), and 500 ng of expression vector (either SP1, pEIT, pEIT-LAP1, pEIT-LAP2, or pEIT-LIP) with Lipofectamine™ 3000 (ThermoFisher L3000015) according to manufacturer’s instructions. Transfection reagent was removed from cells after 8 h. and replaced with complete media to avoid toxicity issues. 48 h. after transfection, cells were washed with DPBS, then lysed with 100ul Passive Lysis Buffer (PLB) and shaken on an orbital shaker for 15 min. at room temperature. Luciferase Assay Reagent II (LARII) was prepared according to manufacturer’s instructions and was 100ul was dispensed into appropriate wells in a 96 well plate. 20ul of sample lysate was added to appropriate wells and firefly luciferase activity was recorded on a BioTek Gen5 microplate reader. To measure the Renilla luciferase activity, 100ul Stop N Glo reagent
was added to each well. Firefly luciferase values were normalized to Renilla internal control luciferase values.

**CHiP-Seq in silico analysis**

Extraction protocol and library preparation for are as described in GSM23330565. FastQ files were downloaded from NCBI Gene Expression Omnibus and uploaded to Galaxy open source online resource (usegalaxy.org). Files were subjected to quality control (FastQC) then converted to BAM using Galaxy standard pipeline. Reads were mapped to genome Hg38 and visualized with UCSC Genome Browser. Mapped read peaks indicate reads per million transcripts (rpm). C/EBPβ antibody used for immunoprecipitation is SantaCruz Biotechnology, catalog #SC-150X, which is specific for the C-terminal region, allowing for detection of all three C/EBPβ isoforms.

**Statistical data analysis**

To determine statistical significance for luciferase promoter assays and RT-qPCR, unpaired two-tailed Student’s T tests were performed using software GraphPad Prism version 9.1.2 (225) in triplicate. For each data set, mean and standard deviation of the mean are reported. P-values of p<0.05(*), p<0.01(**), p<0.001(***), and p<0.0001(****) were labeled and reported as such.
Results

Investigation of C/EBPβ and the Axl promoter

To determine the binding relationship between C/EBPβ and the Axl promoter, we first consulted a published CHiP-Seq dataset to determine if C/EBPβ directly binds the Axl promoter in a relevant model system. CHiP-Seq analysis of human TNBC cell line SUM159 indicated that C/EBPβ binds multiple sites within the Axl coding region, as well as multiple upstream sites that may function as 5’ proximal enhancer regions within 20 kb from the transcription start site (Figure 4). Our results confirmed the physical interaction of C/EBPβ with the Axl coding region. To determine which isoforms activate the Axl promoter, we next designed dual luciferase promoter assays in HEK293T cells which lack C/EBPβ and AXL expression, as shown by immunoblot (Figure 5C). A prior graduate student, Sheng Zheng, cloned the full length and basal Axl promoter sequences as defined by Mudduluru et al.113 into the pGL3-Basic vector (Figure 5A). We confirmed proper integration of promoter sequences by restriction digest (Figure 5B) and continued to co-transfect our promoter plasmids of interest with C/EBPβ-expressing plasmids equipped with dTomato expression cassettes. We confirmed co-transfection of dTomato-expressing C/EBPβ isoform expression vectors by analyzing dTomato fluorescence and immunoblotting for each C/EBPβ isoform (Figure 5C). After performing the dual luciferase assay, we observed that all C/EBPβ isoforms can activate both promoter sequences (Figure 6). LAP2 is generally the most expressed isoform in breast cancer cells, which our data confirmed, as LAP2 bound the basal and full-length promoter sequences to the greatest degree (9.64 and 18.89 R.L.U., respectively). Interestingly, LIP bound
both Axl constructs to a greater statistically significant degree than SP1, a known Axl transcriptional activator (8.66 vs. 2.10 R.L.U. for basal and 9.51 vs. 5.31 R.L.U. for complete, respectively).

C/EBPβ isoform overexpression induces Axl expression

Given that C/EBPβ binds the Axl promoter as determined by our in silico CHiP-Seq analysis and luciferase promoter assay, we sought to determine if each C/EBPβ isoform could induce Axl expression. While LAP1 and LAP2 failed to increase or repress Axl transcription to any significant degree, LIP overexpression in DCIS.com cells increased relative Axl transcripts by 3.4-fold compared to empty vector (pEIT) (Figure 7). Additionally, immunoblotting for AXL and C/EBPβ protein indicates that LIP may increase AXL protein expression compared to pEIT empty vector control, though no densitometry or statistical methods were applied.
ChIP-Seq analysis of SUM159 TNBC cell lines indicates C/EBPβ binds the promoter region as well as the 5’ proximal enhancer region of Axl. Mapped peaks indicate read density defined as units of reads per million mapped reads (rpm). Axl highest mapped peaks 20.2 and 46.9 rpm, respectively. IL-6 gene, a known C/EBPβ target, provided for a positive control with highest promoter and enhancer peaks measuring 46.2 and 29.7 rpm, respectively. Promoter region defined as +/-5 Kb upstream of TSS and 5’ proximal enhancer region defined as 5 Kb – 20 Kb upstream of TSS. GSM2330565

**Figure 4 C/EBPβ binds Axl in TNBC SUM159 cells**
A.) The Axl full length and basal promoter sequences as defined by Mudduluru et al. were cloned into pGL3-Basic luciferase-expressing vectors by a previous graduate student Sheng Zheng. Sites labeled +1 refer to transcription start site. B.) Proper promoter sequence insertions were confirmed by BamHI and NotI restriction digest of 1 ug plasmid. (U = undigested, D = digested). C.) HEK293T cells were then co-transfected with Axl promoter plasmids and dTomato-expressing C/EBPβ expression vectors. Fluorescent images depict transfection efficiency of C/EBPβ vectors (10X magnification) along with immunoblotting to detect C/EBPβ protein expression in transfected cells.

**Figure 5** Axl promoter cloning strategy for luciferase promoter assay
Figure 6 Each C/EBPβ isoform activates Axl promoter constructs

HEK293Ts were transfected with expression vectors (pEIT) expressing either SP1, a known activator of Axl, or individual C/EBPβ isoforms. Basal Axl promoter construct refers to a region -556 bp to TSS, which has been shown to activate a basal-level transcriptional program. The complete Axl promoter construct is -2376 bp to TSS. R.L.U refers to Relative Luminescence Units normalized to Renilla luciferase, an internal control for transfection efficiency. Statistical significance was determined by unpaired Student’s T test where *P <0.05, **P<0.005, ***P<0.0005 and n=3 replicates.
Figure 7 C/EBPβ-LIP induces Axl expression in DCIS.com cells

DCIS.com cells were transduced with C/EBPβ isoform-expressing lentivirus at an MOI of 1. 72 hr. post transduction, RNA was isolated for quantitation of Axl transcripts using RT-qPCR, which showed a 3.4-fold increase upon LIP overexpression. Statistical significance was determined by Student’s T test, P = 0.0281, n = 3 replicates. Only C/EBPβ-LIP yielded significant changes in Axl transcripts. Immunoblotting indicated satisfactory overexpression of each C/EBPβ isoform in transduced DCIS.com cells.
Discussion

The goal of Specific Aim 1 is to determine if C/EBPβ functions as a direct transcriptional activator of the Axl promoter. Constitutive activation of RTK signaling is a common effect driving proliferation, metabolism, and invasiveness in transformed breast cells; therefore, it is imperative to understand the transcriptional regulatory mechanisms dictating RTK function in tumorigenesis. The Axl signaling pathway has been implicated in multiple mechanisms of invasiveness and aggressive disease states in breast cancer. Similarly, C/EBPβ, or more specifically LIP, has been heavily implicated in aggressive breast disease and increases during periods of rapid proliferation within the breast. The expression patterns of both AXL and C/EBPβ are similar in the transition from normal cells to transformed tumor cells which provides rationale to examine the possibility that C/EBPβ can influence Axl activation. Here we characterize aspects of the interactions between C/EBPβ and AXL expression.

We sought to address physical binding of C/EBPβ at the Axl locus by analyzing a publicly available ChIP-Seq dataset. Our analysis demonstrated that C/EBPβ can physically interact with the Axl promoter and 5’ upstream enhancer regions defined as within +/- 5 Kb of the TSS and 5-20 Kb upstream of the TSS, respectively. The data indicate that C/EBPβ can bind the Axl promoter site, though not to the extent of the known C/EBPβ transcriptional target Il6 (Figure 4); however, the results suggest that C/EBPβ can serve as an activator of Axl transcription. This is the first indication of the physical interaction between the two genes within the context of a breast cancer cell line; however, the data provide no context as to which C/EBPβ isoforms are bound to the
promoter, as the antibody used for pulldown identifies all three isoforms. Here, we are interested specifically in the activation of Ax/l by C/EBPβ-LIP, but further studies were necessary to address whether each isoform can activate Ax/l independently.

To address our interests in isoform-specific Ax/l activation, we performed dual-luciferase promoter assays in HEK293T cells which lack both AXL and C/EBPβ expression (Figure 5C). Our data demonstrate that in HEK293T cells, each C/EBPβ isoform can activate both the basal and full length Ax/l constructs (Figure 6). Unsurprisingly, as LAP2 is usually the most abundantly expressed isoform in most cells, LAP2 proved to activate the basal and full-length promoter sequences most strongly (9.6-fold increase and 18.8-fold increase, respectively). Interestingly, LIP was able to bind and activate both Ax/l constructs as well (8.6-fold and 9.5-fold R.L.U., basal and full) in both circumstances inducing transcription to a greater degree than known Ax/l activator SP1 (2.1-fold and 5.4-fold increases, respectively). These results are intriguing as the historical role of LIP mostly recognized its capability to inhibit transcription, with limited examples of its activation potential. While these results were encouraging and demonstrated feasibility of a physical LIP-Ax/l interaction, we recognized the need to determine the transactivational potential of LIP in a model more physiologically relevant to breast cancer.

To establish the relationship between C/EBPβ and AXL within the context of breast cancer, we performed expression studies in cells that express moderate amounts of both proteins. When we overexpressed LIP within DCIS.com cells, a transformed breast cancer cell line, we saw a statistically significant increase in Ax/l transcription (4.6-fold induction), confirming our prior promoter assay results in a more appropriate model
(Figure 7). We then examined AXL protein expression in the same LIP-overexpressing cells and noticed that AXL expression seemed to visibly increase, though no protein quantification techniques were applied. As LIP increased Ax/l transcription in DCIS.com cells, we demonstrated that LIP has the true capacity to activate Ax/l rather than merely causing AXL protein stabilization.

Taken together, here we demonstrated for the first time that C/EBPβ can physically interact with the Ax/l locus in the aggressive TNBC SUM159 cell line. In addition, we functionally demonstrated that the interaction of each C/EBPβ isoform with the Ax/l locus resulted in transcriptional activation as shown by promoter assay and RT-qPCR, despite the typical role of LIP as a transcriptional repressor. These data provide a novel role for LIP in breast cancer with its ability to induce AXL, a known driver of invasiveness and metastasis.
Chapter 3: Generation of C/EBPβΔ/Δ transgenic mouse mammary tumor models

Introduction

The following chapters addressing Specific Aims 2 and 3 seek to identify the contributions of epithelial C/EBPβ to mammary tumorigenesis and how it subsequently influences the surrounding immune microenvironment. To address these aims, we generated two novel transgenic mouse models to genetically ablate C/EBPβ and evaluate resulting tumorigenesis. The two models generated to address the following aims differ in their Cre recombinase delivery, their mechanisms of oncogenesis, and the molecular tumor subtype allowing for a robust examination of the roles of C/EBPβ in very distinct, yet clinically relevant, tumor models.

The first tumor model consists of a spontaneous MMTV-Neu-IRES-Cre (NIC) which allows for a genetic mechanism of Cre recombinase delivery to excise the C/EBPβ locus from the initial tumor-causing oncogenic hits. We crossed in a potent, ubiquitously-expressed fluorescent Cre-recombinase reporter strain, Rosa<sup>mT/mG</sup>, to easily validate Cre recombination in the mammary issues of interest. Animals with our three genotypes of interest were aged and added to tumor progression studies once their genotypes were confirmed.

