Modulation of Triple Negative Breast Cancer Progression by CHA1 Through Epigenetic Mechanisms

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Abstract

Triple-negative breast cancers (TNBC) pose exceptional challenges with Immune Checkpoint Inhibitors (ICIs) and most of them are resistant, or “cold” tumors, due to lack of tumor-resident immune engagement. Maximal ICI efficacy requires Wnt signaling inhibition and Jak/STAT/interferon signaling activation, culminating in the restoration of antigen presentation and a robust immune response. We report a compound combination (CHA1) that does the above-mentioned functions and in addition, likely through reprogramming the epigenetic status of TNBC tumors. CHA1 is comprised of EGCG (epigallocatechin-3-gallate; green-tea compound) and Decitabine (DNA-methyltransferase (DNMT1) inhibitor; 5’deaza cytidine; FDA-approved for hematologic malignancies). We used TNBC tumor cell lines to investigate the tumor-intrinsic reprogramming effects. All the results were maximal with CHA1, but much less with EGCG or decitabine alone, all at probable human dose equivalences with manageable safety profiles. In vitro CHA1 treatment induces a robust tumor-intrinsic JAK/STAT/IFN response 1) to induce PDL1 and 2) to induce antigen presentation and processing genes, including MHC-1, MHC-2, and numerous genes attributed to professional antigen-presenting cells. In addition, it also induces “viral mimicry” genes which augment the adaptive immune responses by increased expression of antigen-presenting components, which increases tumor immunogenicity and likely explains associations between elevated retrotransposon expression, increased T-cell infiltration, and improved tumor response to ICI therapy. We suggest that epigenetic reprogramming primes for “viral mimicry” induction and that epigenetic therapies converge with cancer immunotherapies due to the viral mimicry phenomena. Together, our work supports a model in which CHA1 influences epigenetics, Wnt, and Jak/STAT/IFN signaling mechanisms—all to reprogram an epithelial-mesenchymal TNBC tumor to express

antigen-presenting properties and shows augmentation in adaptive immune response by increased viral mimicry components.

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List of Abbreviations

APCs: Antigen Presenting Cells
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AZA: Azacytidine
BL1: Basal-like 1
BL2: Basal-like 2
cGAS: Cyclic GMP-AMP synthase
CTAs: Cancer-testis antigens
CTLA-4: Cytotoxic T lymphocyte antigen
DAC: Decitabine
DC: Dendritic cell
DCIS: Ductal Carcinoma *in-situ* Invasive Breast Cancers
DNMTi: DNA Methyltransferase inhibitors
dsDNA: Double-stranded DNA
dsRNA: Double strand RNAs
EGCG: Epigallocatechin-3-gallate
EGFR: Epidermal Growth Factor Receptor
ER: estrogen
ERV: Endogenous Retrovirus
EZH2: Enhancer of zeste homolog 2
EZH2i: EZH2 Inhibitor
GAPDH: Glyceraldehyde Phosphate Dehydrogenase
H3K27: Trimethylation of histone 3 lysine 27
H3K9me3: Trimethylation of histone 3 lysine 9
HER2: human epidermal growth factor receptor 2
HK2: Hexokinase
HMT: Histone Methyltransferase
HPV: Human Papillomavirus
IAP: Intracisternal A particles
IBC: Progesterone
ICIs: Immune Checkpoint Inhibitors
IFN: Interferon
IGF1R: Insulin-like Growth Factor 1 Receptor
IHC: Immunohistochemical
IL: Interleukin
IM: Immunomodulatory
IPA: Ingenuity Pathway Analysis
ISGF-3: IFN stimulating gene factor 3
ISREs: IFN-stimulated response element
JAK: Janus Kinases
LAR: Luminal Androgen Receptor
LDHA: Lactate Dehydrogenase
LINE: Long Interspersed Nuclear Elements
LTR: Long Terminal Repeat
MAVS: Mitochondrial Antiviral-Signaling Protein
MET: Mesenchyme to Epithelial Transition
MHCs: Major histocompatibility complex
MSL: Mesenchymal Stem-Like
NK: Natural Killer
PARP: Poly (ADP-ribose) Polymerase

PKM2: Pyruvate Kinase Isoform M2
PR: Progesterone
PRC2: Polycomb Repressive Complex 2
Tet: Tetracycline
TGFβ: Transforming Growth Factor – beta
Th1 & Th2:T-helper 1 and 2
TLR-3: Toll-Like Receptor 3
TNBC: Triple Negative Breast Cancer
Tregs: Regulatory T-cells

Chapter 1: Introduction

Cancer is the second-leading cause of death in the United States. Projections for 2023 estimated nearly 2,000,000 new cancer cases, translating to over 5,000 new patients daily, and over 600,000 cancer-related deaths. Other than prostate cancer incidence, which increased by 3% annually from 2014 to 2019, men fare better compared to women in new cancer incidence: Lung cancer incidence decreased less for women when compared to men from 2015 through 2019 while breast cancer, uterine corpus cancers, liver cancer, and melanoma all continued to increase. Despite these inter-sex discrepancies in cancer incidence, there was a 65% decrease in cervical cancer incidence among women in their early 20s from 2012 through 2019. This trend is thought to be attributed to the Human Papillomavirus (HPV) vaccine, which was first given to this age group. As most of the HPV-associated cancer occur in women, this may bode well for future incidence rates. From 2019 to 2020, the cancer rate declined by about 1.5%, continuing the downward trend from 1991. Importantly, there is an expected increase in advanced-stage cancers and associated deaths soon due to COVID-19 pandemic-rooted system disruptions. In considering the estimates, note that there are several years of lag in the current year for reporting actual incidence and mortality data due to data collection logistics.

Of the nearly 2,000,000 new cancer cases in 2023, about 56,000 are predicted to be new cases of Ductal Carcinoma *in situ* (DCIS) in women [1]. DCIS refers to breast epithelial cells which exhibit abnormally increased growth behaviors, but which remain in their ordinary anatomical locale. Therefore, by definition, DCIS is a nonlethal, noninvasive type of cancer. However, it is the immediate precursor to invasive breast cancers (IBCs), which can be lethal [2]. Our work is focused on one class of IBC: Triple-negative breast cancer (TNBC). When considering breast cancer clinically, there is

usually a focus on three markers: Expression of the endocrine-associated receptors for estrogen (ER) and progesterone (PR or PgR), and abnormal increased expression of human epidermal growth factor receptor 2 (HER2). TNBCs, however, are uniquely defined as tumors that express none of these three receptors, thereby warranting the term “triple-negative.” This invasive subset of breast cancer is particularly challenging in part because of this inherent characteristic, as in other cancer contexts, these three receptors can be useful targets for therapeutic agents.

TNBC most commonly affects premenopausal women under the age of 40, a demographic accounting for up to 20% of all cancer patients. With approximately 46% of patients having a distant metastasis, TNBC has high invasive potential [3]. Crucially, TNBC tends to metastasize to the brain, among other viscera. Following metastasis, median patient persistence is just over 13 months [4]. Post-operative tumor removal, TNBC can recur in up to a quarter of surgery patients in a more rapid timeline relative to other cancer types, and the mortality rate is as high as three quarters within 3 months following TNBC recurrence [1, 5]. TNBC’s aggressive metastatic potential, proclivity for relapse, as well as marked heterogeneity of the disease, has resulted in poor prognosis when compared to other breast cancer subtypes: Mortality rate within 5 years of diagnosis is nearly 40%.

1.1. Pathobiology of Breast Cancer Progression

In recent publications stemming from a massive effort to sequence human cancer genes, it was estimated that cancers carry an estimated 48 genetic mutations in pancreatic cancer specimens to 77 for colorectal cancers and 101 for breast cancers [6, 7]. In addition, it is estimated that cancers carry approximately 100-400 epimutations or alterations that are of epigenetic origin rather than genetic [8].

Cancer metastasis is believed to arise when a small population of cells detach from the primary tumor and travels through the circulation establishing foci in an ectopic site.

This metastasis cascade occurs in distinct stages with distinct molecular circuitries. The earliest stages of cancer cell metastasis involve cells breaking through their normal environments. These cells must acquire invasiveness and be motile, which usually involves undergoing an epithelial-to-mesenchymal transition (EMT). This initial process is termed intravasation. Once in the circulatory system, cells must learn to survive in a hostile environment. These cells have attained the ability to evade normal cell death that would otherwise occur when cells detach from their normal strata; this kind of cell death is termed anoikis. For cells to establish themselves in distant sites, they must first extravasate out of the circulatory vessels and form micrometastases. These cells then undergo a mesenchyme-to-epithelial transition (MET) that allows these cells to aggregate and form macro metastases [9]. This model was challenged by Harold Varmus and colleagues who demonstrated that even non-transformed mammary cells can form foci in the lungs of mice and remain dormant and viable. Upon induction of oncogenes from a tetracycline (Tet)- inducible promoter, these cells can then form aggressive metastatic lesions. These findings imply that even rare, otherwise, normal epithelial cells can break away from their host tissue and survive in an ectopic site [10, 11].

A major focus of clinical breast cancer prevention and management is early detection as it greatly improves chances of survival if the cancer is caught before it invades and spreads. As such, basic science research into the causes of and ultimately treatments for cancer cell invasion and metastasis should be imperative.

1.2. TNBC Subtypes

TNBC is a frighteningly challenging and complex disease. Accurate diagnosis of the triple-negative character requires immunohistochemical (IHC) analysis of ER, PR, and HER2 protein expression levels. Approximately 95% of these diagnosed TNBCs are further classified histologically as either non-specific invasive mammary carcinomas or

as invasive ductal carcinomas [1]. Broadly, breast cancers are classified into subtypes according to intrinsic molecular characteristics: Basal-like, HER2-enriched, luminal A and B, and normal-like. Of these intrinsic molecular (PAM50) subtypes, TNBCs are most often basal-like. There are six further recognized subtypes within the TNBC disease classification based on gene expression: Basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR) [3].

The BL1 genetic profile features amplification of MYC, PI3KA, CDK6, AKT2, KRAS, FGFR1, IGF1R and CCNE1 and deletions of CDKN2A, BRCA2, PTEN, MDM2, RBI, and TP53. MYC, a proto-oncogene that encodes a transcription factor involved in cell proliferation, differentiation, and apoptosis, fuels aggressive cell proliferation including TNBC. PI3KA, part of the PI3K/AKT/mTOR pathway, promotes cell survival and resistance to treatment when it is overexpressed. CDK6, a regulator of cell cycle progression, is a member of the cyclin-dependent kinase family which regulates cell cycle progression and stimulates uncontrolled cell division in TNBC. AKT2, a serine protein kinase, enhances cell survival and drug resistance. KRAS, a proto-oncogene, that encodes a GTPase involved in transmitting signals from cell surface receptors to the nucleus. KRAS mutations are relatively rare in TNBC compared to other cancer types but can drive tumor growth and resistance to therapy. FGFR1, a receptor tyrosine kinase involved in cell proliferation and angiogenesis receptor, is associated with increased cell proliferation and poor prognosis. IGF1R (Insulin-like Growth Factor 1 Receptor), another receptor, fosters cell growth and resistance to therapy. CCNE1, a regulatory protein involved in the G1/S transition of the cell cycle, drives cell cycle progression correlating with aggressive tumor behavior. CDKN2A/B, inhibitors of cell cycle progression, when lost, lead to heightened proliferation in TNBC. CDKN2A encodes the p16INK4a and p14ARF proteins, while CDKN2B encodes p15INK4b. These proteins regulate cell cycle

progression by inhibiting CDKs. Indeed, beyond the overexpression of oncogenes, the repression or inactivation of tumor suppressor genes constitutes a significant mechanism driving the proliferation of TNBC. Tumor suppressor genes such as BRCA2, PTEN, RB1, and TP53 are pivotal in regulating cellular processes essential for maintaining genomic stability, cell cycle control, and apoptosis. Mutations, deletions, or epigenetic silencing of these genes can disrupt their normal functions, leading to uncontrolled proliferation of TNBC cells. For instance, loss of BRCA2 function compromises DNA repair mechanisms, rendering cells susceptible to genomic instability and predisposing individuals to TNBC. Mutations in BRCA2 are associated with an increased risk of breast cancer, including TNBC. TNBC patients with BRCA2 mutations may respond differently to certain treatments, such as platinum-based chemotherapy or PARP inhibitors. PARP, or poly (ADP-ribose) polymerase, enzymes play a critical role in repairing DNA damage through the base excision repair pathway. PARP inhibitors work by blocking PARP enzymes, thus preventing the repair of single-strand DNA breaks. While cells with intact DNA repair mechanisms can tolerate the loss of PARP function, cancer cells, especially those with defects in homologous recombination repair pathways such as BRCA1/2 mutations, become highly sensitive to PARP inhibition. PARP inhibitors induce further DNA damage in TNBC cells with BRCA1/2 mutations, leading to cell death [12] . However, it is essential to note that not all TNBC patients respond to PARP inhibitors, and resistance mechanisms can emerge over time. Among newly diagnosed breast cancer patients, fewer than 10% have a mutation in the BRCA1 or BRCA2 genes, and up to 20% present with TNBC. However, among BRCA1 mutation carriers at least one-third have TNBC [13]. Similarly, PTEN loss enhances PI3K/AKT signaling, promoting cell survival and proliferation. Inactivation of RB1 disrupts cell cycle regulation, while mutations in TP53 compromise its tumor-suppressive functions, allowing aberrant cell growth and survival. TP53 is a well-known tumor suppressor gene

that regulates cell cycle progression, DNA repair, and apoptosis. these genes play critical roles in the development and progression of triple-negative breast cancer, with mutations or dysregulation contributing to tumor growth, treatment resistance, and poor prognosis. Understanding their functions can aid in the development of targeted therapies for TNBC patients.

BL2 type of TNBC refers to a specific molecular subtype identified through gene expression profiling. BL2 subtype is characterized by high expression of genes associated with the "basal-like" phenotype and low expression of genes related to cell cycle and DNA damage response pathways. The BL2 genetic profile features atypical activation of the EGFR, MET, NGF, Wnt/β-catenin, and IGF1R cell signaling pathways. EGFR (Epidermal Growth Factor Receptor), a receptor tyrosine kinase that regulates cell proliferation, survival, and migration, is a common driver for tumor growth and aggressiveness in BL2 TNBC. Targeting EGFR signaling with growth factor inhibitors has shown promise in preclinical studies and clinical trials as a potential therapeutic strategy for BL2 TNBC. Another receptor tyrosine kinase called MET promotes tumor progression, invasion, and metastasis, making it a potential therapeutic target in BL2 TNBC. Dysregulation of MET signaling, including gene amplification or overexpression, has been observed in TNBC, particularly in the BL2 subtype. Emerging evidence suggests that NGF signaling may also contribute to the progression of TNBC, including the BL2 subtype [3]. NGF is a neurotrophic factor that plays a role in the growth, differentiation, and survival of neurons. The Wnt/β-catenin pathway is a key signaling pathway involved in embryonic development, tissue homeostasis, and tumorigenesis. Dysregulation of this pathway, often through mutations or aberrant activation, is associated with various cancers, including TNBC. In BL2 TNBC, aberrant activation of the Wnt/β-catenin pathway promotes tumor growth, invasion, and metastasis, highlighting its significance as a potential therapeutic target. A key mechanism for stem

cell renewal, Wnt signaling probably plays a role in the growth of the cancer stem cell population, which has long been connected to metastasis and recurrence. High Wnt signaling in the tumor has recently been linked to immune evasion and the promotion of an immunosuppressive milieu in the immunological microenvironment of breast and other malignancies. Researchers have shown that immune surveillance systems that prevent decreased T-cell infiltration—a process essential to immune-mediated destruction and the effectiveness of immune checkpoint inhibitors—involve elevated Wnt signaling in a variety of malignancies [14-16]. Improved approaches to reduce Wnt signaling are therefore required. Wnt pathway inhibitors (Wnt 974, PORCN) are currently undergoing clinical studies, yet some side effects, such as bone fractures, have been reported [17]. Wnt signaling is probably necessary for bone regeneration, but it's also critical for the upkeep and regeneration of healthy tissue.

The M subtype features enhanced actin-mediated, cell migration-regulated signaling pathways, as well as extracellular matrix-receptor interaction pathways. Also called metaplastic breast cancer, the mesenchymal subtype also commonly features increased activation of the Wnt, anaplastic lymphoma kinase, and transforming growth factor – beta (TGFβ) differentiation pathways. TGFβ signaling is a potent inducer of EMT, a process by which epithelial cells acquire mesenchymal characteristics, such as increased motility and invasiveness. In M-type TNBC, activation of the TGFβ pathway leads to the downregulation of epithelial markers (e.g., E-cadherin) and upregulation of mesenchymal markers (e.g., N-cadherin, vimentin), facilitating the transition to a more aggressive phenotype capable of invading surrounding tissues and metastasizing to distant sites. TGFβ signaling promotes various steps of the metastatic cascade, including local invasion, intravasation into blood or lymphatic vessels, survival in the circulation, extravasation at distant sites, and colonization of secondary organs. Hence, aberrant activation of the TGFβ pathway enhances the ability of cancer cells to migrate

and invade surrounding tissues, as well as to survive and proliferate at distant metastatic sites. In addition, TGFβ has immunosuppressive effects, inhibiting the function of various immune cells, including T cells, natural killer cells, and dendritic cells [18]. This subtype shares tissue features with sarcomas and squamous epithelial cells. While it may be sensitive to mTOR inhibitors or therapies targeting the epithelial-mesenchymal transition, mesenchymal TNBC often builds resistance to chemotherapies [19].

The immunomodulatory (IM) TNBC subtype is characterized by marked increases in immune cell-derived genes and signaling pathways. These include the T-helper 1 and 2 (Th1 and Th2) pathway, natural killer (NK) cell pathway, B and T cell receptor signaling pathways, dendritic cell (DC) pathway, and interleukin (IL) -12 and IL-7 pathways. Despite TNBC being generally associated with a poorer prognosis, patients with IM subtype TNBC often have a more favorable prognosis compared to other TNBC subtypes. This improved prognosis may be attributed to the heightened immune response within the tumor microenvironment, which could contribute to better tumor control and patient outcomes [20]. The presence of immune cell infiltration and immunomodulatory gene expression signature in IM subtype TNBC suggests potential responsiveness to immunotherapy. Immunotherapeutic approaches, such as immune checkpoint inhibitors targeting PD-1/PD-L1 or CTLA-4, may harness the existing anti-tumor immune response to enhance tumor regression and improve patient survival. In brief, PD-1 is a receptor expressed on activated T cells, while PD-L1 is a ligand expressed on tumor cells and other immune cells. ICIs unleash the immune system's ability to recognize and destroy cancer cells by blocking inhibitory signals that prevent T cell activation and function. By releasing the "brakes" on the immune response, ICIs enhance the ability of T cells to recognize and eliminate tumor cells. The interaction between PD-1 and PD-L1 inhibits T-cell activity, allowing cancer cells to evade immune surveillance. PD-1/PD-L1 inhibitors block this interaction, unleashing the immune system

to attack cancer cells. CTLA-4 is another immune checkpoint receptor expressed on T cells and it competes with the co-stimulatory receptor CD28 for binding to its ligands (B7-1 and B7-2) on antigen-presenting cells, resulting in downregulation of T cell activation. CTLA-4 inhibitors prevent this inhibitory signal, enhancing T cell activation and anti-tumor immune responses [21].

Another TNBC subtype, LAR (Luminal Androgen Receptor), is characterized by high activation of endocrine-derived signaling pathways. As a type of TNBC, LAR still does not express ER, but there are common increases in steroid synthesis, porphyrin metabolism, and androgen and estrogen metabolic cycles. LAR TNBC tumors express high levels of androgen receptor (AR), a steroid hormone receptor that plays a role in the development and progression of breast cancer. AR signaling in LAR TNBC contributes to tumor growth and survival, making it a potential therapeutic target. Targeting AR signaling represents a potential therapeutic strategy for LAR TNBC. Clinical trials investigating the efficacy of AR-targeted therapies, such as anti-androgens or AR antagonists, in LAR TNBC are underway. Additionally, combination therapies targeting AR signaling along with other pathways implicated in TNBC pathogenesis may enhance treatment efficacy in this subtype [22].

1.3. Immunotherapies

Despite the lack of namesake ER, PR, and HER2 cell components, there are several alternative targeted therapy regimens. Immunotherapies have demonstrated efficacy across a wide range of cancer types, including melanoma, lung cancer, bladder cancer, and Hodgkin's lymphoma, among others. They have also shown promise in cancers where traditional treatments have had limited success, such as metastatic melanoma and non-small cell lung cancer. Immunotherapies work by targeting various components of the immune system, including T cells, B cells, and natural killer cells, to recognize and attack cancer cells. One of the most well-known mechanisms is immune

checkpoint blockade, which involves blocking inhibitory signals that prevent T cells from recognizing and destroying cancer cells. This unleashes the immune system's ability to mount an effective anti-tumor response.

Immunotherapy encompasses various approaches aimed at harnessing the body's immune system to target and eliminate cancer cells. There are different types of immunotherapies but my thesis focus would be on ICIs as they offer a promising route to treating TNBC if the mechanisms described next can be harnessed.

1.3.1. Immune Checkpoint Inhibitors

By interacting with and stimulating the local immune system to eradicate malignancies, immune checkpoint inhibitors (ICIs) have completely revolutionized some cancer treatments [16, 21]. Notably, tumor and local T-cell interactions through the PD-L1-PD-1 cell-cell pathway are a major factor in the immune-suppressive local milieu in which malignancies spread. By encouraging local T-cell activation, the common ICIs—anti-PD-L1 (atezolizumab, avelumab) and anti-PD-1 (e.g., pembrolizumab, Keytruda)—disrupt the inhibitory PD-L1-PD-1 connections between tumor and T-cells, respectively, improving immune-mediated tumor killing. A third type, anti-CTLA-4, functions in an inhibitory manner via dendritic cells while simultaneously encouraging the immediate environment's cytotoxic CD8+ T-cell activation. Melanomas and lung cancer were the first cancers to be treated, and the results were initially rather striking. Lung cancer is one instance when anti-PD-1 therapy meets ideal parameters. A strong clinical biomarker for anti-PD1 effectiveness is that the PD-L1 positivity should be more than 50% in the tumor [23, 24].

The grim fact that only around 25% of tumor types are responsive to ICIs, while most cancers have varied efficacy, tempers the success of ICI treatments [23, 25]. Therefore, a key objective is to reduce side effects, which are frequently brought on by

auto-immune reactions, while broadening the spectrum of cancers that are vulnerable and respond well to ICIs. Something relevant to ICI resistance is the term "cold-to-hot transition". The term "cold-to-hot transition" describes the endowment of tumor susceptibility, where "cold" and "hot" generally correspond to low and high levels of inflammation or immune cell infiltration, respectively. Though it is a collection of biologically varied and seemingly unrelated traits including epigenetic status, β-catenin status, epithelial character, etc., the cold-to-hot susceptibility has been addressed in detail [25]. Theoretically, any treatment that transforms "cold" cancers into "hot" ones should increase the range of tumors that ICIs can effectively treat, leading to better patient outcomes.

Because breast cancers frequently lack immune cell infiltration and other molecular signaling features of ICI-susceptible tumors and are in general cold. Based on a significant clinical trial, anti-PD-L1 was granted accelerated approval in March 2019 for use in recurrent or incurable PD-L1 positive metastatic triple-negative breast cancer but was later withdrawn for lack of efficacy [26]. Anti-PD-1 was first licensed for its use as a frontline treatment for metastatic TNBC, but it has now been approved for use in non-metastatic TNBC as well, with improved results in a neoadjuvant setting [26]. Patients with TNBC who had higher levels of PD-L1 expression also had better results, according to post-hoc analysis. The FDA then fully approved anti-PD-1 for use in metastatic TNBC patients whose tumors expressed PD-L1 [26-28]. These TNBC studies demonstrate the necessity of creating standards for better ICI application and for combination therapies that increase PD-L1 to enhance TNBC and treatment for breast cancer [28]. The target tumor's PD-L1 expression may be required, but it is not sufficient to forecast the effectiveness of anti-PD-1 or anti-PD-L1 treatments in the future.

Additional studies showed that many ICI-resistant tumors have elevated Wnt signaling and, dysfunctional JAK/STAT/IFN signaling. Hyperactive Wnt signaling is often

associated with excessive breast cancer growth, recurrence, and metastasis, including fatal brain metastases [4, 29]. Researchers have found out that high Wnt signaling in cancers prevents T-cell infiltration and activation, a process critical for ICIs efficacy and immune-mediated destruction, suggesting ways to limit the Wnt signaling are necessary for ICIs to treat in Wnt-driven tumors [16, 30]. A dysfunctional JAK/STAT interferon (IFN) pathway is a major factor in ICI resistance. In addition, it is also a major factor in regulating the expression of the important bio-marker and checkpoint gene PD-L1, and hence a dysregulation in its expression may lead to resistance to ICIs [16, 21, 31]. JAK/STAT is also involved in triggering the expression of MHCs (major histocompatibility complex-I and -II) and antigen-presenting proteins. So, alterations in JAK/STAT signaling may lead to defects in antigen presentation and processing which may lead to a decrease in tumor immunity [21].

1.3.2. Epigenetic Therapies

Tumor initiation and development are increasingly linked to changes in the epigenome. Selective global DNA hypomethylation combined with selective DNA hypermethylation characterizes the dysregulated aspects of the cancer epigenome [32]. Epigenetic therapies represent a therapeutic vulnerability because epigenetic states are reversible and can differentiate malignancies from their respective cell of origin.

There has been an overlap between epigenetic therapies and cancer immunotherapy approaches via a phenomenon called “viral mimicry.,” a cellular state characterized by an active antiviral response brought on by endogenous stimuli as opposed to an exogenous viral infection. Recently, reports of endogenous retrovirus (ERV) induction after DNMTi (DNA Methyltransferase inhibitors) treatment suggested that DNMTi and antiviral responses may have some molecular connections. Almost half of the human genome is made up of repetitive sequences, and their transcriptional

activation can lead to genomic instability and the pathogenesis of disease [33]. Therefore, these repetitive elements are an integral part of the host defense mechanism which remains suppressed transcriptionally due to DNA hypermethylation.

The first epigenetic drugs that were established were the cytidine analogs 5-azacytidine (azacitidine or AZA) and 5-aza-2′-deoxycytidine (decitabine or DAC) which were synthesized as general cytotoxic agents. Ten years after Taylor and Jones first reported the demethylating properties of AZA and DAC, it was demonstrated that AZA-treated mouse embryonic fibroblasts upregulate ERVs. Similarly, intracisternal A particles (IAP) transposons are seen at higher concentrations in *Dnmt1*-deficient mouse embryos [34]. In human cancer cells, DNMTi therapies also cause repeat sequences like ERVs and Alu (short interspersed nuclear elements) to be expressed [34]. Two groundbreaking studies published in 2015 describe "viral mimicry," in which the host cell interprets aberrantly expressed repetitive element RNAs as a viral infection and activates an IFN response, which leads to direct connections between DNMTi, retrotransposon transcription, and IFN activation [35, 36]. The IFN response initiated by DNMTi treatment corresponds to an Innate immune response against foreign exogenous viruses. Antiviral signaling commences when cytosolic RNA sensors, like MDA5, or endosomal RNA sensors, like Toll-like receptor 3 (TLR-3), recognize retrotransposon-derived double-strand RNAs (dsRNA). When RNA sensors are stimulated, the mitochondrial antiviral-signaling protein (MAVS) aggregates on the mitochondrial surface. This triggers a phosphorylation cascade mediated by TBK1, which phosphorylates, dimerizes, and localizes IRF3/7 in the nucleus to activate type I or type III interferon signaling. Another mechanism of activating type 1 IFN signaling has been reported to happen via sensing of double-stranded DNA (dsDNA). Following cytosolic dsDNA recognition, cGAS (cyclic GMP-AMP synthase) produces cyclic GMP-AMP

(cGAMP) from GTP and ATP. Type I IFN signaling is triggered by the binding of cGAMP to STING, and this process is dependent on both IRF3 and TBK1 [37, 38].

Briefly, IFNs bind to their corresponding receptors, stimulating Janus kinases (JAK) to phosphorylate STAT1/2. IRF9 and STAT1/2 together form the heterotrimeric complex known as IFN stimulating gene SG factor 3 (ISGF-3). After that, ISGF3 moves into the nucleus and attaches itself to IFN-stimulated response elements (ISREs) to start ISG transcription. The consequences of this activation lead to the induction of apoptosis and modulation of tumor adaptive immune response and it self-regulates through positive and negative feedback loops [39].

Epigenetic targets other than DNMT1 may also promote viral mimicry because epigenetic control of the genome requires numerous layers of regulation and cross-talk that permit alteration of both DNA and histone tails. Multifaceted epigenetic approaches target repetitive elements to establish repressive heterochromatin. For example, the epigenetic mechanism responsible for suppressing intergenic long terminal repeat (LTR) regions depends on the evolutionary age of the LTR, with older and intermediate- and younger families repressed by H3K9me3, and DNA methylation, respectively [40]. Facultative heterochromatin mediates compensatory retrotransposon silencing in the event of constitutive heterochromatin disruption. Fascinatingly, several studies show that in both healthy somatic cells and cancer cells with intact constitutive heterochromatin, facultative heterochromatin can be used to mute retrotransposons. These investigations suggested that another possible pharmacological target for viral mimicry induction could be "writers" of facultative or constitutive heterochromatin and "erasers" of euchromatic histone marks. Integration of tumor suppressor protein activity and heterochromatin maintenance with dsRNA silencing implies that viral mimicry might eradicate cells that are susceptible to developing genomic instability and through this thesis, we are also trying to find whether viral mimicry is an endogenous epigenetic checkpoint.

An important challenge for treating TNBC is to develop strategies for converting tumors from “cold” to “hot,” i.e. to engage the local immune environment and increase tumor responsiveness to ICIs. As stated above, maximal ICIs efficacy will require an inhibition of Wnt signalling with an activation of JAK/STAT/IFN signalling. As noted, Yee’s lab CHA1 compound combination inhibits Wnt signaling and promotes the cold-to-hot transition in TNBC tumors. CHA1 consists of EGCG (epigallocatechin-3-gallate; principal compound in green tea) and Decitabine (an FDA-approved DNA methyltransferase (DNMT1) inhibitor for the treatment of hematologic malignancies). They showed that EGCG blocked Wnt signaling through the induction of the HBP1 transcriptional repressor to decrease cellular processes relevant to invasive breast cancer. The studies show that EGCG elevation of HBP1 also resulted in elevation of sFRP1, a Wnt signaling inhibitor. Decitabine (DAC) is an epigenetics-based agent whose principal action is to inhibit DNA methyltransferases (DNMTs) and induce gene hypomethylation. Additionally, the genes for one or more of the Wnt pathway inhibitors such as sFRP1 and HBP1 are themselves subject to suppression by DNA methylation. The Yee lab provided evidence that CHA1-treated tumors exhibit properties of a cold-to-hot transition and additionally, a robust JAK/STAT/IFN response resulting in induction of PD-L1, a critical biomarker for clinical ICI decision-making [41]. In addition, they found that CHA1 orchestrates a re-programming of the epithelial tumor cell to confer antigen-presentation properties that then initiate the local immune interactions.

My thesis is an extension of the following studies on TNBC to use CHA1 at equivalent doses attainable in humans to maximize future clinical trial applications. The focus of my research is focused on the epigenetic aspect and a “viral mimicry” response caused by CHA1 treatments on human MDA-MB-231 breast cancer cell lines. We have proposed a cell-based assay system to identify new compounds that influence epigenetics, Wnt, and Jak/STAT/IFN signaling mechanisms—all to reprogram an

epithelial-mesenchymal TNBC tumor to express antigen-presenting properties and to potentially recruit and activate tumor-resident CD8+-T-cells. The cell treatments have shown robust disruptions in the epigenetic status of the breast cancer cell lines which is one of the reasons for its cold to hot transition. CHA1 treatment in human MDA-MB-231 xenografts in immune-compromised mice effectuated a robust tumor-intrinsic response 1) to induce PDL1 and 2) to induce antigen presentation and processing genes, including MHC-I, MHC-2 and numerous other genes attributed to antigen-presenting cells; 3) to induce CD8+-T-cell infiltration and activation but many of these tumor intrinsic responses where different when seen in the MDA-MB-231 breast cancer cell lines. There was a significant epigenetic disruption in H3K27me3 and H3K9me3 methylation status with induction of “viral mimicry” genes that were pooled out from a human RNA-seq. Through this research, we propose to make a convergence between Epigenetic therapies and cancer immunotherapies through “viral mimicry,” In addition, characterize and expand the “viral mimicry”–inducing approaches in TNBC solid tumors and hope to contribute to current trends in cancer treatments, abating incidence of breast cancer and further decreasing mortality rates.