The second model utilizes an immune-competent transplantable basal-like C3(1)-TAg model which deletes C/EBPβ from existing tumors. This model allowed for
spontaneous tumors to develop in C3(1)-TAg female mice whose tumors were harvested and treated with an adenoviral Cre recombinase to exogenously delete C/EBPβ. The cells were then transplanted into the mammary fat pads of an immunocompetent mouse strain tolerant to C3(1)-TAg tumors, allowing for tumor progression studies in a unique model with fully intact immune compartments.

**Background and generation of the Rosa\textsuperscript{mT/mG};NIC;Cebpb\textsuperscript{Δβ} transgenic mouse model**

*Neu* is the mouse and rat ortholog to HER2 which serves as a protooncogene in mammary cancers. It functions as an RTK that drives mammary carcinogenesis when amplified or mutated to render it constitutively active\textsuperscript{115}. The mechanism of *Neu*-driven mammary oncogenesis under the MMTV long terminal repeat (LTR) was first characterized by Siegel et al. in 1999. MMTV-Neu mice develop multifocal, solid mammary tumors with an average latency of around 7.6 months (228 days). *MMTV-NIC* mice used in our studies are derived, however, from a slightly faster-growing *MMTV-Neu* substrain (NDL2-5) due to a deletion in the coding region in the extracellular domain rendering *Neu* constitutively active in the mammary epithelium through intramolecular disulfide bond stabilization.\textsuperscript{115,116} This substrain, NDL2-5, was used to generate the *MMTV-NIC* model with the addition of a Cre recombinase sequence preceded by an IRES site, allowing for a distinct translational control region resulting in a 1:1 ratio of oncogene to Cre recombinase expression.\textsuperscript{117} This stochiometric ratio ensures that every tumor cell expressing constitutively activated *Neu* will also express Cre recombinase,
preventing the “tumor cell escape” phenomena.\textsuperscript{117} Unlike the slower-growing original MMTV-Neu strain, the two strains NDL2-5 and the Cre-recombinase-expressing MMTV-NIC model have similar mammary tumor latencies in vivo (161 days vs. 146 days, respectively).\textsuperscript{117,118} However, the genetic manipulations to the founding MMTV-Neu strain resulting in the NDL2-5 and MMTV-NIC substrains resulted in no changes in histological phenotype, as all three models are histologically defined as mammary adenocarcinoma solid tumors.\textsuperscript{112,113} An additional benefit to the MMTV-NIC mouse strain is that this substrain only develops mammary tumors compared to the MMTV-Neu mice and NDL2-5 substrains. In all, the development of MMTV-NIC transgenic mice allowed for a mammary tumor model resembling a clinically relevant HER2 activating mutation which allows for precise expression of the rat Neu oncogene and Cre recombinase to the mammary gland.\textsuperscript{115,118,119}

To develop the $\text{Rosa}^{\text{mT/mG}};\text{MMTV-NIC};\text{Cebpb}^{\Delta/\Delta}(\text{NIC};\text{Cebpb}^{\Delta/\Delta})$ transgenic mouse model used in our tumor studies, we crossed $\text{Cebpb}^{\text{fl/fl}}$ mice maintained in-house on the FVB background to the MMTV-NIC mice described previously that were generously gifted by Dr. Bill Muller and provided by Dr. Steve Anderson in 2018 (Figure 8). $\text{Cebpb}^{\text{fl/fl}}$ mice contain lox P sites, the targets for site-specific Cre-recombinase activity, flanking the endogenous $\text{Cebpb}$ locus on both alleles, followed by a neomycin cassette as described in Sterneck et al. in 2006.\textsuperscript{120} In addition, we bred in a transgenic strain that functions as a potent fluorescent Cre-recombinase reporter, $\text{Rosa}^{\text{mT/mG}}$, to easily validate by fluorescence microscopy and immunofluorescence that Cre recombination was confined specifically to the mammary epithelium in tumors.\textsuperscript{121} Because the $\text{Rosa}^{\text{mT/mG}}$ Cre-recombinase reporter integrated at the Rosa26 locus and is
driven by the chicken beta actin (pCA) promoter, fluorescence is both ubiquitously-expressed and constitutively active in all tissues. By breeding the Cebpb^{fl/fl} mouse strain, the Rosa^{mT/mG} transgenic mice, and the previously described MMTV-NIC mice, we generated a novel mouse strain with three genotypes to utilize in subsequent tumorigenesis studies: Rosa^{mT/mG};NIC;Cebpb^{+/+}; Rosa^{mT/mG};NIC;Cebpb^{+/-}; and Rosa^{mT/mG};NIC;Cebpb^{fl/fl}. We determined deletion of C/EBPβ by the replacement of dTomato expression with GFP expression in the mammary epithelium, as shown in Figure 9.

**Background and generation of the Rosa^{mT/mG};C3(1)-TAg;Cebpb^{ΔΔ} transgenic mouse model**

The second model we generated to address how Cebpb contributes to mammary tumorigenesis utilizes the spontaneous C3(1)-TAg tumor strain crossed to the previously described Cebpb^{fl/fl} and Rosa^{mT/mG} strains (Figure 10). Deletion of Cebpb from isolated tumor cells of interest occurred ex vivo upon treatment with an adenoviral Cre recombinase (Ad5.CMV.Cre) (Figure 11). Virus-treated cells were then immediately transplanted into immunocompetent mice tolerized to the SV40 transgene or into athymic immunocompromised mice and subjected to subsequent tumorigenesis studies.

The spontaneous C3(1)TAg mice were described initially by Green et al. in 2000. C3(1)-TAg mice progress through histologically distinct phases of mammary adenocarcinoma development similar to that of DCIS progression in human patients, making it a useful tool for functional DCIS studies and further tumor studies. They genetically resemble basal-like or TNBC in human patients, making them a clinically and
physiologically relevant model for TNBC studies.\textsuperscript{122,124,125} The C3(1) promoter transgene cloned from rats is expressed primarily in the mammary glands of female mice likely due to its shared protein motifs with the mammoglobin family, whose members are characterized by 5’ upstream sequences containing \textit{cis}-acting elements targeting expression specifically to the mammary chain.\textsuperscript{126} The C3(1) promoter drives SV40 transgene expression in this model, thus initiating oncogenesis through the inactivation of \textit{Trp53} and \textit{Rb1} which results in multifocal adenocarcinoma development in the mammary glands.\textsuperscript{127,128} This C3(1)-TAg tumor line was crossed with the \textit{Cebpb}\textsuperscript{0/0} and \textit{Rosa}\textsuperscript{mt/mG} strains to generate either a \textit{Rosa}\textsuperscript{mt/mG};\textit{Cebpb}\textsuperscript{0/0};\textit{C3(1)TAg} or a \textit{Rosa}\textsuperscript{mt/mG};\textit{Cebpb}\textsuperscript{+/+};\textit{C3(1)TAg} spontaneous tumor which was harvested, treated with Ad5.CMV.Cre for genetic ablation of \textit{Cebpb}, and transplanted. These techniques using the aforementioned model allowed us to examine the effect of \textit{Cebpb} ablation after the mammary epithelium had been transformed and progressed to solid tumors, unlike the NIC model which examined the effect of \textit{Cebpb} in the initial transformation process.

A benefit of this C3(1)-TAg transplantable model allowed us to examine the effects of \textit{Cebpb} deletion in tumor cells in an immunocompetent model. Immunocompetent wild type FVB mice are not suitable recipients for the transplantable C3(1)TAg model, as the SV40T antigen causes graft rejection; therefore, we purchased from Jackson Laboratories and bred in house a C3(1)TAg substrain, C3(1)Tag-REAR (abbreviated REAR forward) to serve as transplant recipients. C3(1)TAg mice contain six copies of the C3(1)TAg transgene on chromosome 6 containing the Ki-ras protooncopogene which drives mammary and prostate oncogenesis. The REAR substrain developed as a result of a spontaneous rearrangement at the C3(1)TAg locus, leaving only a single copy
of the C3(1)TAg transgene.\textsuperscript{129} The single copy of SV40T is insufficient to drive any mammary hyperplasias and is indetectable by immunohistology; however, it is sufficient to tolerize the animals to SV40T, allowing for a useful tool for C3(1)TAg transplant studies.\textsuperscript{129}
The three mouse strains containing genetic constructs pictured above (A.) were crossed to generate mice of the genotypes listed in B.

Figure 8 Genetic constructs utilized in Rosa\textsuperscript{mT/mG};NIC;Cebpb\textsuperscript{\(\Delta\Delta\)} transgenic mouse model
A.) Immunofluorescence detecting GFP and RFP in NIC;Cebp√/√ tumors indicates Cre recombination is restricted to the mammary tumor epithelium, while the stroma retains dTomato expression (20x magnification). B.) Fresh whole mount analysis (1x magnification) indicates that NIC;CebpΔ/Δ mammary epithelium has undergone Cre recombination as indicated by endogenous GFP expression.

Figure 9 GFP expression indicates recombination in mammary epithelium in NIC;Cebp√/√ tumors and NIC;CebpΔ/Δ mammary glands.
The three mouse strains containing genetic constructs pictured above (A.) were crossed to generate mice of the genotypes listed in (B.) Spontaneous tumors that developed were then used in transplant studies.

Figure 10 Genetic constructs utilized in Rosa<sup>mT/mG</sup>;C3(1)TA<sup>g</sup>;Cebp<sup>b<sup>+/+</sup></sup> and Rosa<sup>mT/mG</sup>;C3(1)TA<sup>g</sup>;Cebp<sup>b<sup>fl/fl</sup></sup> transgenic mouse model
To determine the best time point to assess deletion of *Cebpb* for future experiments in our model of interest, we harvested *Cebpb^fl/fl;C3(1)Tag* tumor cells, transduced them with Ad.GFP or Ad.Cre, and collected protein lysates every 24 h. beginning at time of transduction (0 hr., 24 hr., 48 hr., 72 hr.).

**Figure 11 Cre recombination is complete in C3(1)Tag tumor cells by 72 hr. post-transduction**
**Discussion**

The major benefits of utilizing these two models are that we can study both the initial contributions of C/EBPβ to the early phases of tumor progression as well as elucidate potential transcriptional programs governed by C/EBPβ in solid tumors by deleting C/EBPβ in different phases of tumorigenesis. While these tumor models differ significantly, they provide us powerful genetic tools to address how C/EBPβ contributes to mammary tumorigenesis in two clinically relevant models representing different molecular subtypes, oncogenic mechanisms, and timing of genetic ablation.
Chapter 4: C/EBPβ is a fundamental driver of mammary tumorigenesis

Specific Aim 2 is designed to address the role of C/EBPβ in mammary tumor initiation and progression. Here we ablate epithelial-derived C/EBPβ by two methods and study resulting tumorigenesis. These experiments utilize the transgenic mouse models described in Chapter 3.

Introduction

As previously mentioned in Chapter 1, C/EBPβ is a ubiquitously expressed transcription factor that dictates a wide array of cellular functions including, but not limited to, proliferation, differentiation, metabolism, and senescence. The C/EBP transcription factor family contains six members, three of which are expressed within the rat and mouse mammary gland and may serve overlapping functions; however, C/EBPβ alone has been proven as essential for normal mammary gland development. As C/EBPβ is a critical regulator of various developmental and homeostatic functions within the mammary gland, interest grew in the roles of C/EBPβ in breast cancer. Previously mentioned in Chapter 1, C/EBPβ consists of three isoforms – LAP1, LAP2, and LIP – the functions of which have been heavily investigated in the developing mammary gland and in cancer. Multiple studies have shown a strong correlation between LIP expression and aggressive breast disease. Gomis et al. found that altering the LIP:LAP ratio in
TNBC cells reduced metastasis in a human xenograft model, demonstrating for the first time in vivo that LIP reduction in aggressive tumors can redirect invasive processes toward cytostasis through a TGFβ-mediated mechanism. While isoform-specific functions within the context of breast cancer are important to understand, there remains a knowledge gap regarding how, and if, C/EBPβ as a whole contributes to mammary tumor initiation and progression. Several studies have tried to address the relationship between breast cancer survival and overall C/EBPβ expression, but have generated conflicting results; therefore, there is a critical need for a genetic model to ablate C/EBPβ and assess resulting mammary tumorigenesis.