Chapter 2: Materials and Methods

2.1. Quantitative RT-PCR

Total RNA was extracted using RNAeasy Mini kits (Qiagen) and the reverse was transcribed with a random hexamer mix (iScript, Bio-Rad). The resulting cDNA was used for real-time qPCR performed using an iCycler (BioRad). The SYBR-Green master mix was purchased from Bio-Rad. All quantifications were normalized to an endogenous 18 S RNA control. The relative quantitative value for each target gene compared to the calibrator for the target is expressed as the comparative Ct (2-(ΔCt-Cc)) method (Ct and Cc are the mean threshold cycle differences after normalizing to 18S). Quantitative RT-PCR experiments were performed in triplicate and the values are means of triplicates ± SEM. The primers and primer sequences are listed in **Table 2.1.**

Primers	Sequences
18s	F – GCCCGAAGCGTTTACTTTG R – CTTAATCATGGCCTCAGTTCC
hHBP1	F – GGCGACGGGTTTGTCAGAG R – TGCCAGATTGGGTAGGATCAC
hSFRP1	F – CTGATAACTGGTTGCTGTGTC R – CATCCATGTCCTGTGTATCTGC
hHLA-DRB5	F – AGCATGGTGTGTCTGAAGC R – CCCGTTGAAGAAATGACACTCA
hHLAB	F – CTAGCAGTTGTGGTCATCGGAG R – GGAGGCGTGAAGAAATCCTG
hIFIT2	F – AACAAAAAGGAACCAGAGGCCA R – TAGTTGCCGTAGGCTGCTCTC
hOAS1	F – TCCGTGAAGTTTGAGGTCCAG R – AGGTTTATAGCCGCCAGTCA
hNYESO	F – TGTCCGGCAACATACTGACT R – AAAAACACGGGCAGAAAGCAC
hMAGE3	F – CCCTGAGCAACGAGCGAC R – GACTCTGGTCAGGGCAACAG

hMAGE6	F – CCCTGAGCAACGAGCGA R – ACTCTGGTCAGGGCAACAG
hERV3	F – AGCCGGAGCTTCTGGTGTAG R – AGTGGGTCCTGGCGTCTTA
hDHX58	F – CTGGACCCTACCTACATCCTG R – GGCATCCAAAAAGCCACGG
hDDX58	F – GGGCCTCCAAACTCGATGG R – TTCTGGGGTGACATGATGCAC
hIFIH2 (MDA-5)	F – GGAGTCAAAGCCCACCATCT R – GGTGACGAGACCATAACGGA
hPDL1	F – TATGCCTTGGTGTAGCACTGA R – CCGATGAACCCCTAAACCACA

Table 2.1. Primer sequences Table

2.2. Total RNA isolation and cDNA synthesis

RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen) and was eluted with RNase-free water. A Nanodrop spectrophotometer (Thermo Scientific) was used to measure RNA concentration by measuring absorbance at OD 260/280. After determining RNA concentration, 500 ng of RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad).

2.3. Western Blotting

Tumor and cell lysates were prepared from frozen tumors and cell pellets respectively by extraction for 30 min at 4˚C in lysis buffer (50 HEPES, pH 7.5, 150 mM NaCl, 1.5mM MgCl2, 1MM EDTA, 10% glycine, 1% Triton X-100, 20mM β-glycerophosphate, 0.1 mM sodium vanadate, 0.1% SDS, 1% deoxycholate, 1 mg/ml leupeptin, 200 mM PMSF, 1 mg/ml pepstatin). All lysates were clarified by centrifugation for 15 min at 14000 rpm and frozen at –80˚C. The supernatants were quantified by using Bradford assay from Bio-Rad and 2X Laemmli SDS sample buffer (Bio-Rad) was added to protein extracts. Protein extracts were normalized between all samples within an

experiment and boiled above 95^{o}C for 5 min.15 µg of total protein samples in each group were subjected to 4–12% Bis-Tris gradient gels (Bio-Rad Mini-PROTEAN TGX gel), then transferred to PVDF membrane. The membranes were incubated with primary antibodies overnight at 4˚C and secondary antibodies for 1 hour at room temperature. All primary antibodies were used at a dilution of 1:2000 and the secondary antibodies from Jakson ImmunoResearch Laboratories, Inc. were used at a dilution of 1:20,000. All the primary antibodies used are listed in **Table 2.2**. The western blot results were developed with Thermo Fisher Supersignal West Femto Chemiluminescent substrate and imaged by the Bio-Rad Chemidoc MP Imaging System. Quantitation of band intensities was performed with ImageJ. All immunoblots were cropped to optimize clarity and presentation.

Reagent	**Source**
β-actin	Sigma-Aldrich
H3K9me3	Cell signaling
H3K27me3	Cell signaling
STAT3	Cell signaling
PSTAT3^{Y705}	Cell signaling

Table 2.2. List of Primary antibodies used for Western blotting.

2.4. Cell Culture and Treatment regimen for *In-vitro* study

MDA-MB-231 cell lines, purchased from ATCC, were used for the *In-vitro* study. DAC and EGCG were dissolved in PBS and ethanol with PBS, respectively. Cells were treated with various combination concentrations of EGCG and DAC, Individual concentrations of EGCG, Individual concentrations of DAC and no treatment as shown in **Table 2.3**. Treatment was conducted for 120 hours and the cells were harvested 24 hours post-last-day treatment. All cells were maintained in the normal media that contained Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), antibiotics, and anti-trypsin during treatment. Media and drugs were replaced every 24 hours (five times during the 120-hour treatment period).

Samples	Treatment concentrations
1	10 µM EGCG
2	5 µM EGCG
3	2.5 µM EGCG
4	10µM EGCG + 1µM DAC
5	5 µM EGCG + 1 µM DAC
6	2.5 µM EGCG + 1 µM DAC
7	10µM EGCG + 0.5 µM DAC
8	5 µM EGCG + 0.5 µM DAC
9	2.5 µM EGCG + 0.5 µM DAC
10	10µM EGCG + 0.25 µM DAC
11	5 µM EGCG + 0.25 µM DAC
12	2.5 µM EGCG + 0.25 µM DAC
13	1µM DAC
14	0.5 µM DAC
15	0.25 µM DAC
16	UT

Table 2.3. Treatment regimen for *In-vitro* cell culture treatments.

2.5. Animals

Balb/c female mice, aged six to eight weeks, were acquired from Jackson Laboratory. The Institutional Laboratory Animal Care and Use Committee (IACUC) at Tufts University-Tufts Medical Center developed rules that the Division of Laboratory Animal Medicine (DLAM) staff followed in maintaining the mice in a sterile environment.

2.6. Cell Preparation for Animal Surgery

The cells were grown to a maximum confluence of 85% in standard growth media (DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 10 mM L-glutamine). The cells were then trypsinized with 0.25% trypsin and

then rinsed with PBS to harvest the cells. After that, the cells were again suspended in Dulbecco's Modified Eagle Medium, which is free of serum and antibiotics. Hemocytometers were used to count the cells after staining them with 0.4% trypan blue solution (Sigma) to accurately measure the cell concentration. The cells were set up for implantation/inoculation into each mouse's mammary fat pad using a 1:1 volume ratio of Matrigel (BD Biosciences). Only the 4T1 cells were prepared for implantation into syngeneic Balb/c mice.

2.7. Drug Treatment Plan for *In-vivo* Study

When tumors reached approximately 0.125 cc, treatments were initiated. Both EGCG (Sigma-Aldrich) and DAC (Sigma-Aldrich) were dissolved in PBS and then filter sterilized. A 28-gauge insulin syringe was used for intraperitoneal injection (i.p). All treatments continued for the indicated time unless tumor size exceeded that allowed by IACUC guidelines or if the animal's health appeared to be deteriorating. The treatment plan was carried out in a syngeneic model with 4T1 tumors implanted into Balb/c mice.

CHA1 treatment in TNBC syngeneic models: mice were randomly assigned to either control or experimental groups with at least five mice in each group. 16.5 mg/kg of EGCG and 0.5 mg/kg of DAC were administrated concomitantly via i.p. injections during cycle 1 and cycle 2**.** The duration of each cycle was 5 days. There was 5 days recovery period between the cycles.

2.8. Tumor Implantation and Resection

The Division of Laboratory Animal Medicine at Tufts Medical Center established rules for animal surgery establishment, and all surgeries were performed in a sterile procedure room. Initially, the animals were anesthetized in an isoflurane chamber. Mice were given a continuous supply of isoflurane through a nasal cannula during the procedure. Surgery: The region surrounding the fourth mammary fat pad was cleaned

with alcohol and iodine, and the hair was shaved. The fourth mammary fat pad was carefully located, and an incision was made to expose the region to allow cancer cells to deposit themselves there. To allow for complete access to the location for cell inoculation, connective tissues were loosened. Thirty to thirty-five milliliters containing 3 x 10^5 – 5 x 10^5 cells suspended in a 1:1 ratio of serum-free, antibiotic-free media, and Matrigel was injected into the mammary fat pad using a sterile Hamilton Syringe. Wound clips were used to heal the incision after the cells were inoculated. The animals were closely observed when they recovered from anesthesia. The analgesic used was buprenorphine (0.06 mg/kg). Three days after surgery, the animals were observed to ensure appropriate healing and to look for any complications. After surgery, the wound clips were taken out 10–14 days later.

The tumors, which had a diameter of between 0.4 and 0.5 cm, were carefully disclosed after the mice were anesthetized and opened. To stop bleeding, the three main blood veins leading to the tumor and breast gland were heat cauterized, and the tumor was gently removed. Wound clips were used to close the wounds. The primary tumor was harvested from each mouse and fixed with formalin. Fixed tissues were preserved in 70% ethanol. A small, unfixed sample from each mouse was stored at -80˚C for further analysis.

2.9. Statistical Analyses

All experiments were performed in triplicate. Student's unpaired t-test with Welch corrections was used to compare the two groups. One-way ANOVA with Tukey's post-hoc test was used for multiple comparisons. A P-value less than 0.05 was considered statistically significant. All analyses were performed by GraphPad Prism 10.

Chapter 3: Results

3.1. The design rationale for CHA1 combination treatment

As was previously mentioned, Wnt signaling either acts as a primary cause of many malignancies or contributes to their progression [42, 43]. Though adverse effects such as bone fractures have caused inconsistent clinical trial results, targeted suppression of Wnt signaling may have some potential [17]. As a result, we thought about alternatives to targeted inhibition. We developed a hypothesis that considered both our prior research and knowledge about the epigenetic regulation of Wnt pathway inhibitors such as sFRP1 and HBP1. Furthermore, an epigenetic approach to block Wnt signaling may have more profound and far-reaching effects (found in retrospect) given the importance of Wnt signaling in tumor-intrinsic immune resistance.

HBP1 was previously known to be a transcriptional repressor of Wnt signaling that interacts with and inhibits the LEF/TCF transcription factors activated by Wnt signaling. Furthermore, DNA methylation can reduce the genes encoding one or more of the Wnt pathway inhibitors, including sFRP1 and HBP1. Previous work showed that HBP1 mRNA is stabilized by EGCG, the primary catechin from green tea (Kim et al). We therefore reasoned that EGCG in combination with an epigenetic disruptor such as decitabine should simultaneously increase both HBP1 and sFRP1. Indeed, this combination, now termed CHA1, successfully increased both HBP1 and sFRP1 levels. My experiments on TNBC cells (MDA-MB-231) confirmed that the CHA1 combination blocked Wnt signaling by activating and stabilizing the HBP1 transcriptional repressor**.** We treated cultured human MDA-MB-231 cells with CHA1 or EGCG monotherapy or DAC monotherapy, using a range of concentrations (refer to **Table 2.3**). As shown in **Figure 3.1**, EGCG or DAC monotherapy treatment increased sFRP1 and HBP1 mRNA levels as hypothesized, while the combination was best at

increasing both inhibitors. Particularly, the sample combination 4 (10 µM EGCG + 1 µM DAC) and 7 (10 µM EGCG + 0.5 µM DAC) showed a discrete synergy in activating HBP1. Further, 1 µM DAC, 0.5 µM DAC, and 0.25 µM DAC show a dose-dependent increase in HBP1 expression. This is not the case with EGCG monotherapies suggesting that 10 µM EGCG (highest EGCG concentration) is cytotoxic and may result in some cell death. In the case of SFRP1, the sample 11 combination (5 µM EGCG + 0.25 µM DAC) and 8 (5 µM EGCG + 0.5 µM DAC) works synergistically relative to the monotherapies to induce SFRP1 expression in human TNBC cell lines **(Figure 3.1 B)**. monotherapies work in a dose-dependent fashion to induce SFRP1 expression **(Figure 3.1 B**).

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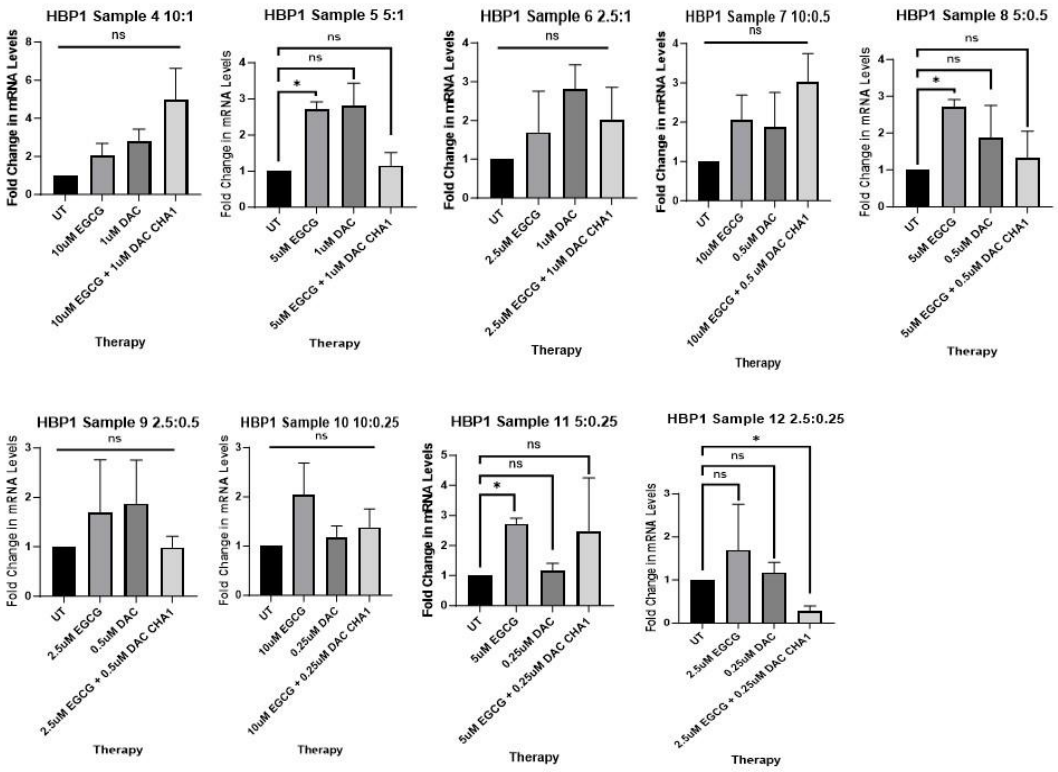

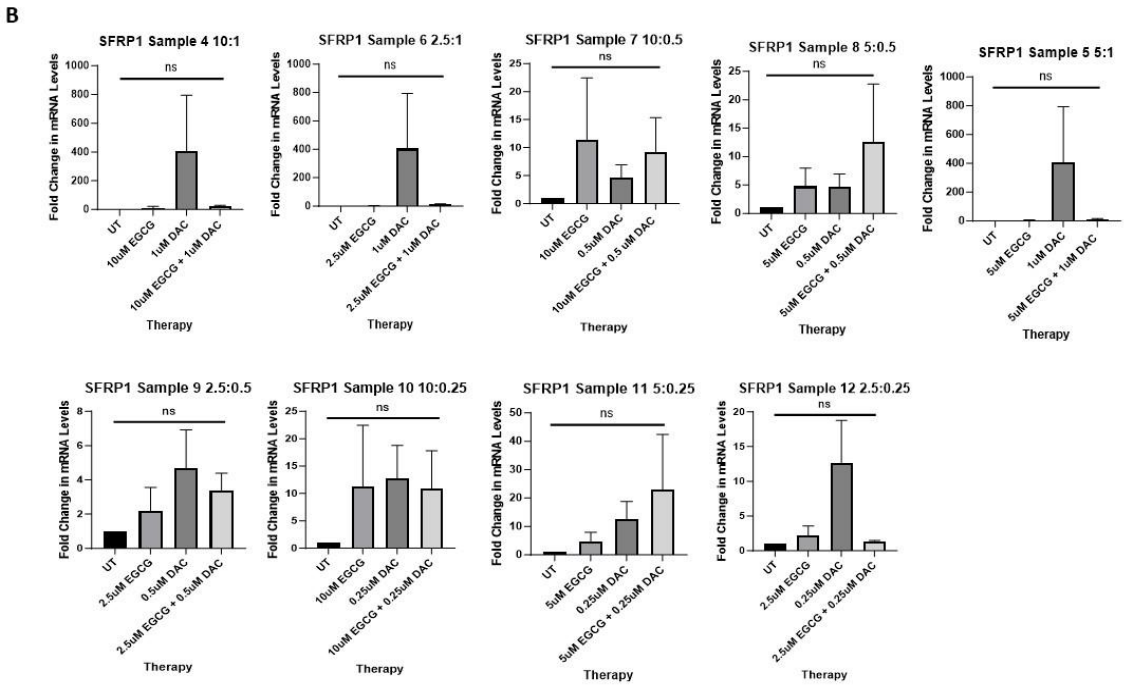

Figure 3.1: The empirical evidence for the combination of EGCG and DAC to suppress Wnt signaling. qRT-PCR results showing gene expressions of HBP1 and SFRP1 in CHA1 treated MDA-MB-231 cell lines. **A**. CHA1 treatment particularly with sample 7 combination (molar ratio 20:1) induces a 3-fold increase in HBP1 expression along with sample 4 combination (molar ratio 10:1) inducing a 5-fold increase in the HBP1 expression in human TNBC cell lines. **B**. CHA1 treatment particularly with sample 11 combination (molar ratio 20:1) induces a 3-fold increase in HBP1 expression along with sample 4 combination (molar ratio 10:1) induces a 20-fold increase in the SFRP1 expression in human TNBC cell lines. **A-B**. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

Given that neither EGCG nor DAC are target molecules and neither drug has demonstrated a significant amount of efficacy in solid tumors on its own [44], it would be advantageous for future human clinical trials if the combination CHA1 demonstrated efficacy in TNBC models, given its established safety. We started a thorough investigation of the CHA1 compounds in cell culture initially, based on a theory about the major regulatory sites for Wnt signaling. Crucially, our work also demonstrated the possibility of expanding and improving the therapeutic uses of immune checkpoint inhibitors, which may lead to better results for patients with breast and other

malignancies. In conclusion, the findings imply that CHA1 engages a broader molecular network that emphasizes a sophisticated biological reaction with practical ramifications.

3.2. Treatment with CHA1 reprograms a variety of cellular processes: Tumor-immune functions

In search of a more mechanistic understanding of the intrinsic effects of CHA1-inhibition of Wnt signaling on the cells, we used an already established unbiased human-specific RNA-seq and bioinformatics approach that was used to analyze human tumor xenografts in an immune-deficient mouse with and without CHA1 treatment. This turned out to be a fortuitous decision because the study of human genes revealed molecular signatures that were tumor-intrinsic due to the absence of a mouse immune system. Ingenuity Pathway Analysis (IPA), **Figure 3.2 A** shows the top 20 canonical pathways that are significantly impacted by CHA1 therapy, one of which was inhibition of Wnt signaling. The strong suppression of multiple Wnt targets found in the RNA-seq (**Fig 3.2 B)** correlated with and supported our previous investigation of Wnt inhibition.

In addition, the RNA-seq data showed an unexpected and extensive cellular reprogramming, with several immune surveillance-related pathways being activated. The antigen-presenting gene sets in these overlapping, tumor-intrinsic gene sets are specific for epithelial cells. A similar analysis of a syngeneic tumor model, i.e., a tumor and immune system from mice, would not have readily shown such features because of the infiltration of immune cells in the tumor microenvironment. MHC class I and class II antigen presentation genes made up the core set. Additionally, MHC-I and MHC-II are IFN-stimulated genes. Although CHA1 seems to have a particularly wide range of functions, other pathways, such as CDK4/6 inhibition and H3K4 methylation by an LSD1 inhibitor, have also allowed others to observe some of these mechanisms [45]. This extensive reprogramming has significant effects on tumor immunology, immune checkpoint inhibitor effectiveness, and vulnerability, as will be discussed later.

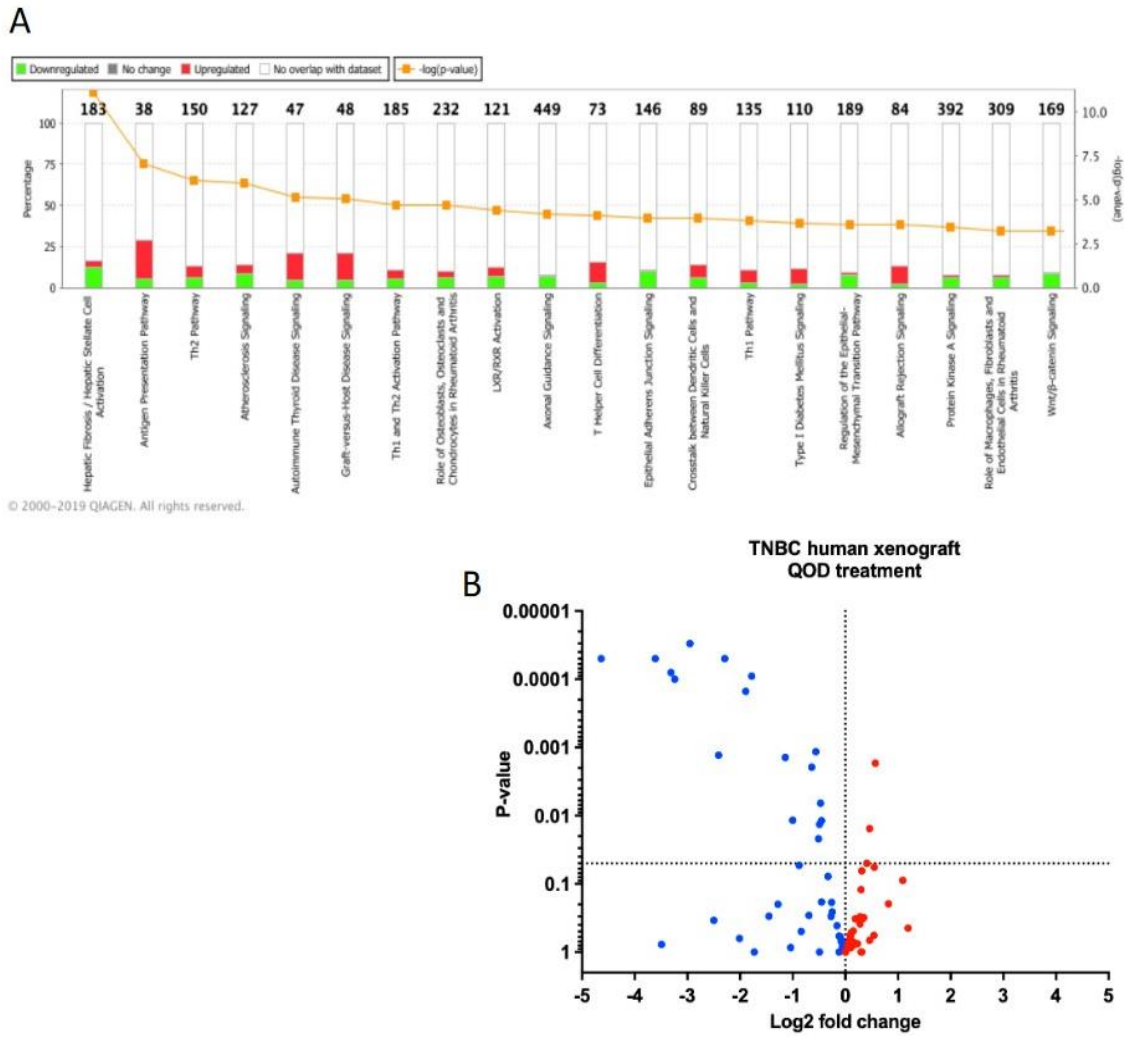

Figure 3.2: The TNBC human xenograft model's bioinformatic study of the CHA1 treated tumor revealed widespread immune-related pathway activation and Wnt pathway downregulation. Saline and CHA1-treated tumors were subjected to RNA-seq. A. The data was analyzed by IPA. The top 20 canonical pathways that underwent significant alteration following CHA1 therapy were shown. Numerous immune system-related signaling pathways were activated, including those linked to antigen presentation, autoimmune thyroid disease, graft-versus-host disease, Th1 and Th2 activation, and allograft rejection. The antigen presentation pathway was triggered, signifying an increase in the expression of genes related to the immune response against tumors. Moreover, Wnt signaling was suppressed. B. Volcano plot showed that CHA1 therapy suppressed the Wnt signaling pathway. Following the CHA1 RNA-seq study, the gene set was retrieved from IPA. 45 out of 83 (54.2%), following CHA1, Wnt target genes were downregulated. Following CHA1, 17 out of 45 (37.7%) Wnt target genes showed downregulation with $P < 0.05$. Reprinted here from [46] with permission from Dr. Amy Yee, unpublished observations.

3.3. CHA1 treatment enhances antigen-presenting properties

For appropriate antigen presentation, including tumor neo-antigens and tumor-associated antigens like cancer-testis antigens (CTAs), MHC-I and MHC-II components are necessary. The results of the bioinformatic analysis strongly implied that CHA1 causes tumors to present antigens. The MHC-I and MHC-II antigen presentation genes made up the core set, as seen in **Figure 3.2 A**. To confirm and differentiate the intrinsic responses of the tumor and to discern the role of CHA1 in the immune milieu, we employed an *In-vitro* TNBC cell line model.

First, CHA1 therapy enhanced MHC-I but not the MHC-II gene expression in the cell line treatment model, shown in **Figure 3.3 A**, unlike what was observed in both immunologically competent and immune-impaired TNBC animals. As shown in **Figure 3.3 A**, not all concentrations work the best in induction of MHC-I (HLAB) but particularly the sample 11 combination (5 µM EGCG + 0.25 µM DAC) works best over the 5 µM EGCG and 2.5 µM DAC monotherapy respectively. In the case of MHC-II (HLA-DRB5), none of the CHA1 combinations or EGCG monotherapy induces MHC-II expression, instead, DAC monotherapy (1 µM DAC gives 4-fold increase) is alone responsible for high MHC-II expressions in the cells as shown in **Figure 3.3 B**. The reason one can think of this discrepancy can be the lack of diversity and complexity of immune cells found *In vitro.* Immune cells play a crucial role in regulating MHC expression through various mechanisms such as cytokine signaling, cell-cell interactions, and antigen presentation. The absence of immune cells *in vitro* can lead to different patterns of MHC expression compared to *In vivo* models.

CHA1 treatment strongly induces expression of MHC-I in TNBC cell lines which is a necessary factor for later activation of CD8+ cytotoxic T-cells [47]. This suggests that both PSMB9 and PSMB10, subunits of immune immunoproteasome that play an important role in processing internal antigens and loading onto MHC-I complex, much

also be upregulated with the CHA1 treatment and can be the next targets to look at. Additionally, to observe and confirm the functions of cytotoxic CD8+ T-cells and natural killer cells, mRNAs for human perforin, normally associated with cytotoxic CD8+ T-cells and NK cells [48], were verified by qRT-PCR and the results were against the hypothesis that we would see an upregulation in the perforin levels. Instead, we were not able to detect perforins in the cell culture models and one of the reasons for this can be that the cell culture model has less involvement from the Immune environment. Even in the human-mouse xenograft which has no mouse B or T cells, there remain both mouse macrophages and NK cells, suggesting there is a residual mouse anti-tumor response, possibly through interferon signaling. According to these findings, CHA1 may activate a process that may be fundamentally responsible for controlling how a tumor interacts with the surrounding immune system and is activated by a wide range of signals.

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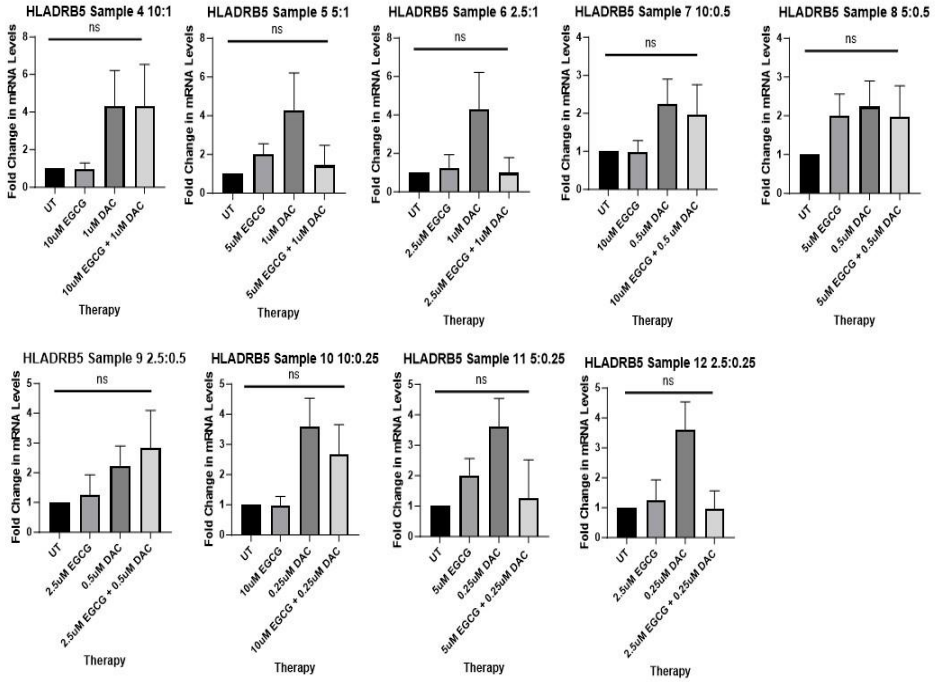


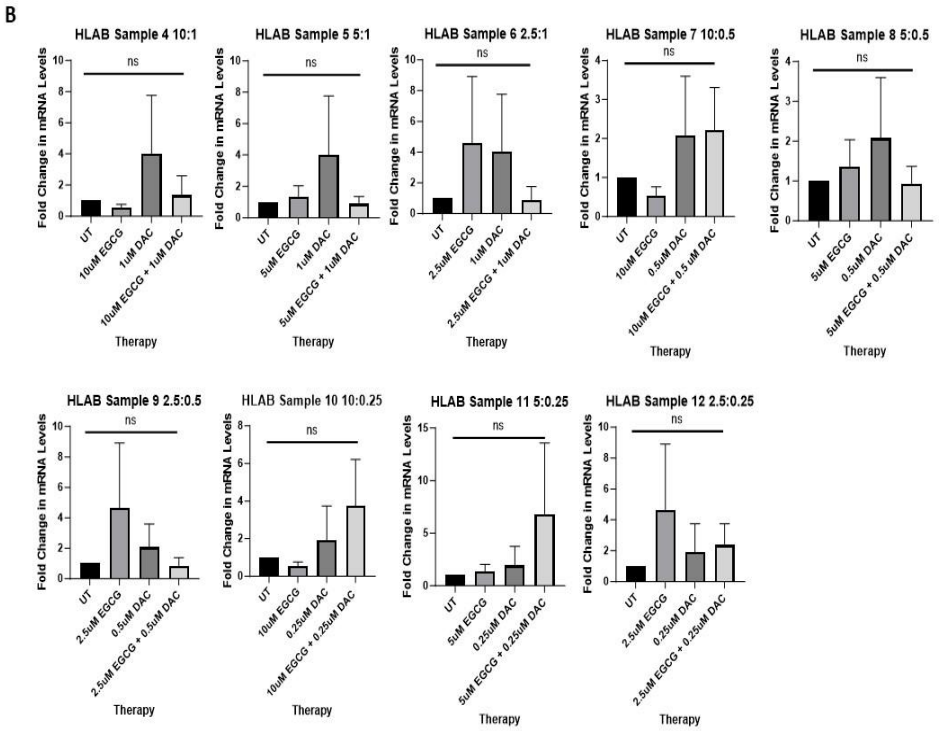


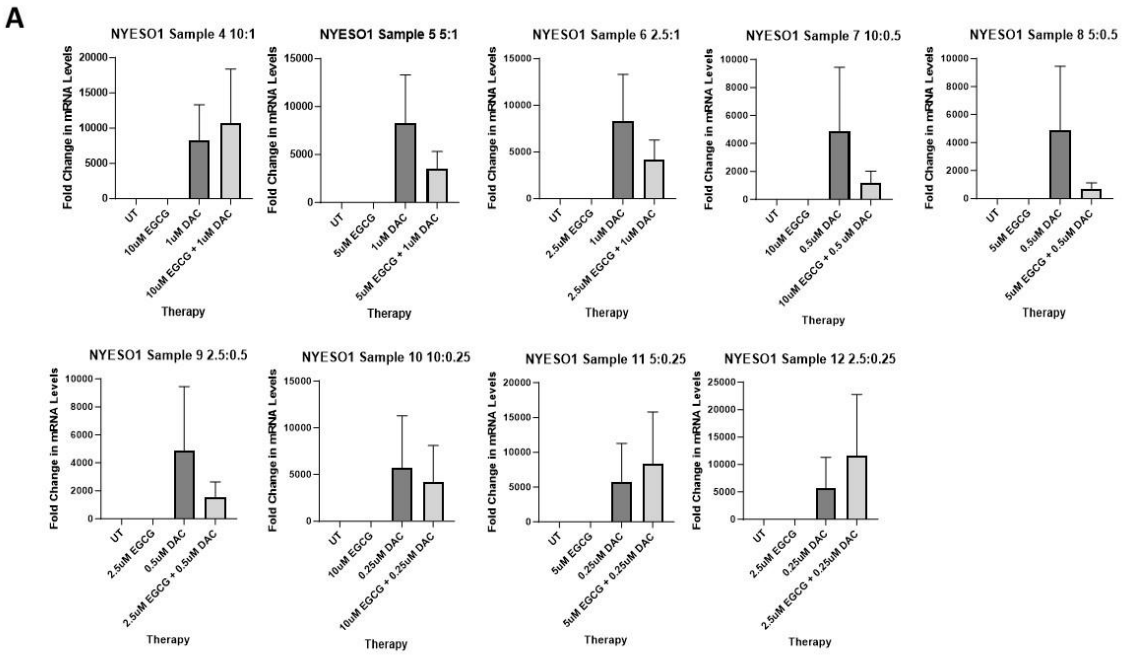
Figure 3.3: CHA1 treatment partially activates antigen presentations *In-vitro.* A. CHA1 treatment at no possible concentration induced MHC-II expression. B. CHA1 treatment particularly with sample 11 combination (molar ratio 20:1) induces a 7-fold increase in MHC-I (HLAB) expression along with sample 10 combination (molar ratio 40:1) induces a 4-fold increase in the MHC-I (HLAB) expression in human TNBC cell lines. A-B. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

3.3.1. An expanded cellular framework for CHA1 reprogramming

We observed the expression of CTAs in addition to the resuscitation of the antigen presentation machinery. Tumor-associated antigens like NY-ESO-1 and the MAGE-A family are a class of antigens that, because of their limited expression in normal adult tissue [49], may enhance immune identification of malignancies. CHA1 re-activates the expression of NY-ESO-1, MAGE-A3, and MAGE-A6 CTAs in human TNBC MDA-MB-231 cell lines as shown in Figure **3.3.1 A-C**. A more thorough comparison of CHA1 in human TNBC cell lines with n CTAs MAGE-A3, MAGE-A6, and NY-ESO-1 with each component (EGCG monotherapy or DAC monotherapy) reveals that DAC

treatment increases CTA expression, which is consistent with earlier reports [49], while EGCG monotherapy has little effect on CTA expression. Even in contrast to the significant DAC response, the CHA1 combination seems to provide a synergistic reaction.