To address the knowledge gap regarding the role of C/EBPβ in mammary tumor initiation, we designed studies to ablate C/EBPβ from mammary epithelium in two tumor models. We hypothesized that the deletion of C/EBPβ will delay onset of mammary tumorigenesis in the NIC and C3(1)TAg models, thereby increasing survival. Surprisingly, the performed studies confirmed that C/EBPβ is a critical driver of mammary tumor formation and the ablation of C/EBPβ in existing tumor cells leads to tumor clearance in vivo.

**Materials and Methods**

*Kaplan Meier survival analysis*

Patient overall survival curves were generated using kmplot.com. For *Cebpb* transcripts, overall survival curve analysis included genechip expression data from all breast cancer subtypes following systemic chemotherapy or neoadjuvant therapy (GSE1456, GSE16446, GSE16716, GSE20271, GSE20685, GSE20711, GSE3494,
GSE37946, GSE42568, GSE45255, GSE7390, n = 1879). Statistical significance was determined using Cox hazard statistical test (p = 2.6E-04, FDR 20%, HR = 1.48 [1.2 – 1.84]), with auto select best cutoff function enabled (cutoff value 5022). For protein values, the patient subset was defined as lymph node-negative, neoadjuvant naïve status with unilateral TNBC primary tumor. C/EBPβ protein expression data was generated by nLC-MS/MS (PMID: 24399849, n = 96, Cox hazard ratio statistical test, p = 0.01, FDR = 50%). C/EBPβ protein expression values are representative of total expression, not individual informs.

**Mouse strain maintenance**

All mice utilized in tumor studies were maintained on FVB/NJ genetic background. Animals were maintained and housed in Tulane School of Medicine Vivarium, in accordance with proper handling and maintenance conditions according to federal laws and the Institutional Animal Care and Use Committee (IACUC, protocol #710). All experiments were performed with prior authorization. Animals were housed in vented racks and fed ad libitum. Males were bred no earlier than 6 weeks of age and females were bred at no earlier than 8 weeks of age. Breeding pairs or harems were provided breeding huts for enrichment to encourage reproductive performance and increase female lactation.

*Cebpb* animals were initially provided by Dr. Esta Sterneck and were backcrossed to the FVB/NJ background. Animals were maintained as homozygous. C3(1)TA (Jackson Laboratory, strain #013591), REAR (Jackson Laboratory, strain #030386) and Rosa26<sup>tm1TmG</sup> (Jackson Laboratory, strain #007576) were purchased from
Jackson Laboratory. C3(1)TAg animals were maintained as hemizygous. C3(1)TAg males were bred to wild type FVB purchased from Jackson Laboratory or from within the colony. REAR and mTmG animals were bred together and maintained as homozygous.

*MMTV-NIC* mice were of mixed strain origin when imported to Tulane University from Dr. Steve Anderson at Colorado University Anschutz in 2019. We therefore backcrossed to wild type FVB/NJ mice (Jackson Laboratories, strain 001800) for six generations to obtain a pure genetic background. Upon the sixth backcross generation, DNA isolated from tail clippings was sent to Jackson Laboratories for strain identification, which resulted in a > 98% sequence alignment with the FVB/NJ background. From these genetic profiling results, we considered the *MMTV-NIC* genotype to be fully fixed to the FVB background and continued tumorigenesis studies from this generation onward.

*Mouse genotyping*

At 18-21 days of age, mice were weaned and ~2 mm tail clippings were collected for genotyping. Tails were digested using the KAPA2G Express Extract and Genotyping kit (Roche catalog #07961804001) kit. Tails were transferred to sterile PCR tubes (USA Scientific #1402-8120, TempAssure PCR tube, 0.2ml) with 5 ul KAPA Express Extract 10X buffer, 1 ul of KAPA Express Extract Enzyme and brought to 50 ul total volume with DI H₂O. Tails and digest mixture were then incubated in the thermal cycler (Applied Biosystems, Thermal Cycler 2720) for 10 min. at 75° for enzyme digestion, followed by 5 min. at 95° for enzyme inactivation.
MMTV-NIC, REAR, and C3(1)TAg genotyping utilized the KAPA2G 2X genotyping kit. To a PCR tube on ice, we added 1 ul of extracted tail DNA, 12.5 ul KAPA2G genotyping mix containing dNTPs and polymerase, 7.75 ul sterile DI H2O, and 1.25 ul of forward and reverse C3(1)TAg primers, and 1.25 ul of forward and reverse IL-12 primers (internal positive control, i.e, IPC). PCR tubes containing genotyping mixture were transferred to a thermal cycler and run with the following program.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°</td>
<td>3 min.</td>
</tr>
<tr>
<td>95°</td>
<td>15 s.</td>
</tr>
<tr>
<td>60°</td>
<td>15 s.</td>
</tr>
<tr>
<td>72°</td>
<td>15 s.</td>
</tr>
<tr>
<td>72°</td>
<td>1 min.</td>
</tr>
<tr>
<td>4°</td>
<td>∞</td>
</tr>
</tbody>
</table>

Cebpb<sup>fl/fl</sup> mouse genotyping required Advantage® GC 2 PCR Kit (TakaraBio, catalog #639120) due to the high GC content across the Cebpb locus. To sterile PCR tubes, we added 2 ul of extracted genomic DNA, 5 ul of 5X GC buffer, 2.5 ul of GC melt, 0.5 ul of dNTPs, 0.5 ul of polymerase, 13.5 ul of sterile DI H2O, and 1 ul of forward and reverse primers. The tubes were transferred to a thermal cycler and run with the following program conditions.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°</td>
<td>2 min.</td>
</tr>
<tr>
<td>94°</td>
<td>30 s.</td>
</tr>
<tr>
<td>68°</td>
<td>30 s.</td>
</tr>
<tr>
<td>72°</td>
<td>30 s.</td>
</tr>
<tr>
<td>72°</td>
<td>5 min.</td>
</tr>
<tr>
<td>4°</td>
<td>∞</td>
</tr>
</tbody>
</table>
PCR products were then separated on agarose gels using gel electrophoresis. All PCR products were run on 1.5% agarose gels except for Cebpb, which was run on a 2% gel. We combined 100 ml of 1X TAE buffer (1X Tris base, Fisher BP1521; acetic acid, Fisher A38500; and EDTA, Fisher BP119500) with either 1.5 or 2 g agarose (Sigma, catalog #A9539) in a 500 ml Erlenmeyer flask and microwaved for 2 min. Molten agarose was cooled to room temperature and 10 ul of ethidium bromide was added. Agarose mixture was poured into gel cassette, comb inserted, then transferred to electrophoresis chamber containing 1X TAE once solidified. PCR products were pipetted into gel wells, along with a 1 KB ladder for band size estimation (Invitrogen, catalog #10787-018), and run at 100V for 1 hr. for all but Cebpb, which ran for 1.5 hr. Bands were visualized using a BioRad ChemiDoc MP imaging system with the Ethidium Bromide setting. Expected band sizes listed in the table above. Gels were disposed of in ethidium bromide waste containers designated by Tulane Environmental Health and Safety.

**Tumor measurement**

Spontaneous and transplanted tumors were measured 1-2x weekly after they reached 5 mm in either height or width. Using calipers, we secured the animal and measured height and width, calculating ellipsoid tumor volume with the below formula.
Regardless of measurement orientation, we defined width as being the smaller measurement.

\[
Tumor\ volume = width^2 \times \frac{length}{2}
\]

**Mouse euthanasia**

Mouse euthanasia was performed in accordance with Tulane University Vivarium protocols. Mice were either placed in CO\(_2\) chambers for 3-5 min. or exposed to inhalation of liquid isoflurane (Fluriso, VetOne, #NDC13985-046-40) until cessation of breathing. Next, we performed cervical dislocation in compliance with the two-step euthanasia method required by Tulane University Vivarium.

**Gland harvesting, fixation, and staining (carmine)**

The abdominal #4 mammary glands were dissected from the animal and spread on a glass slide (FisherScientific, #12-544-7). The slide containing the gland was submerged in Carnoy’s fixative (75% ethanol, 25% glacial acetic acid) in a Coplan histology container overnight at room temperature. Following overnight fixation, glands were transferred to Carmine alum stain (1 g carmine [Sigma, C1022], 2.5 g aluminum potassium sulfate [Sigma A-717] in 500 ml deionized water) overnight at room temperature. The following day, glands were destained and dehydrated in sequential Histoprep ethanol washes (70%, 95%, 100%) (Fisher Scientific #HC8001) for one hour in each ethanol solution. After the final ethanol wash, glands were defatted in xylene (Fisher Scientific #X4-4) for at least one hour at room temperature, then mounted with Permount (Fisher Scientific, #SP15-100) mounting media and imaged 24 h. later.
**Whole mount analysis and imaging (fluorescent)**

Abdominal #4 glands expressing mTmG were harvested from the animal and placed on ice in petri dishes with 500 μl DPBS (ThermoFisher, #14190144) until ready for imaging. Glands were transferred to and pressed between 4” x 3” glass slides and secured with binder clips, then imaged using the Leica M165FC Stereoscope with Nikon DS-Qi-1MC black and white camera. Images were taken at either 0.73x or 1x in both the RFP and GFP channels, then individual channels were merged using Nikon NIS Basic Research software 4.30.01.

**Embedding and sectioning of mammary glands and tumors**

Mammary gland or tumor was dissected from the animal and placed in a plastic cassette labeled with pencil. Mammary glands were fixed for 2 hr. at room temperature in 4% PFA (FisherScientific AC416785000), while tumors were fixed overnight in 4% PFA at 4°C. Tissue cassettes were then transferred to 70% ethanol (700 ml of 100% histoprep ethanol [Fisher Scientific #HC8001], 300 ml deionized water) until embedding in paraffin was in Tulane University Department of Pathology. Embedded glands and tumors were then cut into 5 μM sections using a microtome (Leica, RM2025) and placed in a heated water bath at 42°C (Premiere Lighted Tissue Bath, XH-1003) until section flattened and straightened. Sections were then placed on charged microscope slides (Denville, Cat #M1021), dried overnight at room temperature, and stored until staining.
**Immunofluorescence**