DAC alone showed robust induction in NY-ESO-1 and MAGE-A3 gene expression following treatments on TNBC cell lines. Samples 11 and 12 (2.5 µM EGCG + 0.25 µM DAC) of CHA1 combinations showed a synergistic effect inducing NY-ESO-1 gene expression even in contrast to a significant DAC response as shown in **Figure 3.3.1 A.** Sample 11 of the CHA1 combination alone showed a synergistic effect in MAGE-A3 expression following treatment **(Figure 3.3.1 B).** In the case of MAGE-A6, sample 4 (10 µM EGCG + 1µM DAC) of CHA1 combination showed a robust increase in the expression of MAGE-A6 following treatments on the TNBC cell lines **(Figure 3.3.1 C).** DAC monotherapies had a dose-dependent effect on MAGE-A6 gene expression with 1 µM DAC having the highest induction of the three DAC concentrations.



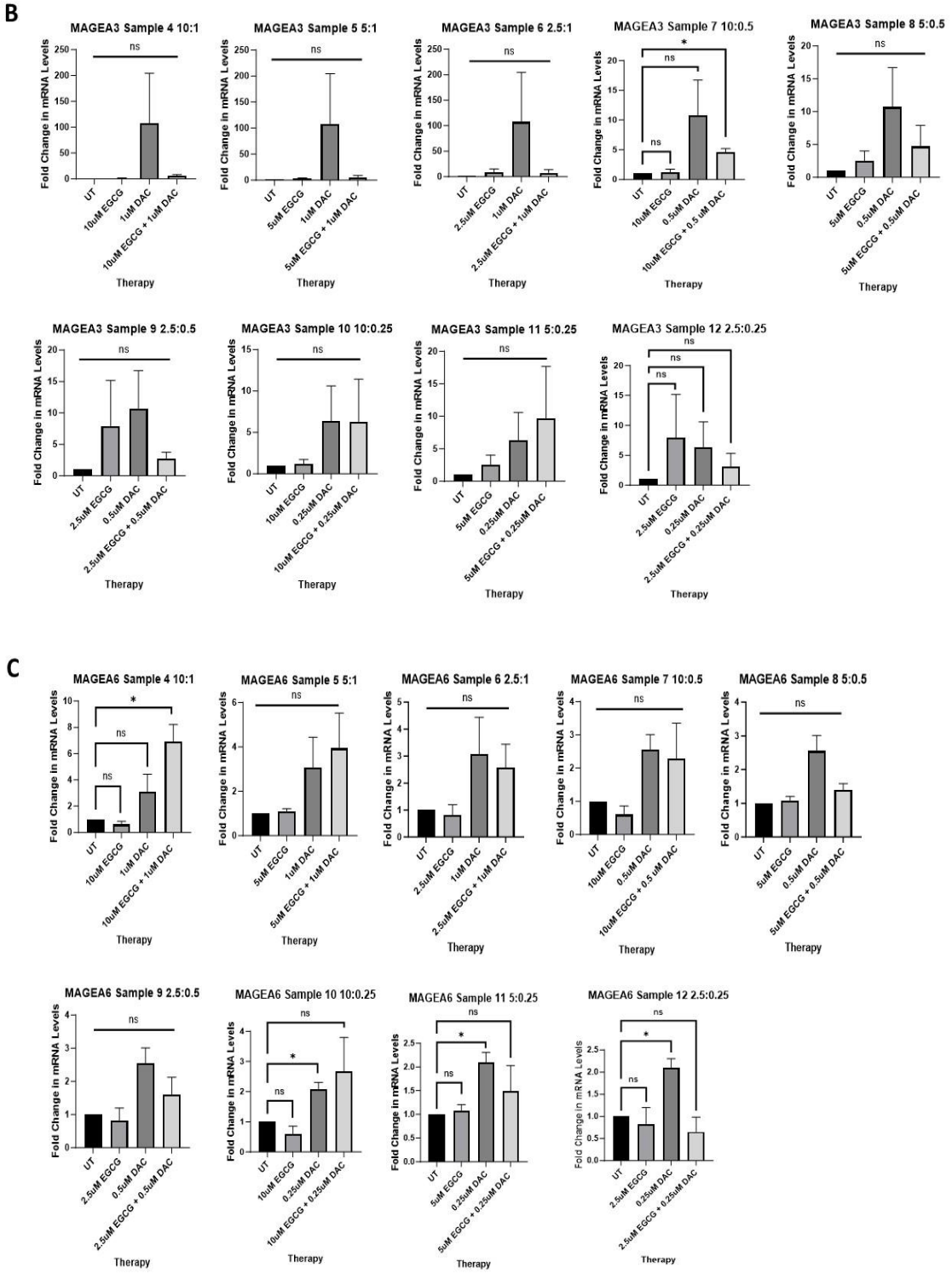


Figure 3.3.1: Upregulation of CTAs with CHA1 combination treatment. qRT-PCR results showing gene expressions of NY-ESO-1, MAGE-A3 and MAGE-A6 in CHA1 treated MDA-MB-231 cell lines. A. CHA1 treatment particularly with samples 4

combination (molar ratio 10:1) and sample 11 combination (molar ratio 20:1) induces 10^5-fold and 8000-fold increase in expression of NY-ESO-1 in human TNBC cell lines. No statistical significance has been made since the UT group had no signal. B. CHA1 treatment particularly with sample 11 combination (molar ratio 20:1) induces a 10-fold increase in MAGE-A3 expression along with 1 µM DAC and 0.5 µM DAC alone induces 100-fold and 10-fold increase in the MAGE-A3 expression in human TNBC cell lines. C. Sample 4 CHA1 combination gives a 6-fold increase in MAGE-A6 expression in human TNBC cell lines. In most of the cases, DAC monotherapy worked the best in inducing MAGE-A6 expression. A-C. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

3.4. The IFN signaling pathway is triggered by CHA1 in Tumors

Next, we investigated how CHA1 might cause a partial and significant antigen presentation response. There is some evidence that Wnt and IFN signaling interact [50]. Notably, the induction of MHC and antigen presentation genes is linked to interferon-γ [51]. The production of several ISGs and the JAK-mediated tyrosine phosphorylation of a STAT transcription factor are the hallmark processes for both IFN α/β signaling and IFN γ signaling.

Using western blot analysis, we first evaluated if CHA1 stimulation of IFN signaling was achievable in CHA1-treated TNBC tumors. The necessity of both CHA1 components for maximal $STAT3^{Y705}$ phosphorylation was demonstrated in tumors treated with DAC, EGCG, or CHA1 (**Figure 3.4 A**). We also looked at $STAT3^{Y705}$ phosphorylation in the cells treated with CHA1 and found a different response compared to the tumors. The cells showed a phenotype where inhibition of $STAT3^{Y705}$ phosphorylation was dominant (**Figure 3.4 A)** and this concluded that the $STAT3^{Y705}$ phosphorylation is not directed by CHA1 treatment but is a result of the treatment dependence on other tumor extrinsic factors absent in cell models.

Secondly, we established the expression of specific genes that overlap IFN α/β signaling and IFN γ pathways. We specifically looked at expression levels of IFIT2 and OAS1 which are the overlapping genes between the two pathways. The induction of IFN

α/β and IFN γ IFN-stimulated genes requires CHA1 therapy. DAC monotherapy treatment enhanced the expression of IFN-stimulated genes; in contrast, the gene expression stimulated by some CHA1 combination treatments was greater (**Figure 3.4 B-C).** In the case of IFIT2 (IFN α/β specific target gene), sample 4, sample 6 (2.5 µM EGCG + 1µM DAC), and sample 7 of CHA1 combination show an induction following treatments on TNBC cell lines. With OAS1, sample 4, sample 11, and sample 12 (2.5 µM EGCG + 0.25 µM DAC) shows robust induction in OAS1.

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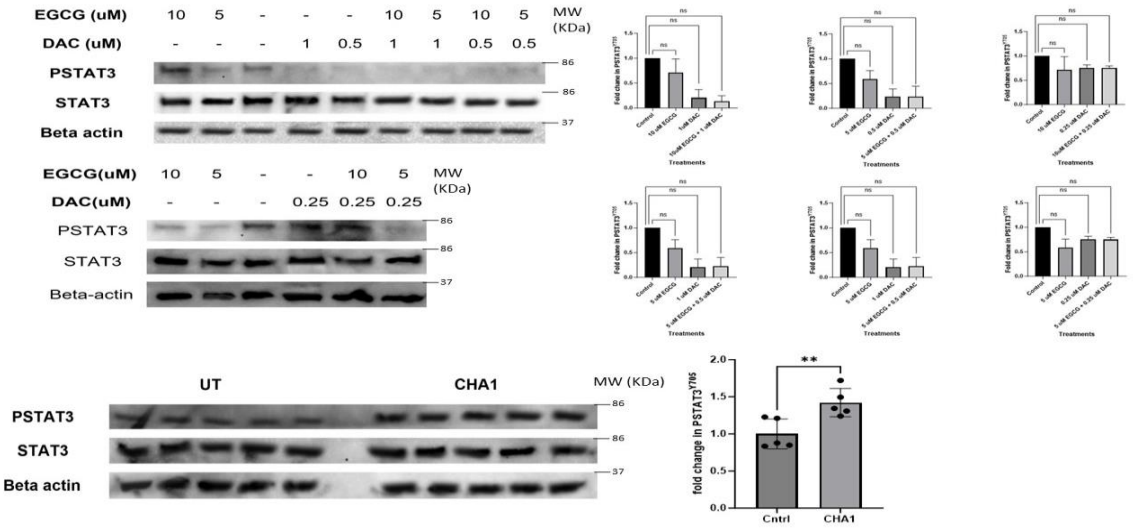

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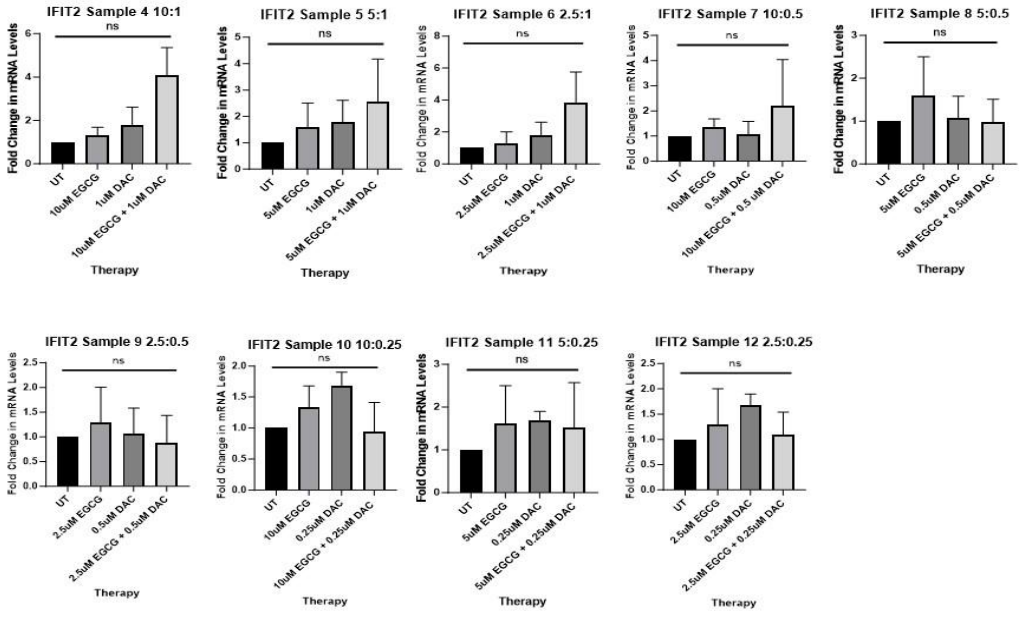

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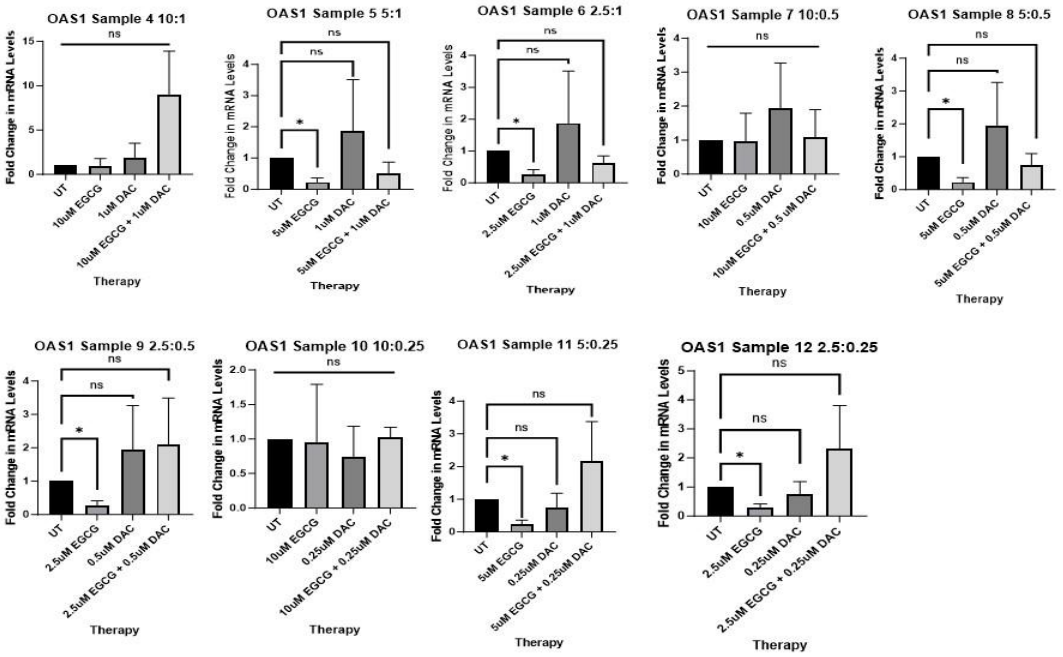


Figure 3.4: CHA1 treatment stimulated IFNα/β and IFNγ pathways in TNBC syngeneic mouse model but not in TNBC cell lines. Significantly, CHA1 decreased STAT3^{Y705} protein expression. A. Representative western blot (Top-left) and quantification (Top-right) of cells treated with different concentrations of CHA1 combinations and their respective monotherapies. CHA1 treatment, to some extent, induced STAT3^{Y705} protein expression in Tumor mice syngeneic model. Representative western blot (bottom-left) and quantification (bottom-right) of syngeneic tumors treated with CHA1 and control are shown. qRT-PCR results showing gene expressions of IFIT2 and OAS1 in CHA1 treated MDA-MB-231 cell lines. B. CHA1 treatment particularly with sample 4 (molar ratio 10:1) and sample 6 (molar ratio 2.5:1) combinations induces a 4-fold increase in IFIT2 expression along with sample 7 combination (molar ratio 20:1) inducing a 2-fold increase in the IFIT2 expression in human TNBC cell lines. C. In the case of OAS1, sample 4 (molar ratio 10:1) gives a 10-fold increase in expression along with samples 11 (molar ratio 20:1) and 12 (molar ratio 10:1) gives a 2-fold increase in OAS1 expressions in human TNBC cell lines. A-C. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Western blot quantification was the combination of two different experiments in cells (n=4 for EGCG monotherapies and UTs/experiment, n=2 for rest of combinations and DAC monotherapies/experiment), and a single experiment for syngeneic tumors (n=5 for both control and CHA1 treated/experiment). Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