Immunofluorescence was performed per standard protocol with sodium citrate antigen retrieval. Slides were deparaffinized and rehydrated through a series of xylene and ethanol washes. Slides were incubated for 3 minutes 3x in xylene (Fisher, X4-4). They were transferred to 100% ethanol 3x for 3 min., then rehydrated by incubation for 3 min. in an ethanol (Fisher, HC8001) series (95%, 80%, 75%). Finally, they were washed for 5 min. in PBS on an orbital shaker. Antigen retrieval was performed in a 2L beaker. Citrate buffer consisted of 60 ml of 100 mM sodium citrate dihydrate (Fisher, cat. #BP327-1) diluted in 540 ml distilled water with 600 ul of Tween-20 (Sigma, P-1379) to a 0.1% final volume. We adjusted the pH to 6.0, and microwaved at full power for 20 min., checking every 7 min. to ensure slides remained submerged in retrieval buffer. Buffer and slides were cooled to room temperature, then washed 2x for 5 min. in distilled water, followed by PBS washes 3x for 5 min. Tissue sections were circled with PAP pen liquid blocker (Fisher, cat. #NC0552848) and nonspecific binding was blocked using the M.O.M. Immunodetection kit (Vector Laboratories, BMK-2202) for 1 hr. at room temperature. Primary antibodies (table below) were diluted using the M.O.M. kit and incubated overnight in a humidification chamber. The following morning, antibodies were drained from slides and rinsed 3x in 1x PBST (PBS + 0.1% Tween-20) for 10 min. Secondary antibodies were diluted in M.O.M. protein concentrate and incubated with slides for 2 hrs. in a black humidification chamber. Antibodies were drained from slides, washed 3x for 10 min. each in PBST, and incubated with DAPI for 10 min. Slides were washed 3x for 10 min. in PBST then mounted with Permount (Fisher Scientific, #SP15-100) and left to dry overnight in the dark before imaging.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Manufacturer</th>
<th>Catalog</th>
<th>species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>1°</td>
<td>Abcam</td>
<td>13970</td>
<td>chicken</td>
<td>1:1000</td>
</tr>
<tr>
<td>RFP</td>
<td>1°</td>
<td>Rockland</td>
<td>600-401-379</td>
<td>rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>K8</td>
<td>1°</td>
<td>DSHB</td>
<td>TROMA-1</td>
<td>rat</td>
<td>1:250</td>
</tr>
<tr>
<td>K14</td>
<td>1°</td>
<td>Biolegend</td>
<td>PRB-155P</td>
<td>rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-chicken IgY</td>
<td>2°</td>
<td>Invitrogen</td>
<td>A11039</td>
<td>Chicken</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-rat IgG</td>
<td>2°</td>
<td>Invitrogen</td>
<td>A11006</td>
<td>rat</td>
<td>1:50f0</td>
</tr>
<tr>
<td>Alexa Fluor 555 goat anti-rabbit IgG</td>
<td>2°</td>
<td>Invitrogen</td>
<td>A32732</td>
<td>rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit mAb IgG isotype control</td>
<td>1°</td>
<td>Cell signaling</td>
<td>3900S</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse IgG Isotype control</td>
<td>1°</td>
<td>Invitrogen</td>
<td>31903</td>
<td>mouse</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

**Hematoxylin and Eosin staining**

Mammary and tumor tissue sections were stained by hematoxylin and eosin (H&E). Sections were subjected to a progression of xylene and ethanol washes to deparaffinize and rehydrate tissue. The slides were washed 3x for 3 min. in xylene followed by washes in 100% ethanol. Tissues were rehydrated by a single 3 min. wash in 95% ethanol, followed by 80% ethanol, and finally 70% ethanol. They were transferred to deionized water for 3 min., then dipped 10x in freshly filtered hematoxylin (Poly Scientific, S212A-1GL) followed by a quick rinse in deionized water. Slides were transferred to a 5 min. incubation in tap water, then exposed to acid ethanol for 10 seconds (70% ethanol + 0.25% hydrochloric acid [Fisher. A144500]). After the acid ethanol dips, the tissues were subjected to three water washes for 2 min. – tap water, distilled bottled water (Ozarka), then deionized water. Slides were counterstained in eosin (Poly Scientific S176-1GL) for 45 s., then quickly rinsed in deionized water. Tissues were then dehydrated by dipping 10x in three separate 95% ethanol containers, followed by dipping 10x in two separate 100% ethanol containers. Slides were then incubated for 2 min. in 100% ethanol. Tissues were then incubated 2x for 5 min. in xylene, followed by
another 15 min. incubation in xylene. Slides were then mounted with Permount and a coverslip and left to dry overnight before imaging.

**Tumor cell isolation**

Tumors were excised from animals at 1.2-1.5 cm³ total volume. Digestion medium consisted of DMEM/F12 containing 1 mg/ml Collagenase A (Roche, cat. #57981821) and 1 ug/ml of DNase I (StemCell Technologies, cat. #07900). For every 1 g. of tumor tissue digested, 10 ml of digestion medium was added. Excised tumors were chopped using two razor blades in petri dishes until finely minced (pieces estimated ~1x1mm in diameter) and transferred to sterile 50 ml conical tubes for 2 hr. digestion. Tubes were shaken in a 37°C rotary shaking incubator set to 125 rpm, with tubes placed at a 45° angle. Every 30 min., tubes were removed from incubation and vigorously hand shaken to further disrupt epithelial clumps. After 2 hrs., we removed the tumor digest tubes and continued to enrich for tumor organoids by removing stromal and immune cells through differential centrifugation. All tubes, pipet tips, filter strainers, and plates from this point forward were coated in 3% BSA in DPBS (Bovine serum albumin, SigmaAldrich, cat. #A7906-500G, DPBS ThermoFisher, #14190144) to prevent epithelium from sticking to the polypropylene tubes. Digestion mixture was transferred to a 15 ml conical (multiple tubes if digestion mixture exceeded 15 ml) and centrifuged in an Eppendorf centrifuge (5810R) at 1500 rpm for 5 min. The supernatant was aspirated and the cell pellet was washed with 10 ml DPBS (ThermoFisher, #14190144). Upon DPBS addition, tubes underwent short centrifugation steps to enrich for epithelium. The digested pellets were centrifuged at 450 x g (~1500 rpm) for about seven seconds. We
started the centrifuge, allowed it to reach desired speed (450 x g), counted for seven
seconds, then stopped the spin. Supernatant was aspirated, then we repeated the DBPS
wash 2-3 more times until the supernatant turned clear. By this point, stromal and red
blood cells were largely depleted while tumor organoids were enriched in the pellet. To
generate single cells, we added 2 ml of pre-warmed 0.25% Trypsin-EDTA with 0.1
mg/ml DNase I (StemCell Technologies, cat. #07900) to each pellet and transferred the
organoid-trypsin mixture to a BSA pre-coated 6 well dish to monitor dissociation to
single cells. Trypsinization at 37°C continued for 15-18 min., with a gentle pipetting up
and down at 10 min. to aid in mechanical disruption of tumor organoids and to observe
dissociation. Trypsinization was neutralized with 7 ml of serum-containing primary cell
media (table below) and filtered into a 50 ml conical tube fitted with a 70 μM cell strainer
(Avantar, 732-2758). Tubes containing single cell suspensions were spun at 1000 rpm x 5
min., then washed 2x with 10 ml primary cell media. After final wash, cells were
resuspended in 5-10 ml primary cell media. To obtain viable cell count, 10 ul of cell
mixture and 10 ul of 0.4% trypan blue stain (Invitrogen, cat. #T10282) were loaded onto
a Countess™ cell count chamber slide and loaded into the Countess™ cell counter.
Viable cell count was obtained 2x and the final cell counts were determined by the
average.

<table>
<thead>
<tr>
<th>Primary cell media</th>
<th>REAGENT</th>
<th>COMPANY</th>
<th>CATALOG NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>Gibco</td>
<td>11320033</td>
<td></td>
</tr>
<tr>
<td>10% FBS</td>
<td>R+D Biosystems</td>
<td>S115504</td>
<td></td>
</tr>
<tr>
<td>5 ug/ml insulin</td>
<td>Sigma</td>
<td>I-5500</td>
<td></td>
</tr>
<tr>
<td>1 ug/ml hydrocortisone</td>
<td>Sigma</td>
<td>H0888</td>
<td></td>
</tr>
<tr>
<td>10 ng/ml EGF</td>
<td>Sigma</td>
<td>SRP3196</td>
<td></td>
</tr>
</tbody>
</table>
**Viral transduction and transplantation**

Adenoviral transduction using Ad5.CMV.GFP or Ad5.CMV.Cre for C3(1)TAgs injections was performed at MOI of 50 according to the below formula. Previously aliquoted adenovirus was thawed on ice. A total of 5.0x10^6 tumor cells were transferred to 50 ml conical tubes containing 3 ml of primary cell media. Volume of adenovirus to add to cells was calculated as below and added directly into cell-containing media. Tubes were incubated at 37°C, 5% CO_2 for 1 hr. and were placed at a 45° angle to ensure maximum surface area for viral transduction. Every 15 min., tubes were homogenized by hand with gently swirling. At 30 min., cells were mixed by gently pipetting up and down with a P1000 pipet, then returned to the incubator. After the hour incubation, cells were washed 3x with 10 ml primary cell media to remove virus. Viable cell counts were obtained, and transduced cells were resuspended in 1:1 DPBS:Matrigel® ratio at a concentration of 5.0x10^5 cells/10 ul diluted Matrigel mixture (Matrigel® Growth Factor Reduced basement membrane matrix, Corning, cat. #CLS354230).

\[
ul \text{ of virus to add } = \frac{(5.0 \times 10^6 \text{ cells})(MOI \ 50)(1000x)}{PFU \ of \ adenovirus}
\]

Transduced C3(1)TAgs cells were kept on ice in the diluted Matrigel® suspension until mice were anesthetized for injections. Mice were transferred to induction chamber and were administered 2.5% vaporized isoflurane (Fluriso, VetOne, #NDC13985-046-40) supplemented with O_2 flow rate of 1.5 L/min. Once induced, they were removed from the induction chamber and transferred to surgical pads outfitted with extended nosepieces to maintain anesthetic delivery. LubiFresh P.M. (Major) was applied to eyes of anesthetized
mice to maintain moisture during anesthesia and 2 mg/kg of Meloxicam was administered via sub cutaneous injection for pain control. Surgeons utilized sterile technique for all injections as dictated by our IACUC protocol.

For hair removal and sanitation, hair depilatory crème (Nair, Amazon) was applied to the abdomen covering the abdominal and inguinal glands and was removed with ethanol after hair dissolution. Betadine antiseptic was applied to the area then removed with ethanol after sanitation. A horizontal midline incision was made between the fourth nipples using sterile surgical scissors, carefully avoiding puncture of the peritoneum. Fascia was gently pushed aside to reveal the fourth mammary fat pad. A total volume of 10 ul of cell mixture containing $5.0 \times 10^5$ cells in diluted Matrigel® were drawn up in a Hamilton syringe (Hamilton, MICROLITER TM #710) outfitted with a 26.5G needle (BD, Precision Glide) and injected into the exposed #4R mammary gland. Sterile saline (0.3 cc/10g mouse) was provided as a single intraperitoneal injection to maintain hydration. Surgical incision was closed using 1-2 AUTOCLIP 9 mm wound clips (Clay Adams, cat. #427631) which were removed 7-10 days post surgery. Animals were recovered on heating pads to prevent hypothermia. Animals were administered another dosage of Meloxicam the day after surgery for continued pain control.

**FACs analysis of cultured tumor cells**

Transduced tumor cells were maintained in 800 ul of primary cell media at a concentration of $1.0 \times 10^6$ cells/well in a super-low attachment 24 well dish (Costar, cat. #3473) for 72 h. post adenoviral transduction. After 72 h., cells were transferred to 15 ml conical tubes and pelleted by centrifugation at 450 x g for 5 min. Primary media was
removed from cells, followed by 2x wash with pre-warmed DBPS. Cells were trypsinized with pre-warmed 0.25% trypsin-EDTA for 10 min., then trypsin reaction was neutralized with 3 ml primary cell media. Primary cell media was removed after pelleting cells and pellet was resuspended in 500 ul DPBS. Cells were filtered into FACs tubes through strainer cap lids (Corning, Falcon, cat. #08-771-21) and brought for FACs analysis (BD Symphony).

**Immunoblotting**

Immunoblotting was performed as previously described in Chapter 2. Antibodies used for the following chapters are listed in the table below.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration</th>
<th>Manufacturer/Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPβ</td>
<td>1:200</td>
<td>Santa Cruz SC79-62</td>
</tr>
<tr>
<td>β actin</td>
<td>1:1000</td>
<td>Cell Signaling #4967</td>
</tr>
<tr>
<td>α/β tubulin</td>
<td>1:1000</td>
<td>Cell Signaling #2148</td>
</tr>
<tr>
<td>Goat anti-mouse IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32230</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32260</td>
</tr>
</tbody>
</table>

**Establishment and culture of C3(1)TAG cell lines**

*C3(1)TAG;Cebpb*<sup>fl/fl</sup> tumor cells were isolated from the 3R glands of animals #1478 and #1479 and transduced with either Ad5.CMV.GFP or Ad5.CMV.Cre as previously described in Chapter 4. Cells of each condition were plated at 1.0x10<sup>6</sup> cells per 6 cm. tissue culture dish. We transferred the cells from the 6 cm. dish to a 12 well plate (p1) due to cell death from adenoviral transduction and low confluency. We passaged cells to expand into larger vessels – first a 3.5 cm dish (p2) followed by a 6 cm dish (p3) – before trypsinization (0.125% trypsin-EDTA x 3 min.) and FACs sorting. Cells were sorted into 100% FBS-containing DMEM. We sorted on the highest
expressing dTomato+ population for the Cebp<sup>+/+</sup> group and recovered 5.0x10<sup>5</sup> cells which we expanded in a 3.5 cm tissue culture dish. Similarly, we sorted on the highest expressing GFP+ population for the Cebp<sup>Δ/Δ</sup> group, of which we recovered 5.0x10<sup>4</sup> cells in a 24 well tissue culture dish. Cells of each group were re-sorted using the same FACs parameters 4 passages later.