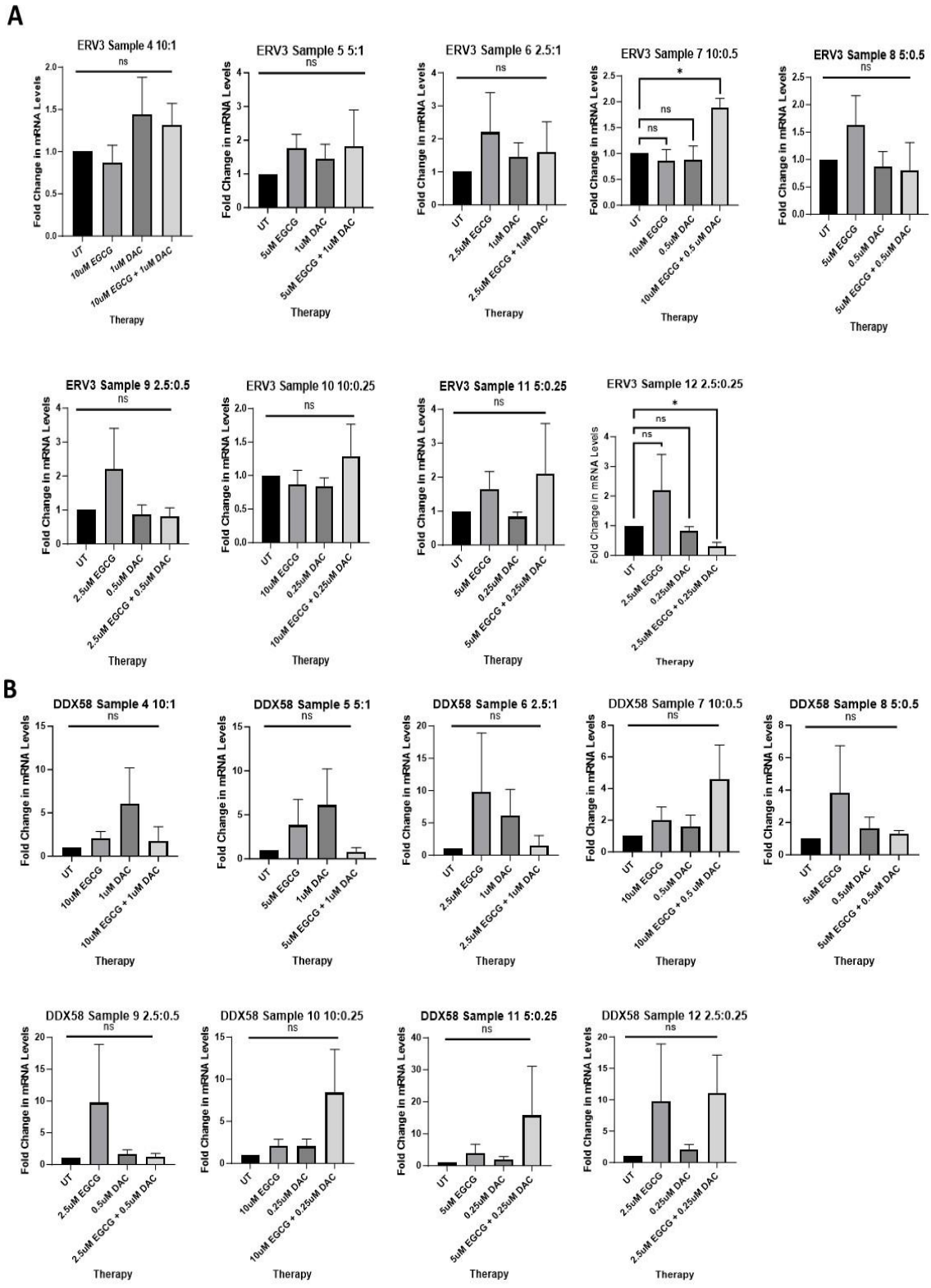
3.4.1. CHA1 activated “viral mimicry” status in tumors

Next, we wanted to know how IFN was produced. A major clue came from the *In-vitro* tumor model, which is grown in an environment where Immune cells cannot be a source of IFN of any type. One of the possible mechanisms was “viral mimicry.” Prior

research has demonstrated that DNMT inhibition might cause endogenous retroviruses (ERVs) to re-express, increasing the amount of dsRNA and triggering receptors that recognize dsRNA patterns, including RIG-1 (DDX58), LGP2 (DHX58), and MDA5 (IFIH1) [52]. We therefore investigated relative expression levels of ERV3-1, DDX58, DHX58, and IFIH1 gene expression. In the human TNBC cell lines, CHA1 generated endogenous retrovirus (ERV3-1) mRNA and triggered the gene production of dsRNA pattern recognition receptors **(Figure 3.4.1 A-D).** With samples 7 and 11 CHA1 combinations we observed a 2-fold increase in ERV3-1 expressions in the TNBC cell lines (**Figure 3.4.1 A).** A dose-dependent increment was seen with DAC monotherapies and a reverse was observed with EGCG monotherapies in ERV3-1 expression levels. The same sample combinations that worked with DDX58 showed a 5-fold and 15-fold increase in DDX58 expressions respectively in TNBC cell lines (**Figure 3.4.1 B)**. With DHX58, sample 10 and sample 11 worked the best in enhancing DHX58 levels in the cells (**Figure 3.4.1 C)**. There was a 5-fold and 12-fold increase, respectively, in the levels of DHX58 compared to the control groups. Again, a dose-dependent increment in levels of DHX58 was observed with DAC monotherapies. In all the above three genes, 2.5 µM EGCG showed an unusual induction in the viral mimicry status. In the case of IFIH1, DAC monotherapies in most of the groups worked the best over the combination treatment. A 2-fold increase in IFIH1 expressions with sample 4 (10 µM EGCG + 1µM DAC) and a 3-fold increase in sample 9 (2.5 µM EGCG + 0.5 µM DAC) CHA1 combination were observed (**Figure 3.4.1 D).** 1µM DAC and 0.5 µM DAC each showed a 1.5-fold and 1.25-fold increase in expression of IFIH1.

Overall, these results suggest that the path from DNA methylation inhibition to viral mimicry, interferon induction, and acquisition of antigen presentation in an epithelial cell

may be a component of basic cellular functions that can be used to respond to various signals through epigenetic rearrangements, as will be discussed in more detail later.



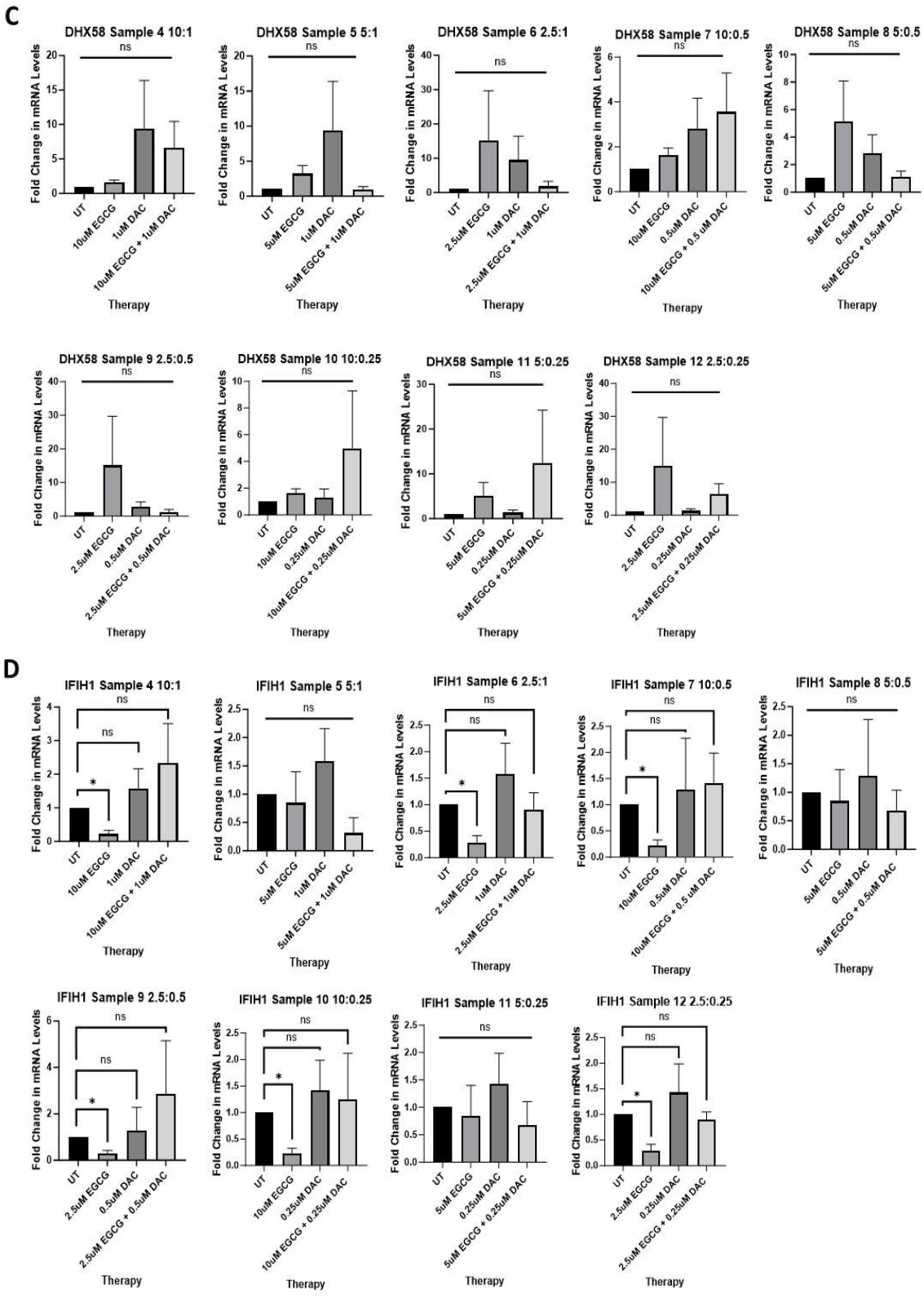


Figure 3.4.1: "Viral mimicry" status induced with CHA1 treatment. qRT-PCR results showing gene expressions of ERV3-1, DDX58, DHX58 and IFIH1 in CHA1 treated MDA-MB-231 cell lines. A. CHA1 treatment with sample 7 (molar ratio 20:1) and sample 11

(molar ratio 20:1) combinations induce a 2-fold increase in ERV3-1 expression in human TNBC cell lines. B. In the case of DDX58, the same sample CHA1 combination worked with a 5-fold and 15-fold increase in DDX58 expression in human TNBC cell lines. C. sample 10 (molar ratio 40:1) and sample 11 (molar ratio 20:1) worked the best in enhancing DHX58 levels in the cells with 5-fold and 12-fold increases respectively. D. DAC monotherapy worked the best over combination in most of the cases. Sample 4 (molar ratio 10:1) and sample 9 (5:1) gave a 2-fold and 3-fold increase in the expression of IFIH1 in the cells following the treatments. A-D. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

3.5. "Viral mimicry": an intersection of Epigenetic therapies with cancer immunotherapies.

The complex, modified cancer genome exhibits aberrant dysregulation with more widespread hypomethylation and specific hypermethylated regions as discussed previously. The major marker of facultative heterochromatin is H3K27me3. Enhancer of zeste homolog 2 (EZH2), a catalytic component of Polycomb repressive complex 2 (PRC2) that forms the three histone methyl groups that make H3K27me3, has been linked to the growth of tumors in several malignancies. Furthermore, it has been demonstrated that H3K27me3 partially compensates for hypomethylated areas of the cancer epigenome. Although the tumor benefits from this mechanism, which stops the viral mimicry pathway from being induced, it also creates a vulnerability that an EZH2 inhibitor (EZH2i) can exploit to cause the expression of dsRNA [34]. We predict that an enhanced response to ICIs will follow the induction of the viral mimicry pathway through dsRNA expression and detection, which will boost tumor-infiltrating lymphocytes (TILs).

With this, we investigated the methylation status of H3K27me3 in the treated TNBC cells and syngeneic tumors. We see a significant drop in the methylation status of H3K27 with the specific CHA1 combination in the TNBC cell lines (**Figure 3.5 A**). Additionally, we see EGCG monotherapy (10 µM EGCG and 5 µM EGCG) having a significant effect on the methylation status of H3K27 suggesting that it acts as an EZH2i. In syngeneic tumors, we see a drop in H3K27 methylation status in CHA1-treated

groups compared to untreated groups (**Figure 3.5 B).** The most potent effect is shown by 5 µM EGCG and 0.25 µM DAC followed by 10 µM EGCG and 1 µM DAC combination (1µM DAC has a dominant effect reflected in the combination treatment). This explains that the effect of CHA1 on tumor reprogramming and alteration in tumor-immune cell interaction may in part be due to epigenetics of the solid tumor**.**

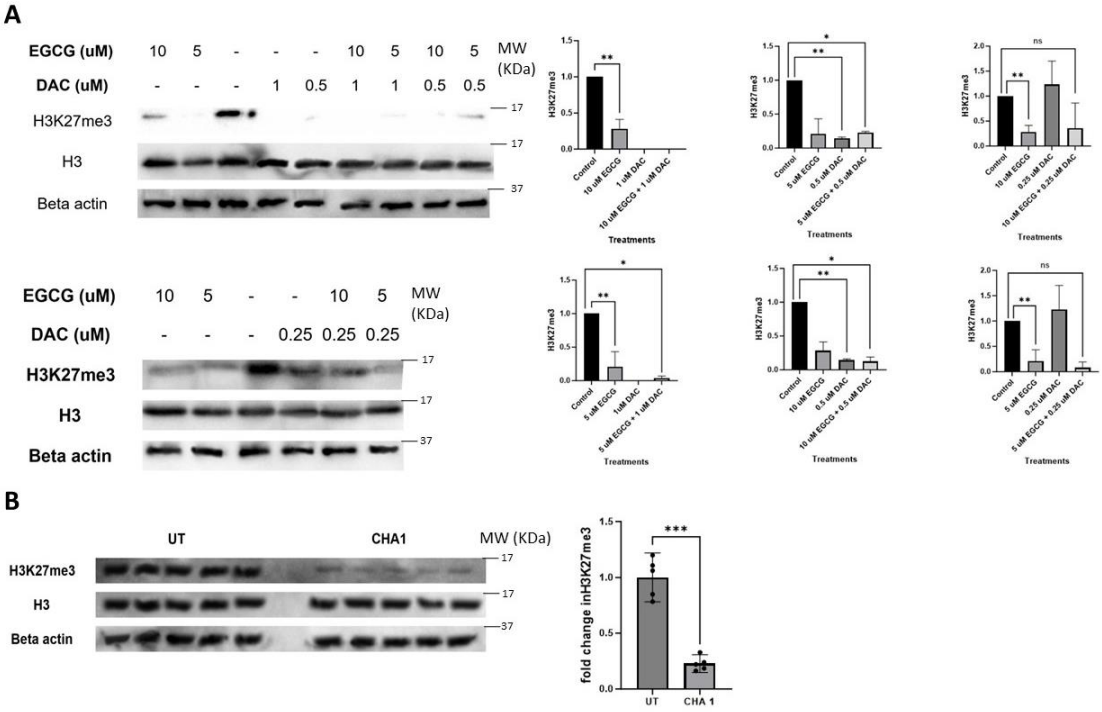


Figure 3.5: Disruption of Methylation status of H3K27me3 by CHA1 treatment. Significantly, CHA1 decreased H3K27me3 levels across TNBC cells and syngeneic mice tumors. A. Representative western blot (Top-left) and quantification (Top-right) of cells treated with different concentrations of CHA1 combinations and their respective monotherapies. B. CHA1 treatment, prominently, decreased H3K27me3 levels in Tumor mice syngeneic model. Representative western blot (bottom-left) and quantification (bottom-right) of syngeneic tumors treated with CHA1 and control are shown. A-B. Western blot quantification was the combination of two different experiments in cells (n=4 for EGCG monotherapies and UTs/experiment, n=2 for rest of combinations and DAC monotherapies/experiment), and a single experiment for syngeneic tumors (n=5 for both control and CHA1 treated/experiment). Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001**.**

3.5.1. Expanding on CHA1 Epigenetic disruption effect.

Next, we looked at methylation status at H3K9 methylation status, which is commonly associated with gene silencing or transcriptional repression**.** Like H3K27me3, when histone H3 is trimethylated at lysine 9, it promotes a compacted chromatin

structure that inhibits access of transcriptional machinery to the DNA, thereby reducing gene expression. This mechanism is often utilized by cells to silence repetitive DNA elements, such as transposable elements and endogenous retroviruses, which can otherwise be detrimental if activated.

The contributions of histone methylation to constitutive heterochromatin formation and retrotransposon silencing are best illustrated during early mammalian development. The mammalian genome undergoes two waves of widespread DNA demethylation during early embryogenesis and germline development. During these periods, the histone methyltransferase (HMT) SETDB1 is critical to establish H3K9me3 to silence transposable elements such as long interspersed nuclear elements (LINE) and ERVs [53]. Beyond early development, SETDB1 broadly silences ERVs in subsets of normal somatic cells [53] and AML to prevent IFN activation. H3K9me3 is regulated through two enzymes, SUV39H1 and SETDB1 [54]. Restoring H3K9me3 is necessary to stop inappropriate transcriptional activation and DNA replication stress since repressive H3K9me3 modifications are halved during DNA replication. As an epigenetic reader, the F-box protein FBXO44 binds residual H3K9me3 at the replication fork to draw a repressive complex comprising SUV39H1 to develop chromatin. When the FBXO44/SUV39H1 system is disrupted in cancer cells, it increases the production of repetitive elements, triggers an IFN response downstream that limits the growth of tumors, and overcomes ICI resistance [55].

Since trimethylation of histone 3 lysine 9 (H3K9me3) and lysine 27 (H3K27me3) are the best-known histone modifications associated with gene repression and heterochromatin we next looked at the methylation status of H3K9. We used the same tumor TNBC cell lines and the syngeneic mice model for this study. We observed that some CHA1 combinations had a significant effect on the methylation status of H3K9 in addition to H3K27 (**Figure 3.5.1).** Interestingly, EGCG alone had no effect on H3K9

methylation status unlike seen in H3K27 methylation which suggests that EGCG has a selective effect on H3K27 methylation. Amongst the CHA1 combination treatment, 5 µM EGCG + 1 µM DAC worked the best in complete demethylation of H3K9 (**Figure 3.5.1 A).** In the case of syngeneic mice tumors, a significant reduction in H3K9me3 was observed compared to the untreated groups (**Figure 3.5.1 B).** This suggests that the CHA1 combination has an epigenetic disruption effect on tumors which might be the reason behind the induction of genes related to tumor immune microenvironment.

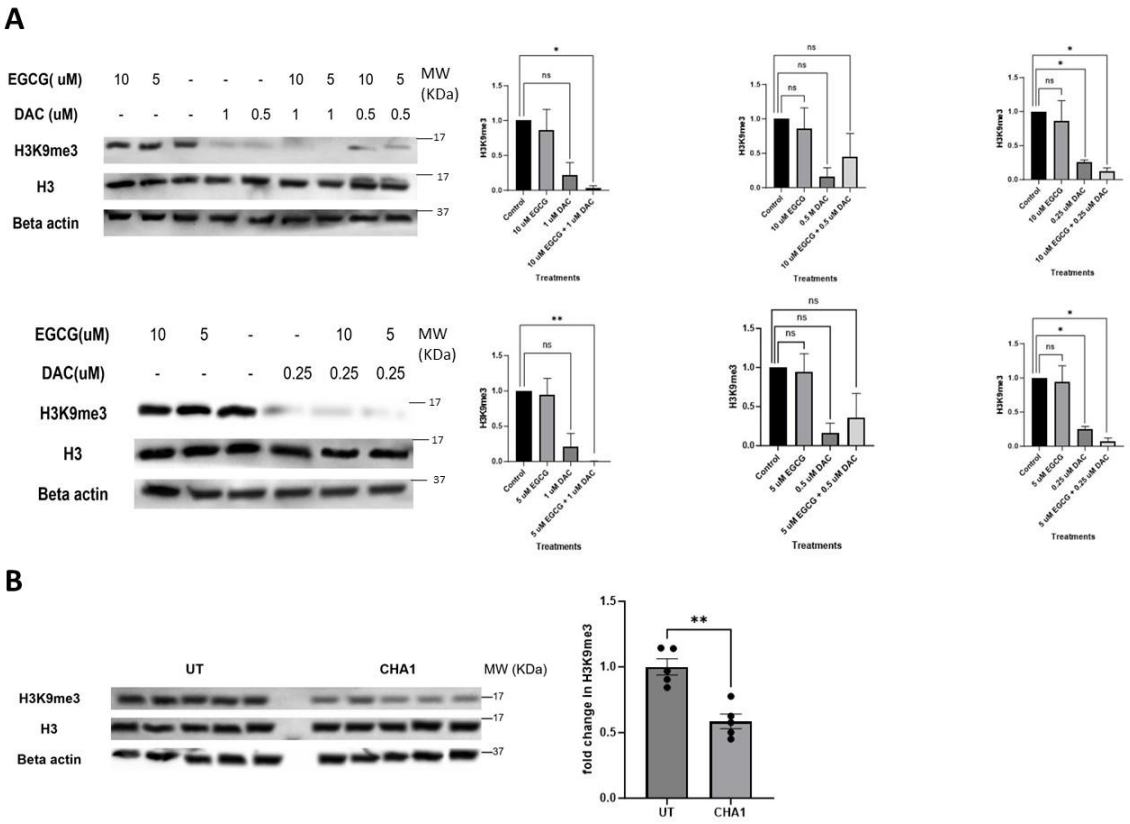


Figure 3.5.1: Disruption of Methylation status of H3K9me3 by CHA1 treatment. Significantly, CHA1 decreased H3K9me3 levels across TNBC cells and syngeneic mice tumors. A. Representative western blot (Top-left) and quantification (Top-right) of cells treated with different concentrations of CHA1 combinations and their respective monotherapies. B. CHA1 treatment, prominently, decreased H3K9me3 levels in Tumor mice syngeneic model. Representative western blot (bottom-left) and quantification (bottom-right) of syngeneic tumors treated with CHA1 and control are shown. A-B. Western blot quantification was the combination of two different experiments in cells (n=4 for EGCG monotherapies and UTs/experiment, n=2 for the rest of combinations and DAC monotherapies/experiment), and a single experiment for syngeneic tumors (n=5 for both control and CHA1 treated/experiment). Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

3.6. Extending the mechanism of CHA1 function to the tumor-immune environment

Tumors use a variety of tactics to avoid the endogenous immune surveillance systems, which makes immunotherapy treatments even more difficult. Immune checkpoint effectors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 ligand (PD-L1) can be expressed by them [56]. Activated T cells express PD-1, which binds to PD-L1, which is typically present in antigen-presenting cells (APCs). The reversible “exhausted” T cell state is brought on by this binding. T cells produce CTLA-4, which binds to pAPCs' B7 receptors to stop T cell activation. Tumors can use regulatory T cells (Tregs), which are essential to tumor immunological tolerance, by enlisting them to create a tolerogenic immune milieu that suppresses inflammation and immunity [56, 57].

The subsequent work was guided and focused by the connection made between the divergent outcomes of an unbiased investigation and the regulatory mechanisms that specify tumor-immune interactions, or the "cold to hot" transition. We hypothesized that CHA1 could be a helpful instrument to increase vulnerability to immune checkpoint inhibitor-induced breakdown of the immune system and efficacy. While these studies produced many fascinating results, one important finding was that CHA1 treatment in the cells increased the expression of the important clinical biomarker PD-L1 with some specific CHA1 combinations, which frequently indicate how well ICIs will work in treating a patient. Sample 4 CHA1 combination gives a close to 5-fold increase in the PD-L1 expression as observed with qRT-PCR on the TNBC cell lines. In addition, high concentration DAC i.e., 1µM DAC, itself gives a close to 4-fold increase in PD-L1 expression, and on the other hand, the highest concentration of EGCG i.e., 10 µM EGCG, does not have any effect in the induction of PD-L1 but when combined with DAC it shows synergy and a significant increase in the levels are observed (**Figure 3.6).** In

sample 11 of CHA1 combinations, 0.25 µM DAC and 2.5 µM EGCG have no significant effects on levels of PD-L1 but when combined they show a synergistic effect and induce a 2-fold increase in the PD-L1 expressions (**Figure 3.6).** Moreover, one feature of hot tumors is an increase in PD-L1, which is frequently utilized as a therapeutic biomarker to identify individuals who may benefit from ICI treatment. Furthermore, we suggested that PD-L1 status might shed light on how CHA1 might affect tumor-immune cell infiltration and activity.

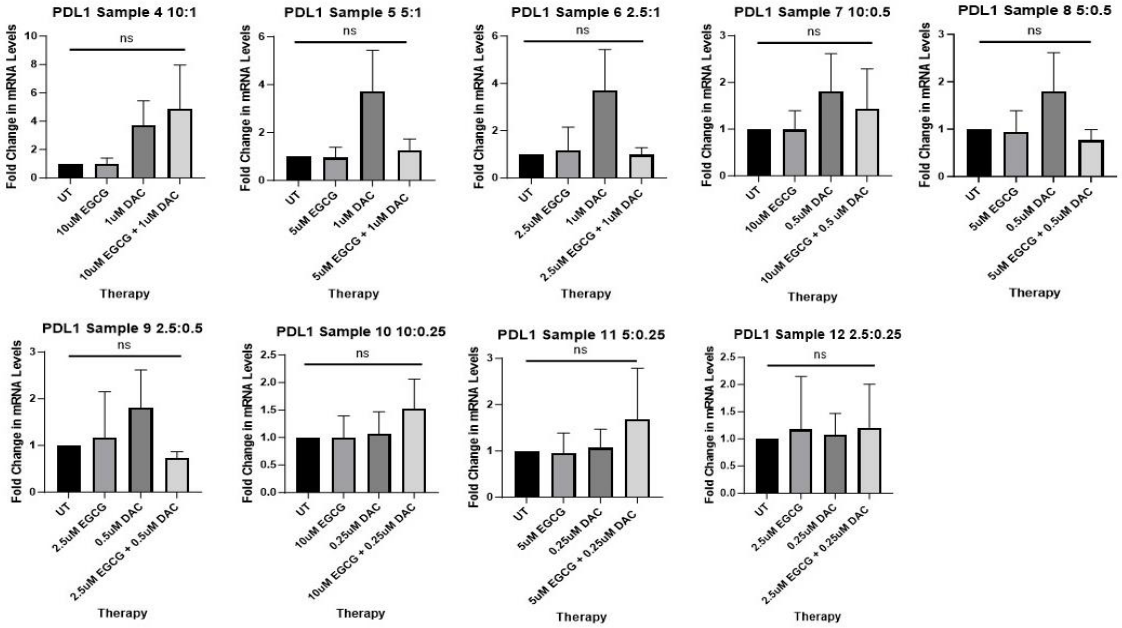


Figure 3.6: Slight PD-L1 induction with CHA1 treatments. qRT-PCR results showing gene expressions of PD-L1. DAC shows a dose-dependent effect on PD-L1 expression in TNBC cell lines with 1 µM DAC showing a 4-fold increase. Sample 11 (molar ratio 20:1) of CHA1 combinations gives close to a 2-fold increase higher than the respective monotherapies. EGCG has no significant effect on PD-L1 expression at any concentrations. An unpaired two-tailed t-test was used for the comparison. qRT-PCR experiments were done in triplicates in three independent experiments. Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

3.7. Effect of CHA1 on tumor metabolism.

As discussed above, Hyperactive Wnt signaling is a prevalent feature of many TNBC tumors. The growth of breast cancer cells and the recurrence of the disease have been related to Wnt signaling activation. Reduced T-cell infiltration into tumor locations is

a consequence of high Wnt signaling, and this is essential for the effectiveness of ICIs [16, 58, 59]. Furthermore, appropriate anti-tumor immune response, ICI effectiveness, and stimulation of tumor antigen presentation depend on active IFN signaling [59]. JAK/STAT/IFN system has been shown to modulate PD-L1 [60], and mutations in the interferon receptor pathway have been connected to resistance to anti-CTLA-4 or anti-PD-1 in patients with metastatic melanoma.

In addition to hyperactive Wnt signaling, it has been reported that tumors usually show upregulation in proteins responsible for uptake of the glucose and preferential production of lactate by the cells. An increase in glucose uptake by the cells correlates with tumor glucose requirements for growth. With HBP1 knocked out, which is a Wnt signaling suppressor, there is increased proliferation with the formation of lactate from excess glucose intake, termed the “Warburg effect”. It has been suggested that the Warburg Effect serves as an adaptive mechanism to meet the needs of uncontrolled proliferation in terms of biosynthesis [61]. In this case, the increased consumption of glucose serves as a supply of carbon for anabolic reactions that promote cell division. This excess carbon is used for the de novo generation of nucleotides, lipids, and proteins and can be diverted into multiple branching pathways that emanate from glycolysis. Another proposed mechanism to account for the biosynthetic function of the Warburg Effect is the regeneration of NAD+ from NADH in the pyruvate to the lactate step that completes aerobic glycolysis. In this scenario, NADH that is produced by glyceraldehyde phosphate dehydrogenase (GAPDH) must be consumed to regenerate NAD+ to keep glycolysis active.

Further, tumor microenvironments have limited availability of glucose and undergo competition for nutrients with stromal cells and the immune compartment [62]. Additional support is found in a recent study that showed when changes to the cellular environment were induced to greatly increase ATP demand by altering the demand of

ATP-dependent membrane pumps, aerobic glycolysis increased rapidly and oxidative phosphorylation remained constant [63]. This finding provides additional rationale for the function of the Warburg Effect to support the rapid production of ATP that can be rapidly tuned to support the demand for ATP synthesis. Elevated glucose metabolism will decrease the pH in the microenvironment due to lactate secretion. An acid-mediated invasion hypothesis suggests that H+ ions secreted from cancer cells diffuse into the surrounding environment and alter the tumor stroma interface allowing for enhanced invasiveness [64].

Since CHA1 has the effect of inhibiting Wnt signaling by inducing expressions of Wnt suppressor, HBP1, and SFRP1, it can alter the tumor metabolism and eventually prevent its growth and metastasis. Previously in Yee's lab, it has been observed that CHA1 has the potential to decrease the levels of glycolytic enzymes which are usually up during tumor metastasis, suggesting that CHA1 treatment has potential in preventing tumor growth and metastasis. Next, we wanted to know the role of HBP1 in tumor metabolism and whether CHA1 has an independent effect on the altered metabolism of tumors. For this, we used HBP1 KO 4T1 mice TNBC cell lines. HBP1 KO clones were prepared by one of the PhD candidates who worked in Yee's lab. We investigated the levels of 3 main enzymes that are involved in the glycolysis cycle. The first is pyruvate kinase isoform M2 (PKM2), an alternatively spliced variant of the gene resulting in an enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis and plays a role in regulating cell metabolism. Second, Lactate dehydrogenase A which in humans is encoded by the LDHA gene and catalyzes the conversion of pyruvate to lactate step in the glycolysis cycle. Lastly, the Hexokinase 2 (HK2) enzyme phosphorylates glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. HK2 is uniquely localized to the mitochondrial ATP translocase, yielding maximal enzyme activity due to elevated ATP concentrations [65].

We see a significant spike in the levels of these proteins in HBP1 KO clones compared to the control groups (**Figure 3.7).** This aligns we our hypothesis that CHA1 inhibits Wnt signaling via inducing HBP1 and that it requires HBP1 to prevent tumor growth and metastasis.

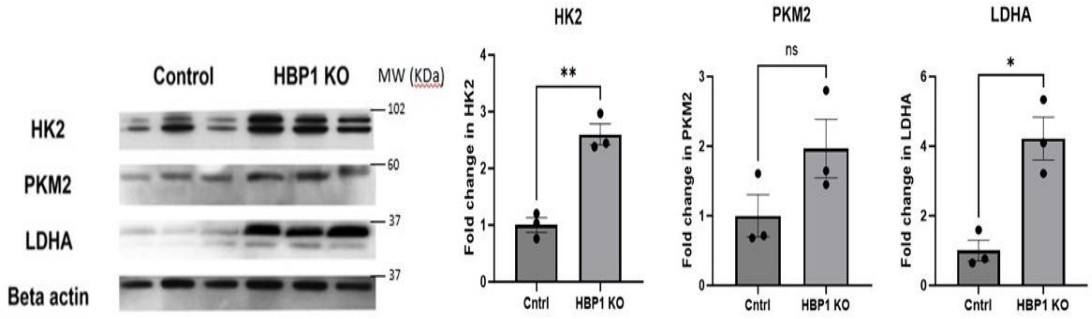


Figure 3.7: Induction of glycolytic enzymes with CHA1 treatment in HBP1 KO clone. Significantly, CHA1 up-regulated glycolytic enzyme expression in HBP1 KO clone. Representative western blot (left) and quantification (right) of cells both control and HBP1 KO treated with CHA1 combination. CHA1 treatment, to some extent, induced a 2.5-fold, 2-fold, and 4-fold increase in HK2, PKM2, and LDHA enzymes respectively. Western blot quantification was the combination of a single experiment in cells (n=3 for CHA1 treated and control groups). Data were represented as mean ± SEM. ^{ns} P>0.05, * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001.