**Results**

**High C/EBPβ expression indicates worse overall survival in breast cancer patients**

To determine the relationship between C/EBPβ expression and overall survival, we generated Kaplan-Meier survival curves of available patient data as displayed in Figure 12. We analyzed the overall survival following chemotherapy or neoadjuvant therapy across all breast cancer subtypes and found that patients with high Cebp mRNA transcripts have a statistically worse overall survival (p = 2.6E-04) than the low expression cohort. Upper quartile survival for the low expression and high expression cohorts are 143.74 months and 79.2 months, respectively. Similarly, for the limited publicly-available C/EBPβ protein expression data, we generated overall survival curves for lymph node-negative, neoadjuvant naïve TNBC patients. Patients with high C/EBPβ protein expression also had a statistically worse overall survival (p = 9.9E-03). These results suggest that C/EBPβ expression at can serve as an overall prognostic indicator in not only TNBC patients, but also across all other subtypes of breast cancer.
**Genetic ablation of Cebp inhibits tumorigenesis in NIC transgenic mice**

As discussed in **Chapter 3, Figure 8** characterizes the genetic model utilized to delete C/EBPβ from tumor epithelium in the NIC spontaneous tumor model. As demonstrated in **Figure 13**, NIC;Cebp⁺/+ mice reached clinical endpoint with a median survival of 5.53 months (166 days) compared to NIC;Cebp⁺Δ mice, whose median survival was 7.63 months (229 days). We terminated the experiment at 12 months after NIC;CebpΔ/Δ mice failed to form tumors. Tumors from NIC;Cebp⁺/+ and NIC;Cebp⁺/Δ mice were measured to determine changes in growth rate upon deletion of Cebp. We demonstrated no statistical differences in growth rates for NIC;Cebp⁺/+ and NIC;Cebp⁺Δ tumors. As NIC;Cebp⁺Δ tumors have the same growth rate but a longer median survival, we concluded that tumor onset is delayed with the deletion of a Cebp allele in NIC mice. Tumor burden, defined as the number of tumors per animal, was determined at clinical endpoint for each genotype. NIC;Cebp⁺/+ and NIC;Cebp⁺Δ animals developed on average 5.33 and 4.82 tumors per animal, respectively, which was not statistically significant. These results suggest that deletion of a single C/EBPβ allele delays mammary tumorigenesis in this model, but does not affect tumor growth once tumors have formed (**Figure 16A-B**).

**Characterization of NIC;Cebp⁺/+ tumors and NIC;CebpΔ/Δ mammary glands**

In **Figure 9A**, we performed immunofluorescence on sections of formalin-fixed, parrafin-embedded (FFPE) mTmG;NIC;Cebp⁺/+ tumors. We demonstrated that Cre recombination, as determined by GFP expression, is strictly confined to the mammary epithelium, while tumor stroma retains dTomato expression. Whole mount analysis of
endogenous GFP expression in the mammary ducts of mTmG;NIC;Cebpb\(^{AA}\) glands indicated that Cre recombination was restricted to the mammary epithelium as anticipated (Figure 9B). Carmine whole mount analysis and hematoxylin and eosin staining (H&E) of NIC;Cebpb\(^{AA}\) glands in Figure 14 indicated deletion of Cebpb caused mammary ductal distension and dilation, as well as reduced secondary and tertiary budding as compared to the age-matched, estrus stage-matched wild-type control. Immunofluorescence for basal and luminal keratin markers (K8 and K14, respectively) in mTmG;NIC;Cebpb\(^{AA}\) glands appeared similar to controls (Figure 15), suggesting similar ductal architecture and normal epithelial differentiation in mTmG;NIC;Cebpb\(^{AA}\) glands.

**Cebpb\(^{AA}\);C3(1)TAg tumor cells fail to form tumors in vivo**

As shown in Figure 11 in Chapter 3, we tested Ad.Cre recombination activity in isolated tumor cells by immunoblot and confirmed Cebpb deletion begins at 24 h., but is mostly completed by 72 h. After confirming complete recombination at 72 h., we used this time point to assess recombination efficiency for the following studies. Utilizing the genetic model described in Figure 10, we transplanted either Cebpb\(^{AA}\);C3(1)TAg or Cebpb\(^{+/+}\);C3(1)TAg cells into immunocompetent REAR mice according to the graphical schematic outlined in Figure 17. As Ad5.CMV.Cre transduction is not a 100% efficient process, we anticipated the transduced tumor cells to be mostly mosaic consisting of a mixture of both dTomato+ Cebpb\(^{+/+}\) cells and GFP+ Cebpb\(^{AA}\) cells as represented in Figure 18. FACs analysis of tumor cells transduced with Ad.Cre or Ad.GFP indicate that 72 hr. post transduction, recombination had occurred as demonstrated by GFP expression (Figure 19, top row). In addition, we confirmed Cebpb deletion in the experimental
group by immunoblot, validating that the 67.91% GFP-expressing \(Cebp_\Delta;C3(1)TAg\) tumor cells had undergone recombination (Figure 20). Similarly, in the resulting tumors, we addressed the GFP expression and C/EBP\(\beta\) protein expression by immunoblot as they reached clinical endpoint of 1.2-1.5 cm\(^3\). After gating out the stromal cells from the tumor digest (dTomato\(^\ast\);GFP\(^\ast\) population), the \(mTmG;Cebpb^{+/+}\) Ad.Cre control tumors retained GFP expression (56%) as determined by FACs. Less than 0.05% of the resulting \(Cebp_\Delta;C3(1)TAg\) tumors retained GFP expression, but did express dTomato (Figure 19, bottom). Upon immunoblotting, as determined by the return of C/EBP\(\beta\) protein bands, we observed that the injected \(Cebp_\Delta;C3(1)TAg\) tumors were outcompeted by the contaminating untransduced \(mTmG;Cebpb^{+/+}\) tumor cells in the initial injection mixture (Figure 20). These results suggest that the \(Cebp_\Delta;C3(1)TAg\) cells were either outcompeted by \(Cebp^{+/+}\) tumor cells or were cleared by the immune system.

\textbf{\(Cebp_\Delta\) tumor cells outcompete \(Cebp^{+/+}\) cells in vitro}

We established \(Cebp^{+/+};C3(1)TAg\) and \(Cebp_\Delta;C3(1)TAg\) cell lines as described in Methods. We confirmed their C/EBP\(\beta\) protein expression status by immunoblot (Figure 21) after FACs sorting on dTomato and GFP for \(Cebp^{+/+}\) and \(Cebp_\Delta\), respectively. The FACs-sorted GFP\(^\ast\) \(Cebp_\Delta\) cells retained a contaminating dTomato\(^\ast\) \(Cebp^{+/+}\) population which we tracked by FACs analysis after multiple passages. The contaminating dTomato\(^\ast\) \(Cebp^{+/+}\) population decreased over time in adherent culture from 4.19% at passage 6 to 1.32% at passage 10 (Figure 22), unlike the
results we saw in vivo. These results demonstrate that $Cebpb^{+/+};C3(1)TAg$ cells do not outcompete $Cebpb^{+/};C3(1)TAg$ cells in adherent cell culture conditions.
A.) Increased *Cebpb* expression at the mRNA level indicates worse overall survival across all subtypes in systemically untreated patients and in patients following chemotherapy or neoadjuvant therapy. Kaplan-Meier curves of mRNA data were generated using gene chip expression values (GSE1456, GSE16446, GSE16716, GSE20271, GSE20685, GSE20711, GSE3494, GSE37946, GSE42568, GSE45255, GSE7390. Untreated n = 397, treated n = 1879. Cox hazard ratio statistical test, \( p = 3.5 \times 10^{-3} \) and \( p = 2.6 \times 10^{-4} \), respectively).

B.) Patient subset defined as lymph node-negative, neoadjuvant naïve status with unilateral TNBC primary tumor. C/EBPβ protein expression data was generated by nLC-MS/MS (PMID: 24399849, n = 96, Cox hazard ratio statistical test, \( p = 9.9 \times 10^{-3} \)). C/EBPβ protein expression values are representative of total expression, not individual informs.

**Figure 12** High C/EBPβ correlates with worse overall survival in breast cancer patients

A.) Increased *Cebpb* expression at the mRNA level indicates worse overall survival across all subtypes in systemically untreated patients and in patients following chemotherapy or neoadjuvant therapy. Kaplan-Meier curves of mRNA data were generated using gene chip expression values (GSE1456, GSE16446, GSE16716, GSE20271, GSE20685, GSE20711, GSE3494, GSE37946, GSE42568, GSE45255, GSE7390. Untreated n = 397, treated n = 1879. Cox hazard ratio statistical test, \( p = 3.5 \times 10^{-3} \) and \( p = 2.6 \times 10^{-4} \), respectively). B.) Patient subset defined as lymph node-negative, neoadjuvant naïve status with unilateral TNBC primary tumor. C/EBPβ protein expression data was generated by nLC-MS/MS (PMID: 24399849, n = 96, Cox hazard ratio statistical test, \( p = 9.9 \times 10^{-3} \)). C/EBPβ protein expression values are representative of total expression, not individual informs.
Median survival of NIC;Cebpb animals is 166 days, while NIC;Cebpb animals were sacrificed at 365 days after failing to form tumors. (p<0.0001, Log-rank (Mantel-cox), n= 6,9,6).

Figure 13 Genetic ablation of Cebp inhibits tumorigenesis in NIC transgenic mice
Whole mount (2x magnification) and H&E analysis (10x magnification) of embedded mammary sections indicates NIC;Cebpb\textsuperscript{\textDelta/\Delta} glands appear distended with reduced secondary and tertiary branching compared to age-matched wild type controls.

Figure 14 NIC;Cebpb\textsuperscript{\textDelta/\Delta} mammary glands display altered ductal morphology
Figure 15 NIC;Cebpb\textsuperscript{ΔΔ} mammary glands retain normal keratin expression patterns

Immunofluorescence detecting expression patterns of luminal keratin 8 (K8) and basal keratin 14 (K14) in NIC;Cebpb\textsuperscript{ΔΔ} appears similar to age-matched wild type controls. Matched isotype controls in inset, 10x magnification.
A.) Animals were measured 1-2x weekly once tumor volume exceeded 0.5 cm³. Growth rates were plotted, and linear regression was performed on slopes of tumor growth rates. (p=0.2674, n=3 and 5 for NIC; Cebpb +/+ and NIC; Cebpb +/Δ, respectively.) B.) Number of tumors present throughout the mammary chain was counted upon euthanasia at 1.2-1.5 cm³ endpoint. Student’s unpaired T test of tumor burden showed no statistical significance in tumor burden between NIC; Cebpb +/+ and NIC; Cebpb +/Δ animals, with each group developing an average of 5.33 and 4.82 tumors/animal, respectively. (p=0.3929)

Figure 16 Deletion of one Cebpβ allele does not alter tumor growth rate or tumor burden
When tumors of the indicated genotypes arose, tumor epithelial cells were isolated and transduced in vitro with Ad5.CMV.GFP or Ad5.CMV.Cre virus. Virus was removed post-transduction and immediately injected into 8–14-week-old female REAR mice tolerized to SV40T antigen. Resulting tumors were harvested at 1.2-1.5 cm³ analyzed for GFP and C/EBPβ expression.
These transplant studies utilized two control groups. Ad.GFP was used with \textit{mTmG};\textit{Cebpb}^{fl/fl} tumor cells to estimate viral transduction efficiency, while Ad.Cre was used with \textit{mTmG};\textit{Cebpb}^{+/+} tumor cells to estimate recombination efficiency. Potential outcomes for each fluorescent tumor group are indicated above.