Chapter 4: Discussion

The initial design of CHA1 as a therapeutic was to inhibit Wnt signaling as a means of inhibiting tumor growth, which was successful. Careful analysis of dosing combinations of the two components, EGCG and DAC, we found that not all CHA1 treatment combinations worked in inhibiting Wnt signaling in the TNBC cell lines. In addition, in vivo analyses showed that CHA1 did far more in altering the tumor microenvironment and improving immunotherapy for TNBC. Our objective was to carefully weigh our options and reduce the patient's known negative effects. When used alone, the individual components of CHA1 (EGCG and DAC) have well-established safety profiles but have almost no effect on reducing tumor growth. However, when EGCG and DAC were used in combination (20:1 molar ratio, CHA1), the effects were significantly stronger. Together with the earlier findings of Yee lab [41] and my work, the summary shown in **Figure 4**, indicates that CHA1 treatment has broad effects that influence the fundamental biology of TNBC tumor cells and have equally broad biological and clinical ramifications.

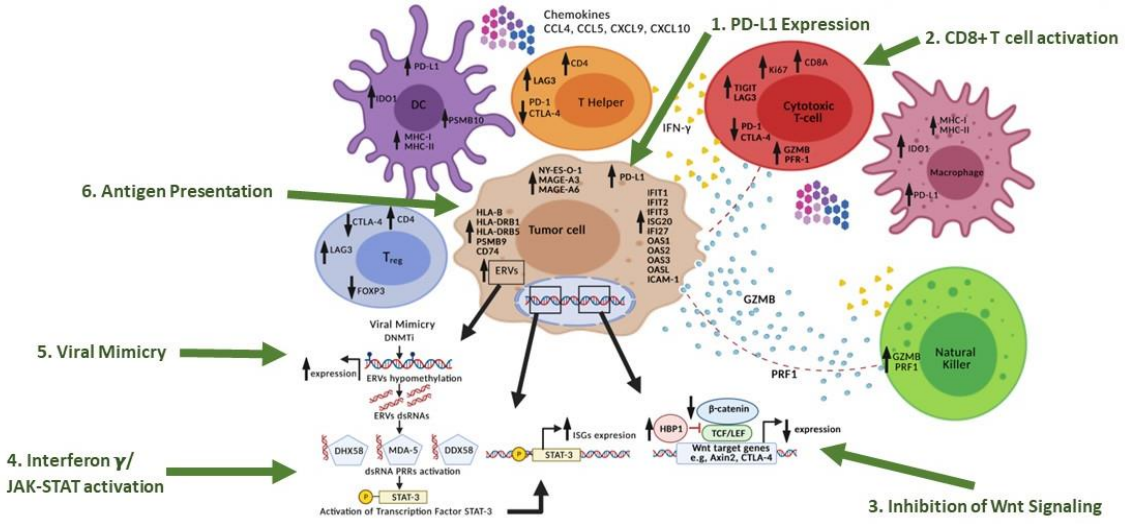


Figure 4: CHA1 modifies the tumors and its interaction between tumor cells and immune cells. By modifying important signaling pathways and cellular processes, CHA1 can transform cold tumors into hot tumors. It can also reprogram a tumor cell to exhibit antigen presentation, upregulate PD-L1, increase the infiltration of effector immune cells, decrease that of immune suppressor cells, and induce an inflammatory signature in T cells. Therefore, CHA1 may be the perfect working partner to improve the immune

checkpoint inhibitor (ICI) response. CHA1-induced Wnt inhibitors, such as sFRP1 and HBP1, decrease the Wnt/α-catenin pathway, a tumor intrinsic factor that is critical in determining the response to ICIs. Furthermore, CHA1 elevated interferon-stimulated genes (ISGs) and activated the IFNα/β and IFNγ pathways, which were associated with successful treatment with inhibitory compounds. Viral mimicry activation was one potential mechanism. Also, CHA1-induced CTAs on tumor cells can result in increased immune recognition of tumor cells and therefore, activation of T-cells to secrete IFNγ. Important downstream signaling molecules were stimulated because of the production of IFNγ by activated cytotoxic T-cells and other cells, including T-helper cells and natural killer (NK) cells. MHC-I, MHC-II, and other elements of the immunoproteasome and antigen-presenting apparatus can all have their expression levels increased by IFNγ. Antigen presentation activation is crucial for T-cell activation, which eliminates tumor cells. Active CD8+ T cells and NK cells can secrete PRF1 and GZMB which play important roles in apoptosis. Furthermore, IFNγ induces a variety of checkpoints, including PD-L1, on the surface of tumor cells, DCs, and macrophages. In addition to PD-L1 overexpression, T cell activation, and IFN-signaling can also homeostatically upregulate TIGIT, LAG3, and IDO1 as checkpoint molecules to limit the antitumor immune response. Overall, CHA1 works via a variety of effector molecules to create a T-cell-inflamed tumor microenvironment (TME), which is essential for the efficacy of ICIs. Fig 4 was made using BioRender.com.

Although CHA1 is not a single target therapeutic, the collection of its various mechanisms (**Figure 4**) offers novel biological and clinical implications for accessing an underutilized biological network, inducing tumor cell reprogramming, and opening new applications as an ICI enhancer agent. The CHA1 combination modulates two key signaling pathways that determine the transition from an immune-suppressive (cold) tumor to an immune-active (hot) tumor: Wnt signaling and interferon signaling. These signaling pathways fall under the broad category of cold-to-hot transition. Epithelial cancer undergoes a fundamental biological reprogramming into a tumor with a loss of antigen presentation function, loss of cancer testes antigen expression, and a reversal of epithelial properties into a mesenchymal phenotype. Significant evidence suggests that the process is initiated through epigenetic deregulation. Further, restoration of antigen presentation, etc in a cold-to-hot transition can, at least partially be achieved through a viral mimicry mechanism – which in turn activates IFN signaling. This activation of IFN signaling is crucial for the re-expression of antigen presentation genes and critical immune checkpoint proteins like PD-L1.

Hyperactive Wnt signaling is a common feature of many TNBC tumors as well as other “cold” tumors. The growth of TNBC and the disease's recurrence are correlated with elevated Wnt signaling [29]. Wnt signaling causes an increase in β-catenin levels, resulting in downstream gene activation, which may be a factor in the poor prognosis of TNBC patients [66]. Moreover, elevated Wnt signaling reduces T-cell infiltration into tumor locations, which is essential for the effectiveness of ICIs [16].

The data from my analysis of the TNBC cell line revealed that the specific CHA1 combination induces Wnt inhibitors HBP1 and sFRP1. Numerous investigations revealed that EGCG inhibited Wnt signaling in breast, lung, and colon cancer stem cells [67, 68]. Furthermore, prior research demonstrated that in AML cell lines and a mouse model, DAC monotherapy was unable to downregulate Wnt target genes [69]. My results confirm some of these prior observations and show that the combination therapy when at a specific ratio, is greater than either compound alone (**Figure 3.1).**

The malfunction of the JAK/STAT/IFN pathway is the second reason that ICI therapy fails to cure melanoma [16, 58]. Both the innate and adaptive immune responses are enhanced by IFN signaling activation [51]. Furthermore, appropriate anti-tumor immune response, ICI effectiveness, and stimulation of tumor antigen presentation depend on active IFN signaling [16]. According to reports, the JAK/STAT/IFN system regulates PD-L1, and mutations in the interferon receptor pathway have been connected to resistance to anti-CTLA-4 or anti-PD-1 in individuals with metastatic melanoma [60]. Our data shows that CHA1 increases the phosphorylation status of STAT3 in syngeneic models but not in human TNBC cell lines, suggesting that the $STAT3^{Y705}$ phosphorylation is not activated by CHA1 treatment but instead may be dependent on tumor extrinsic factors absent in cell models. Despite the lack of $pSTAT3^{Y705}$ induction, we examined downstream targets. CHA1 therapy significantly raised IFN-stimulated gene (ISG) expression (**Figure 3.4).** We investigated

potential sources of IFN stimulation, and one important one is epigenome modification-induced viral mimicry. Numerous findings from research on decitabine indicated that the hypomethylation of ERVs may contribute to some of its effectiveness [35]. Therefore, we examine whether CHA1 induces human endogenous retroviruses (ERVs) and other associated “viral mimicry” genes. Indeed, human ERVs and associated “viral mimicry” genes are induced by CHA1 (**Figure 3.4.1).**

CHA1's ability to access biological reprogramming suggests an underappreciated alternate cellular phenotype that arises when the equilibrium of cellular epigenetics is upset through pharmacological or genetic means. The results of our work here and several other published studies support the discovery of epithelial plasticity that may also change the immunological milieu within tumors to prevent immunosuppression. When taken as a whole, our research in this area offers crucial biological context of the characteristics of a "hot" tumor and the significance of controlling or altering the cancer epigenome. Numerous signals, such as DNMT1 inhibitors, CDK4/6 inhibitors, CDK9 inhibitors, LSD1 inhibitors, or LINE regulation [52, 70, 71], were responsible for similar phenotypes to our CHA1 experiments here. Further, these reports all noted that although the immunological phenotypes the drugs elicited were initially unexpected, they also activated similar mechanisms, which we have expounded upon for CHA1. Together, we make the case that CHA1 interacts with a complex and fundamental network based on epigenetics that leads to tumor-derived antigen-presenting cells. This highlights the plasticity of epithelium and has implications for immune interactions and function within tumor-resident tissues.

We observed that the disruption of the cancer epigenome results in the tumor cell acquiring novel and unanticipated characteristics related to the presentation of antigens. All epigenetic modifications in the cancer genome, including DNA methylation, chromatin remodeling, histone methylations, and histone acetylation, are called the "cancer

epigenome." Furthermore, an epigenetic alteration that was brought about by a variety of unconnected causes re-activates tumor-localized antigen presentation and processing. Different compounds result in broad disruption of the hypermethylated phenotype of tumor cells, leading to the re-expression of silenced heterochromatic regions such as endogenous retrovirus (ERVs) and the viral mimicry response to produce interferons that then increase MHC genes and other antigen presentation and processing genes.

Chiappinelli, Baylin, and associates' groundbreaking research was the first to investigate how the DNMT inhibitor DAC inhibited DNA methylation in cells, re-activating highly methylated endogenous retroviruses, and thus causing interferon signaling and the stimulation of genes linked to antigen presentation and processing [35, 72]. APC reprogramming was also observed in breast cells in a different study that used a CDK4/6 inhibitor intended to function at G1 in the cell cycle, most likely resulting from a decrease in DNMT1 gene expression, which in turn causes a global decrease in DNA methylation, the repression of ERVs, dsRNAs, and IFN signaling activation [52]. Other results suggest that the same outcome can be attained by interacting with additional epigenetic modulators. According to one study, CDK9, a transcription factor regulator and cell cycle kinase inhibitor, can induce reprogramming to express APC genes and mechanisms that improve tumor-resident T-cell infiltration. There was also evidence of a viral mimicking mechanism, although a chromatin remodeling protein was responsible for the epigenetic disruption [73]. In seminal investigations, Shi and coworkers discovered LSD1 (KDM1) as the first histone demethylase [45]. Since H3K4me3 is a marker of active gene transcription, LSD1 inhibition is expected to disrupt silent and/or heterochromatic regions—where ERVs may be found—by promoting H3K4 methylation (by blocking demethylation) or regions of active transcription. One subtlety of these exquisite experiments is that LSD1 is attached to the promoter of ERVs rather than any IFN gene promoters, suggesting that H3K4 demethylation plays a role in the transcriptional

silencing mechanism of ERVs [74]. Subsequently, LSD inhibitors would disrupt this balance, encouraging transcriptional activation and H3K4 methylation. Lastly, re-expression of LINEs and other repetition components has been the subject of some studies. A particular H3K9 methylase, which is known to be essential for the creation and maintenance of heterochromatin, was implicated by Classon and associates [75]. Lastly, PRC2 and EZH2 are implicated in a comparable process by another research [76]. H3K27me3 is frequently linked to DNA methylation and silenced genes. These investigations, along with our own using CHA1, span a wide range of epigenetic alterations and biological causes. The key finding is that cellular destiny and reprogramming with a phenotype for the acquisition of APC characteristics can be specified by control of the cancer epigenome. The unexpected flexibility and innate capacity of the epithelial-like tumor cells to reacquire antigen-presentation and other characteristics subsequently determine their interactions with the immune cells that reside in the tumor.

Confirming the epigenetic basis of CHA1 function, CHA1 treatment stimulated the expression of CTAs, most notably NYESO1 (**Figure 3.3.1).** Prior research has demonstrated that DAC therapy causes CTAs to be upregulated in hematologic tumor cells as well as in solid tumors [49]. Furthermore, it has been documented that a patient treated with dendritic cell (DC) vaccination based on CTA and DAC had complete remission from a solid tumor relapse [77]. Following immunization, the patient's immune system recognized tumor cells and there was an increase in MAGE-A3-specific T cells. CTA expression is limited to solid tumors and immune-privileged locations including the testis, placenta, and throughout fetal development. Examples of these sites include the MAGE, SSX gene families, PRAME, NY-ESO-1, and SP17. By repressing immune-stimulatory components epigenetically, malignant cells avoid immune identification. DNA methylation controls the expression of CTAs, MHC-I, and MHC-II [78]. In cancer, the

transcription of these genes is often silenced by hypermethylation. Demethylating agent such as DAC can reactivate these methylation-silenced genes [49, 78]. Our data show that CHA1 induces MHC-I and to some extent MHC-II in human TNBC cell lines. The upregulation of MHC-I and MHC-II reveals that CHA1 manipulated the tumor cells to become APCs. Collaboration of both components, EGCG and DAC, are important for some gene's full re-expression.

Based on the literature and our results with CHA1, which show essentially similar results with other epigenetic disruptors, it is conceivable that the appearance of antigen-presentation and processing genes and protein in an epithelial tumor is a useful and functional indicator of an epigenetic reorganization that is being influenced by a variety of signals. Furthermore, CHA1 and the combined results reinforce the requirements needed to achieve a "hot" tumor. Like this, the outcomes of our methodical mechanistic dissection provide a framework for the discovery of treatments that can cause a tumor to turn from cold to hot and confer ICI susceptibility.

The responsiveness to immunotherapy is determined in part by several significant parameters. Some elements that originate from tumor cells or are present in the micro- or macroenvironment around the tumor, such as the enrichment of prognostic immune gene signatures, the diversity of the immune infiltrate, the expression of immunological checkpoints, and the load of neoantigens. For immune checkpoint inhibitors to be effective, a tumor must transform from a cold, non-inflammatory state to a hot, inflammatory state. An efficient reaction to immune checkpoint inhibitors has been linked to inflammatory tumors, which are identified by T-cell infiltration, IFNα in the tumor microenvironment, and PD-L1 expression [79]. One approach to achieving a durable clinical response in patients with a cold tumor is the use of collaborative agents to create an immunogenic tumor microenvironment [80]. As a surrogate biomarker for immune checkpoint inhibitor efficacy in clinical trials, PD-L1 may be a factor in treatment success

due to intrinsic PD-L1 expression [81]. Although anti-PD-1 and anti-PD-L1 have many uses, Roche (anti-PD-L1 Atezolizumab) voluntarily withdrew its indication for triple-negative breast cancer (TNBC) in August 2021 as continued patient monitoring showed little improvement in overall survival. Although ICIs continue to be a viable choice for patients with TNBC, the inconsistent clinical outcomes suggest that some enhancements are required to optimize effectiveness and reduce adverse effects in existing treatment plans.

Epigenetic inhibitors have been identified as potential options for enhancing cancer immunotherapy due to their ability to modulate global hypomethylation and influence PD-L1 status. To express antigen presentation machinery and produce a functioning compartment with infiltration and active (not fatigued) CD8+ T-cells, a recent screen concentrated on epigenetic inhibitors that express antigen presentation features in TNBC organoids [82]. My and previous Yee's lab research suggests processes and frameworks that could offer valuable insights into enhancing ICIs for TNBC. Strong induction of PD-L1 and potentially other markers is a minimum prerequisite for a collaborating agent, but substances that rewire and activate the tumor-resident milieu may be useful in creating an environment in which ICIs can operate at lower concentrations. The incidence of tumor reprogramming towards MHC expression and antigen presentation through a viral mimicry to the IFN pathway as a result of epigenetic disruption is a startling discovery from our work and the literature. Our studies here set a solid foundation for thoughtful compound development to improve the current ICIs and look toward newer immune checkpoint drugs.

Chapter 5: Bibliography

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