Figure 18 Graphical schematic of the expected resulting C3(1)TAg tumors
Tumor cells of each genotype were isolated, treated with corresponding adenovirus, and injected into fat pads of 8–14-week-old female REAR mice. Fluorescence was assessed at 72 h. post-transduction, indicating transduction efficiency in the Ad.GFP control and recombination efficiency in the Ad.Cre groups. Expected results and actual results indicated in the schematic pictured above. (N = 12, 14, 18 for Cebpb<sup>+/+</sup> + Ad.Cre, Cebpb<sup>fl/fl</sup> + Ad.GFP, and Cebpb<sup>fl/fl</sup> + Ad.Cre, respectively.)

Figure 19 GFP+ Cebpb<sup>ΔΔ</sup> tumors fail to grow in REAR mice
Western blot analysis displaying C/EBPβ expression in injected C3(1)TAg tumor cells 72 hr. post transduction and in the resulting tumors. Interestingly, injected Cebpβfl/fl + Ad.Cre cells at 72 h. show significant reduction in C/EBPβ expression, but expression returns in the resulting tumors for that group.

Figure 20 C/EBPβ expression in injected C3(1)TAg cells and in the resulting tumors
Figure 21 Cebpb is deleted in C3(1)TAgtumor cell lines transduced with Ad.Cre

A.) GFP expression indicates Ad.GFP viral infection has occurred within 24 hr. of transduction. GFP expression in the Ad.Cre-treated groups indicate Cre recombinase activity and deletion of C/EBPβ. Brightfield insets indicate cell confluence. (20X magnification for 24 and 48 h., 10X for 72 h.) B.) Confirmation of C/EBPβ deletion in adenovirus-treated mTmG;Cebpβ/';C3(1)TAgtumor cell lines by immunoblot.
Transduction of \textit{Cebpb}^{fl/fl} cell lines with Ad.Cre resulted in \(\sim75\%\) of cells expressing GFP, indicating recombination. \textit{Cebpb}^{\Delta\Delta} cells were sorted on the highest GFP\(^{+}\) population and re-plated. The contaminating dTomato\(^{+}\) \textit{Cebpb}^{+/+} cell population remaining after sorting was tracked with each passage by FACs analysis.

**Figure 22** \textit{Cebpb}^{+/+} cells do not outcompete \textit{Cebpb}^{\Delta\Delta} cells in vitro

Transduction of \textit{Cebpb}^{fl/fl} cell lines with Ad.Cre resulted in \(\sim75\%\) of cells expressing GFP, indicating recombination. \textit{Cebpb}^{\Delta\Delta} cells were sorted on the highest GFP\(^{+}\) population and re-plated. The contaminating dTomato\(^{+}\) \textit{Cebpb}^{+/+} cell population remaining after sorting was tracked with each passage by FACs analysis.
Summary and conclusions

In Specific Aim 2, we demonstrated that Cebpb expression correlates with poor survival outcomes in breast cancer patients at both the transcriptional and translational level, demonstrating its potential as a prognostic indicator. In addition, utilizing two distinct genetic mouse models ablating Cebpb from existing tumor cells and before the onset of tumorigenesis, we established that Cebpb serves as a critical driver of mammary tumor formation and maintenance.

In the NIC model analogous to luminal HER2 tumors, we concluded that Cebpb is essential for tumor initiation as NIC;Cebpb^/+ females developed no tumors. Interestingly, their mammary glands histologically resembled germline Cebpb^-/- mammary glands with distended ducts and a loss of secondary and tertiary side branching. Additionally, we observed that deletion of one Cebpb allele is insufficient to inhibit tumorigenesis but does delay tumor onset. Ultimately, loss of one Cebpb allele in tumors did not affect tumor growth or tumor burden.

In the basal-like C3(1)TAg model, we established that Cebpb is required to sustain tumor cell growth in vivo. After exogenous deletion using adenoviral Ad5.Cre recombinase, Cebpb^/+ cells injected into immunocompetent REAR hosts failed to form mammary tumors and were outcompeted by Cebpb^-/- cells. The competition phenotype was lost in vitro, suggesting potential exogenous factors regulating Cebpb^/+ tumor cell growth in the animal.

Our studies demonstrated in two mouse models that epithelial-derived Cebpb serves as a critical regulator of tumorigenesis from time of tumor initiation through
tumor growth and progression. The downstream mechanisms affected by loss of $Cebpb^{A/d}$ tumor cells were addressed in the following aim.
Chapter 5: C/EBPβ regulation of immune evasion

The goal of Specific Aim 3 is to investigate whether tumor-derived C/EBPβ influences immune clearance. The experiments described in this chapter utilize the C3(1)TAgt transplantable model previously described in Chapters 3 and 4.

Introduction

A classic hallmark of cancer is the ability of transformed cells to evade the anti-tumor response\(^{95,135}\). Many tumor-intrinsic mechanisms contribute to this effect, including sustaining tumor-promoting inflammation, downregulation of tumor-specific antigens, and reprogramming the local tumor stroma to support tumor growth\(^{92,94,95,135}\). Several studies have hinted that tumor-derived C/EBPβ has the potential to influence immune clearance. In 2014, Kurzejamska et al. found that inhibition of C/EBPβ in transplantable 4T1 tumors reduced tumor growth, increased MHCII expression, and induced infiltration of CD4+ lymphocytes\(^{132}\). C/EBPβ knock down driven by the K14 promoter had similar effects in a DMBA-induced squamous papilloma model, causing tumor regression and upregulation of type I interferon response; however, the study failed to show significant changes in CD4+ or CD8+ immune infiltrates in the regressing knock out tumors\(^{108}\). More recently, Sterken et al. demonstrated that C/EBPβ deletion in a human TNBC cell line, BT-20, induced gene programs associated with antigen
presentation via MHCI/MHCII\textsuperscript{109}. Their findings provide further evidence that tumor-derived C/EBPβ promotes tumorigenesis by silencing tumor antigen presentation that would induce a CD8+ anti-tumor response, though no mechanisms were tested in the scope of their studies.

The findings of our previous studies demonstrated that C/EBPβ is required for the onset of mammary tumorigenesis in the NIC model and also for growth of C3(1)TAg tumors transplanted into immunocompetent recipients. As C/EBPβ is not required for C3(1)TAg cell growth in vitro, we were led to the hypothesis that tumor-derived C/EBPβ suppresses the anti-tumor lymphocyte response in vivo. To address that question, we designed experiments to investigate the potential mechanisms by generating bulk RNAseq data on \textit{C3(1)TAg;Cebpb}\textsuperscript{Δ/Δ} cell lines and performing transplant studies in immunocompromised mice lacking CD4 and CD8 lymphocytes. Our findings confirm our hypothesis that tumor-derived C/EBPβ suppresses immune recognition and clearance likely through downregulation of interferon-regulatory factors (IRFs) involved in antigen processing and presentation.
Materials and Methods

Mice

8-week-old athymic nude mice (strain #002019, Jackson Laboratories) were purchased and stored at Tulane School of Medicine Vivarium, in accordance with proper handling and maintenance conditions according to federal laws and the Institutional Animal Care and Use Committee (IACUC, protocol #710). C3(1)TAg tumor cell isolation, transduction, and injections were performed as previously described in Chapter 4 and injected into the animals at 9 weeks of age. Mouse euthanasia and tumor analysis by FACs were performed as previously described in Chapter 4.

Tumor tissue fixation and imaging

Tumors were excised, immediately placed in disposable base molds (FisherSci, #22-363-555), and frozen in Optimal Cutting Temperature Compound (OCT compound, FisherSci, #23-730-57) on dry ice. Fang-Yen “Mark” Chiu, a student in the lab of James Jackson, PhD, graciously cut the tissue into 5 μm sections on the cryostat and captured endogenous fluorescence using a Nikon TiE-2 Inverted Research Microscope Nikon A1rSi Laser Point Scanning Confocal microscope equipped with the Plan Apo λ 60× oil objective. Image brightness was minimally altered for images using NISElements software.
**Lentiviral transduction of C3(1)TAg cell lines**

Lentiviral transduction of C3(1)TAg cell lines for RT-qPCR was as performed as described in Chapter 2 at an MOI of 3, only the lentiviral construct for pEIT-LIP contained the mouse, rather than human, LIP sequence.

**RT-qPCR**

Transcript expression of genes of interested in C3(1)TAg cells was assessed by RT-qPCR as described in Chapter 2. Primer sequences used in this chapter are listed in the table below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
</thead>
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<tr>
<td>Stat1</td>
<td>For GCCCTCTCATTTGCACCGAAGAAC</td>
</tr>
<tr>
<td></td>
<td>Rev TGCTGACGTTGGAGATCACCA</td>
</tr>
<tr>
<td>Ccl5</td>
<td>For CCTGCTGCTTTGCCTACCTCTC</td>
</tr>
<tr>
<td></td>
<td>Rev ACACACTTGGCGGTTCTCTCA</td>
</tr>
<tr>
<td>18s</td>
<td>For GTAACCCGTTGAACCCATT</td>
</tr>
<tr>
<td></td>
<td>Rev CCAATACGAGTGAGCG</td>
</tr>
</tbody>
</table>

**Immunoblotting**

Protein expression by immunoblotting was performed as described in Chapter 2. Antibodies used in this chapter are listed in the table below.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration</th>
<th>Manufacturer/Catalog #</th>
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<tr>
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<td>Cell Signaling #94205</td>
</tr>
<tr>
<td>p-STAT5</td>
<td>1:1000</td>
<td>Cell Signaling #9351</td>
</tr>
<tr>
<td>Goat anti-mouse IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32230</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (H+L) Poly-HRP</td>
<td>1:5000</td>
<td>ThermoFisher #32260</td>
</tr>
</tbody>
</table>

**RNAseq**

RNA from C3(1)TAg cell lines was isolated with Trizol as described in Chapter 2. Purity RIN scores were determined by TapeStation with special thanks to the Jay Kolls.
Lab at Tulane University School of Medicine. RNAseq was performed by BGI Americas Corporation at a read depth of 20 million reads per sample on the DNBseq platform. Bioinformatic analysis was kindly performed by the Melody Baddoo with the Tulane Cancer Center NGSA core according to their standard pipeline.

**CHiP-Seq analysis**

The in silico ChIP-Seq data analysis methods performed in this chapter are identical to those described in Chapter 2.

**Results**

**Athymic mice support CebpbΔΔ;C3(1)TAg tumor cell growth**

We performed similar C3(1)TAg transplant studies described in Chapter 4 in athymic mice (Figure 23). We assessed GFP expression by FACs analysis at 72 hr. post-transduction and found 87.1% and 63.5% of cells expressed GFP in the Cebpb+/+ and CebpbΔΔ cells, respectively, indicating the vast majority of tumor cells had undergone recombination (Figure 24, left). At clinical endpoint, Cebpb+/+ and CebpbΔΔ tumors were 22.6% and 26.0% GFP+, indicating athymic mice support CebpbΔΔ tumor growth, unlike immunocompetent REAR recipient mice (Figure 24, middle). The percent of GFP+ cells in both the control and experimental groups were statistically insignificant (data not shown), suggesting that recombination occurred to the same degree in both tumor groups. The endogenous fluorescence of harvested tumors indicated CebpbΔΔ tumor cells were interspersed randomly among Cebpb+/+ cells within the tissue sections and did not appear to form spatially isolated clusters (Figure 24, middle). CebpbΔΔ tumors exhibited a slight
delay in growth at day 21 (P < 0.05, Student’s T test, n = 10 each group), but the tumor volume between groups was insignificant from that point until harvest at 1.2 cm³ endpoint suggesting that the deletion of C/EBPβ had no effect on tumor cell proliferation in athymic recipient mice (Figure 25).

**C/EBPβ suppresses total STAT and CCL5 signaling**

To address changes in chemokine signaling relevant to T cell migration and cell-mediated immunity, we performed qPCR on C3(1)TAg;Cebpβ+/+ and C3(1)TAg;Cebpβ-/- tumor cell lines and found that C/EBPβ significantly suppressed Stat1 and Ccl5 expression (Figure 26A); interestingly, re-expression of LIP in Cebpβ-/- cells rescued the suppression phenotype of Ccl5 (Figure 26B) (Student’s T test statistical method, n = 3 biological replicates, p<0.05*, p<0.01**). As CCL5 is potent chemokine involved in recruitment of CD4+ T cells to mammary tumors in vivo, the deletion of C/EBPβ may alter T cell recruitment to C3(1)TAg tumors. We addressed STAT signaling by western blot and found that C/EBPβ appears to slightly repress total STAT3 and STAT5 expression but noticed negligible changes in phosphorylation of either protein (Figure 26A). These data suggest that although stabilization of each protein may occur with the loss of C/EBPβ, functional changes in STAT signaling are likely negligible given the minor changes in phosphorylation status.
**C/EBPβ suppresses genes involved in interferon type I signaling, antigen processing, and antigen presentation via MHC I**

RNAseq analysis indicated that the most differentially expressed genes between C3(1)TAg; Cebpb^{+/+} and C3(1)TAg; Cebpb^{ΔΔ} tumor cell lines are GO signatures involved in type I interferon signaling. The GO terms most significantly enriched in C3(1)TAg; Cebpb^{ΔΔ} tumor cells were plotted by their normalized enrichment scores (NES). Similarly, the REACTOME signatures most enriched in C3(1)TAg; Cebpb^{ΔΔ} tumor cells displayed the most significant upregulations in interferon alpha/beta signaling, the unfolded protein response, and antigen processing, as displayed by their graphed NES (Figure 27A). Gene set enrichment analysis (GSEA) of C3(1)TAg; Cebpb^{ΔΔ} tumor cells showed significant enrichment of antigen processing GO terms (NES = -1.69, p = 0.00) and REACTOME unfolded protein response (NES = -1.73, p = 0.00) (Figure 27B).

**C/EBPβ suppresses IRF signaling genes in C3(1)TAg cells**

The DEGs most significantly upregulated upon loss of C/EBPβ belong to the IRF transcription factor family as graphed by log2FC in Figure 28A with Cebpb as a reference point. Although STAT1 and STAT2 appear enriched in C3(1)TAg; Cebpb^{ΔΔ} tumor cells based on their rank metric scores (Figure 28B), the p values for their respective log2FC failed to reach statistical significance (p = 0.14 and 0.15, respectively). We graphed the average TPM from each IRF family gene that was statistically differentially expressed to examine the magnitude of potential effects of
changes in IRF signaling. The greatest increases in overall transcript number from greatest to least were as follows: *Irf7, Irf1, Irf9, Irf5*, and *Irf8* (Figure 28C).

*C/EBPβ binds IRF regulatory elements in SUM159 cells*

To determine if C/EBPβ interacts with any of the IRF family promoters, we analyzed a published ChIP-Seq dataset generated from TNBC SUM159 cells. The promoter regions defined by the authors describe a region +/- 5 Kb from the transcription start site (TSS). Enhancer regions were defined as 5-200 Kb upstream from the transcription start site. Read density at each genetic locus was indicated by mapped peaks which directly correlated to units of reads per million mapped reads (rpm). We examined the promoter and enhancer loci up to 25 Kb from the TSS for several IRF family genes that were differentially expressed in our RNAseq data, including *Irf1, Irf5, Irf7, Irf8*, and *Irf9*. C/EBPβ bound each of the promoter sites, but the promoter sites with the highest read densities belonged to *Irf7, Irf1, and Irf9* (9.4, 8.7, and 8.7 rpm, respectively) (Figure 29). Similarly, C/EBPβ interacted with all enhancer regions, but mapped the most heavily to the *Irf8, Irf9*, and *Irf7* enhancer sites (23.5, 21.4, and 18.4 rpm, respectively). These data suggest that C/EBPβ can physically bind the enhancer and promoters of relevant type I interferon response effector genes, which can potentially serve as a suppressor in a LIP^inh^ model.
Tumor epithelial cells were isolated and transduced in vitro with Ad.Cre. Virus was removed post-transduction and immediately injected into 9-week-old athymic mice (n = 10, each group). Resulting tumors were harvested at 1.2 cm³ and analyzed for GFP expression.
Figure 24 Athymic mice support *Cebpb*Δ/Δ tumor cell growth

*Cebpb*+/+ and *Cebpb*Δ/Δ cells were assessed for GFP expression by FACs analysis at 72 h. post-transduction and at clinical endpoint. Tumors were harvested at 1.2cm³ representing clinical endpoint (N = 10 for each group.) Representative images of frozen tumor sections were imaged by confocal microscopy. Endogenous GFP fluorescence indicates recombination.
Athymic mice were injected with $5.0 \times 10^5$ cells in the 4R and 4L gland. Tumors were measured twice weekly until 1.2 cm$^3$ endpoint. T test for statistical significance,* corresponds to $p < 0.05$ ($n = 10$ WT and KO tumors at day 21, then $n = 6$ WT and KO tumors all other time points).

**Figure 25** *Cebpb*Δ/Δ* tumors exhibit initial delay but recover growth rate compared to *Cebpb*+/+* tumors
Figure 26 C/EBPβ suppresses total STAT expression, while C/EBPβ and C/EBPβ-LIP suppress Ccl5 expression

A.) RT-qPCR of CebpbΔΔ basal-like C3(1)TAg cell lines indicating induction of Stat1 (Student’s T test statistical method, n = 3 biological replicates, p<0.05*, p<0.01**) and Western blot of CebpbΔΔ triple-negative C3(1)TAg cell lines indicating STAT protein expression. B.) Cebpb+/+ and CebpbΔΔ basal-like C3(1)TAg cell lines were transduced with pEIT (EV) or pEIT-LIP lentiviral expression plasmid at MOI 3. (Student’s T test statistical method, n = 3 biological replicates, p<0.05*, p<0.01**)
A.) RNAseq of $Cebpb^{+/+}$ and $Cebpb^{ΔΔ}$ C3(1)TAg cell lines (n = 3 biological replicates) indicates significant enrichment of the above genetic signatures. REACTOME and GO terms were plotted by normalized enrichment score (NES).

B.) GSEA shows significant enrichment of antigen processing genes and unfolded protein response in $Cebpb^{ΔΔ}$ cells.

Figure 27 Tumor-derived C/EBPβ suppresses interferon type I and antigen processing/presentation via MHC I gene signatures
A.) The most significant DEGs in the Type I Interferon response GO term list are the IRF family, as shown by log2FC, graphed with Cebpb for point of reference.  
B.) Heatmap of DEGs indicates statistical significance of genes of interest.  
C.) Table of raw average of transcripts per million (TPM) of each IRF family member of interest to indicate global effect of transcriptional changes, graphed with Cebpb for reference.

Figure 28 C/EBPβ suppresses type I interferon response and genes responsible for initiating immune signaling in tumor cells
ChIP-Seq analysis of SUM149 TNBC cell lines indicates C/EBPβ binds the 5’ upstream enhancer region and promoter of multiple IRF genes. Highest mapped peaks in promoter region and within proximal 5’ enhancer region (~25 Kb upstream of TSS) listed in table above. Mapped peaks indicate read density defined as units of reads per million mapped reads (rpm). Promoter region defined as +/-5 Kb upstream of TSS and enhancer region 5 Kb – 200 Kb upstream of TSS. GSM2330565
Summary and conclusions

Here we demonstrated through the experiments performed in **Specific Aim 3** that athymic mice lacking functional CD4 and CD8 T cells support \( C3(1)TAg;Cebpb^{A/A} \) tumor cell growth. Given this result, we hypothesized that tumor-derived C/EBP\( \beta \) represses transcriptional mechanisms involved in T cell recognition of tumor cells. We investigated those potential transcriptional mechanisms through RNAseq of \( Cebpb^{+/+} \) and \( Cebpb^{A/A} \) \( C3(1)TAg \) tumor cells. The most differentially expressed genes belonged to type I interferon signaling and antigen processing and presentation genetic signatures which were repressed in the \( Cebpb^{+/+} \) group. IRF family genes were the most upregulated upon loss of C/EBP\( \beta \) in our tumor cell line. To determine if C/EBP\( \beta \) regulated any of these individual IRF genes, we performed an in silico analysis of ChIP-Seq data in TNBC cell line SUM159. The published data showed that C/EBP\( \beta \) bound both the promoters and enhancers of five IRF family genes, suggesting that genetic suppression of IRF genes by C/EBP\( \beta \) in our \( C3(1)TAg \) is feasible. We further discuss the conclusions, implications, and future studies based on the results of **Specific Aims 2** and **3** in Chapter 6.
Chapter 6: Discussion, Conclusions, and Future Directions

In this chapter, we discuss the implications of our findings in Chapter 4 and Chapter 5 and future directions to further probe the role of C/EBPβ in tumor immunoregulation. Our data cemented the status of C/EBPβ as a negative prognostic indicator in patient survival regardless of tumor subtype. Through our genetic studies outlined in the previous chapters, we firmly established tumor-derived C/EBPβ as an essential modulator of mammary tumorigenesis. In addition, our mouse transplant studies coupled with the results of our RNAseq data demonstrated a novel role of C/EBPβ in suppression of anti-tumor immunity.

Discussion

Given the associations of C/EBPβ with invasive breast disease\textsuperscript{84,85,136}, many have attempted to delineate its significance as an individual prognostic indicator. The published patient survival data addressing C/EBPβ expression specifically in TNBC indicates that patients with high expression have worse overall survival\textsuperscript{83}. When expanded to include other subtypes of breast disease, the published findings are inverted – patients expressing low amounts of C/EBPβ have worse overall survival\textsuperscript{132}. Our Kaplan-Meier survival data utilized an expanded patient data set and our results challenged previous findings. We discovered that overall survival decreased in the high-
expressing C/EBPβ patients across all subtypes (Figure 11A), aligning with TNBC-specific data, suggesting that C/EBPβ contributes to mammary tumorigenesis regardless of subtype. In addition, our survival findings based on C/EBPβ protein expression in TNBC patients upheld the results seen at the mRNA level – high C/EBPβ in TNBC decreases survival (Figure 11B). Considering the established role of C/EBPβ contributing to chemotherapeutic resistance, we addressed the overall survival of high-expressing C/EBPβ patients of all subtypes before and after chemotherapy or neoadjuvant therapy. The upper quartile survival in the high C/EBPβ expression group drops (Figure 11A), suggesting an association with treatment resistance. The significance of the generated survival data is limited as none of the results indicate specific isoform expression. As we know LIP and LAPs often work in opposition to each other, isoform-specific studies are needed to further delineate their individual roles in breast disease.

Several other groups have addressed epithelial-derived C/EBPβ in mouse models of tumorigenesis, but none had performed the genetic deletion experiments to definitively determine the role of C/EBPβ in mammary tumorigenesis. To that end, we performed C/EBPβ deletion experiments utilizing two clinically relevant tumor models (Figure 8, Figure 10). By deleting C/EBPβ at different points – before tumor formation and from existing tumors – we established in vivo that C/EBPβ is a critical molecular driver of mammary tumorigenesis.

In our NIC model, analogous to the luminal molecular subtype in human patients, we discovered that C/EBPβ is an essential driver for spontaneous mammary tumor formation. NIC;Cebpβ+/+ animals developed tumors and reached clinical endpoint at 5.53
months, while \( \text{NIC};Cebpb^{+/A} \) animals reached endpoint at 7.63 months. \( \text{NIC};Cebpb^{A/A} \) animals failed to form tumors for the year that we followed the study, suggesting that in this model, C/EBP\( \beta \) is required for mammary tumorigenesis (Figure 13). The fourth, or abdominal, mammary glands of \( \text{NIC};Cebpb^{A/A} \) animals appeared histologically similar to the mammary phenotype witnessed in \( Cebpb^{-/-} \) animals\(^{79} \). \( \text{NIC};Cebpb^{A/A} \) glands displayed distended and dilated ducts with reduced side branching and budding compared to age-matched diestrus wild type glands (Figure 14) and retained similar ductal differentiation patterns as determined by cytokeratin expression (Figure 15). A limitation of the study is that we did not address the potential hyperplasias in thoracic (second and third) mammary glands in the \( \text{NIC};Cebpb^{A/A} \) animals. Generally, these glands develop tumors first in wild type spontaneous tumor models; therefore, it is possible that the thoracic \( \text{NIC};Cebpb^{A/A} \) glands underwent hyperplastic changes or could have developed tumors beyond one year or with pregnancy challenge. While the complete abrogation of tumor formation up to one year was a surprising result, this model was insufficient to determine the molecular mechanisms preventing or suppressing tumor formation; therefore, we turned to another genetic model utilizing an exogenous Cre-recombinase delivery to study the molecular changes induced by C/EBP\( \beta \) deletion.

The results from our C3(1)TAg transplant model confirmed the data generated by our NIC model, as transplanted \( Cebpb^{A/A} \) tumor cells failed to grow out in immunocompetent hosts (Figure 19). We considered three potential reasons for the loss of \( Cebpb^{A/A} \) tumor cells in vivo – senescence, out-competition, or immune clearance. As the \( Cebpb^{A/A} \) tumor cells grew at the same rate as wild type cells (data not shown) and did not outcompete wild type cells in vitro (Figure 22), immune clearance remained the
likely option. To test this hypothesis, we performed the same C3(1)Tag transplant in nude mice, which lack functional CD8+ and CD4+ T cells (Figure 23). As athymic mice supported Cebpβ+/− cell growth, we confirmed our hypothesis that tumor-derived C/EBPβ likely suppresses transcriptional programs in C3(1)Tag cells that influence T cell recruitment or cell mediated immunity. Interestingly, the Cebpβ+/− tumor cells from athymic mice were dispersed randomly through the resulting tumors and did not form isolated clusters, which could potentially have provided specific survival advantages (Figure 24). The Cebpβ+/− cells grew in contact with Cebpβ+/+ cells, suggesting similar exposure to the cytotoxic intratumoral innate immune cells in athymic mice, which further led us to suspect the involvement of antitumor T cells in Cebpβ+/− cell death. The Cebpβ+/− cells expressed the same percentage of GFP as the Cebpβ+/+ group (data not shown), suggesting that cell death due to Cre recombinase activity is not responsible for the failure of Cebpβ+/− cells to grow in the immunocompetent REAR mice. Instead, the data pointed to a cell-mediated event involving anti-tumor cytotoxic CD8+ T cells.

To better understand the mechanisms responsible for the immune phenotype from our in vivo experiments, we turned to RT-qPCR and RNAseq from Cebpβ+/+ and Cebpβ+/− C3(1)Tag cell lines C3(1)Tag. Wang et al. discovered that in TNBC cells, C/EBPβ activates STAT5A signaling which is involved in suppression of anti-tumor immunity and survival in tumor epithelium83,139. While we saw a slight increase in total STAT protein expression in the Cebpβ+/− cells, we detected no changes in phosphorylation. Additionally, neither STATs we addressed by RT-qPCR were differentially expressed according to our RNAseq expression data of the same cell lines, indicating STAT signaling pathways are unlikely involved in the clearance of
CebpbΔ/Δ cells in vivo (Figure 26A). We also probed Ccl5 expression by RT-qPCR and RNAseq. While previously thought of as a negative prognostic indicator in breast cancer patients, it was recently shown that CCL5 secreted by tumors and intratumoral myeloid cells recruits CD4+ T cells that serve in a supporting role necessary for cytotoxic CD8+ T cell expansion and renewal\textsuperscript{140}. We found by RNAseq that not only does C/EBPβ suppress Ccl5 expression to a significant degree (p = 1.2E-03), but our RT-qPCR results indicate that re-expression of LIP alone in CebpbΔ/Δ cells can drive the suppression phenotype (Figure 26B). As C3(1)TAg is a LIP\textsuperscript{hi} model, these results indicate that suppression of Ccl5 in Cebpb\textsuperscript{+/+} cells may potentially reduce CD4+ T cell recruitment in vivo that may aid in tumor survival, though we did not test those hypotheses in vitro or in vivo.

Our RNAseq results provided us with many potential mechanisms responsible for CebpbΔ/Δ clearance in vivo. Of the standard REACTOME and Gene Ontology – Biological Processes (GO) gene sets, containing 255 and 1054 gene signatures, respectively, 53 and 111 signatures were statistically significant (p<0.05) suggesting massive changes in transcriptional programs upon the loss of C/EBPβ. There was much overlap in the differentially expressed genes (DEGs) within the REACTOME and GO genetic signatures, but the top three most differentially regulated pathways in both gene sets in the CebpbΔ/Δ cells were interferon type I (α and β) signaling, unfolded protein response, and antigen processing and cross-presentation (Figure 27A), further evidencing our hypothesis that C/EBPβ serves as a pro-tumor transcriptional suppressor that shields tumor cells from CD+ T cell cytotoxicity. The p values of the gene set enrichment analysis (GSEA) of the top three differentially expressed signatures indicate strong enrichment, as p = 0.0 for all, but we provided the normalized enrichment scores (NES)
to better visualize the most-enriched pathways (Figure 27B). Upon further analysis of the DEGs in the interferon type I GO term, we discovered that interferon regulatory factors (IRFs) were among the most significantly upregulated in Cebpb\(^{Δ/Δ}\) cells (Figure 28A-B). IRFs play significant tumor suppressor roles in multiple epithelial cancers by enhancing proteasomal degradation and upregulating MHC I and MHC II expression, which induces more potent antigen presentation responses, often mediated through STAT signaling\(^{141-144}\). Interestingly, none of the upstream components of the known STAT-IRF signaling axes described recently by Jiang et al. were differentially expressed in Cebpb\(^{Δ/Δ}\) cells\(^{142}\). STAT signaling was effectively unchanged, as was interferon α/β receptor expression, genes that are heavily represented in the Type I interferon response GO and REACTOME signatures. As type I interferon response gene signatures were very clearly the most differentially expressed genes, it was surprising that the majority of the genes upstream of IRF signaling (STATs, IFNARs, Tyk2) were unchanged. Additionally, these cells were sequenced in the absence of IFNα/β stimulation, indicating C/EBPβ may play a direct role in IRF suppression independent of STAT and interferon α/β signaling, though we did not test this in vitro. We examined TPMs of IRF family genes to gather more insight into the global transcriptional influence IRF changes exerted. While Irf8 was the most statistically significant (p = 1.13E-07) family member upregulated in Cebpb\(^{Δ/Δ}\) cells, the total TPM increased from only 1.11 to 8.80 (Figure 28C), deeming it an unlikely candidate in driving the antigen processing and presentation phenotype in our Cebpb\(^{Δ/Δ}\) cells\(^{141}\). As Irf7 is known to activate MHC I antigen presentation in epithelial cells\(^{142}\), and displayed the greatest change in TPM (138.32 to 1907.31) in Cebpb\(^{Δ/Δ}\) cells, we reasoned that it serves as the likely candidate driving the phenotype antigen
processing phenotype. As we showed that C/EBPβ most strongly bound *Irf7* in TNBC cells, we demonstrated that C/EBPβ can physically regulate IRF family transcription in a relevant TNBC model (Figure 29).

The data generated from our C3(1)TAg transplant models taken together with our RNAseq data indicated that C/EBPβ suppresses anti-tumor immunity, potentially through its direct transcriptional suppression of the IRF family genes responsible for antigen presentation to cytotoxic CD8+ T cells. As we did not perform ChIP-Seq or address changes in antigen processing or IRF signaling in our NIC model, these results suggesting C/EBPβ directly suppressing IRF signaling (Figure 30), and therefore antigen presentation, may be limited to the basal-like or TNBC-relevant models and remain to be tested functionally. Given that this C3(1)TAg model expresses significant amounts of LIP, and we showed that LIP alone can suppress *Ccl5* expression, we speculate that LIP is functioning as per its generally accepted function – as a transcriptional repressor – which may be sufficient to drive the immunosuppressive phenotype alone. This data demonstrates a novel function of tumor-derived C/EBPβ in tumor immunoregulation.

**Future directions and considerations**

To strengthen our claims regarding the role of tumor-derived C/EBPβ in immunosuppression, in vitro functional antigen presentation assays would definitively confirm our findings. In addition, demonstrating this phenotype in another model with lower LIP expression would delineate whether this is a LIP-specific phenomena, or if LAPs are indirectly involved by activating repressors of IRF transcription. An ideal
experiment to test the role of C/EBPβ suppressing the cytotoxic CD8+ T cell response is to perform thymectomies or antibody T cell depletion experiments in NIC;CebpbΔ/Δ mice and assess spontaneous tumor development. Given that these NIC mice have difficulties breeding, obtaining the number of mice necessary for an in vivo experiment may not be feasible. RNAseq of NIC;Cebpb+/+ and NIC;CebpbΔ/Δ tumor cell lines may be informative to confirm this immunosuppressive phenotype, but without a NIC;CebpbΔΔ cell line to submit in addition, the transcriptional changes may be too slight to provide useful results.

The intended goal of these studies was to shed light on C/EBPβ as a molecular driver of mammary tumors. Given that transcription factors are notoriously difficult therapeutic targets due to their ubiquitous expression, we did not design experiments for the purpose of addressing C/EBPβ as molecular target. However, given the substantial immunosuppression phenotype it exerts in tumor cells, the idea of C/EBPβ as a druggable target in solid tumors is tempting. In fact, a recent publication has shown in vivo that cell-penetrating peptides that selectively inactivate C/EBPβ can work in combination with other therapeutic agents to increase overall survival without affecting healthy cell survival145. Additionally, a phase 1-2 clinical trial (NCT04478279, estimated completion October 2024) is recruiting metastatic breast cancer patients for ST101 therapy, a peptide inhibitor of C/EBPβ. Given the results of our powerful genetic studies and the aforementioned peptide inhibition projects and trials, further exploration of C/EBPβ as a therapeutic target across breast cancer subtypes and in combination with other therapeutics is warranted.
Jiang et al. demonstrated that a STAT1-IRF7 pathway regulates MHC I expression and antigen processing in response to type I interferon stimulation in islet β cells in type I diabetes. We hypothesize that in breast tumors C/EBPβ negatively regulates IRF family expression, and therefore antigen processing and presentation, ultimately holding CD8+ T cell engagement and clearance in check.

Figure 30 Proposed mechanisms of immune evasion phenotype in C/EBPβ+/+ tumor models
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BIOGRAPHY

Caitlin Marie Burke was born and raised in Baton Rouge, Louisiana, as the youngest of three siblings. Her early childhood was spent deeply immersed in competitive elite gymnastics, even attending Bela Karolyi’s Ranch, the USA National Gymnastics training facility. She also played club travel sports – fastpitch softball and volleyball – throughout high school but left sports to focus on her academic studies. Her love of science was fostered by Dr. Anna Bourgeois, DVM, her high school biology teacher, who introduced her to veterinary medicine and her first veterinary technician job. She continued to work in animal medicine from high school through her studies at Louisiana State University with the intention of applying for veterinary school. In May 2012, she obtained her degree in Microbiology with two minors in Chemistry and Classical Studies. She briefly moved to Dallas, Texas to look for work in industrial science, but ultimately moved to New Orleans, Louisiana. She took an analytical chemistry technician position at Eurofins in 2015, where she was introduced to method development and SOP validation, sparking her interest in research and development. With her experience in veterinary medicine and her new interest in research, she applied and was accepted to the Biomedical Sciences PhD program at Tulane in 2016, where she obtained her PhD in October of 2022.