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**The Role of the Nucleosome Remodeling Factor NURF in Inhibiting T and Natural
Killer Cell Mediated Antitumor Immunity by Suppressing Tumor Antigenicity and
Natural Cytotoxicity Receptor Co-ligands**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

5mC: methylation of the fifth carbon of cytosine

ACT: adoptive cell transfer

ADCC: antibody-dependent cellular cytotoxicity

APC: antigen presenting cell

AR: androgen receptor

β_2m : β_2 -microglobulin

BPTF: bromodomain containing phd finger transcription factor

BTLA: B- and T-lymphocyte associated protein

CAR: chimeric antigen receptor

CCND1: cyclin D1

cDNA: complementary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

CM: complete media

CPA: cyclophosphamide

CpG: cytosine-phosphate-guanine

CRS: cytokine release syndrome

CTCF: CCCTC-binding factor

CTL: cytotoxic T lymphocyte

CTA: cancer testis antigen

CTLA-4: cytotoxic T-lymphocyte associated antigen 4

DAC: 5-aza-2'-deoxycytidine

DAMP: damage-associated molecular pattern

DC: dendritic cell

DN: double negative

DNMT: DNA methyltransferase

DNMTi: DNA methyltransferase inhibitor

DMEM: Dulbecco's Modified Eagle Medium

DP: double positive

ECM: extracellular matrix

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

ELISPOT: Enzyme-Linked ImmunoSpot

EMT: epithelial-to-mesenchymal transition

ER: estrogen receptor

FADD: Fas-associated via death domain

FAIRE: Formaldehyde Assisted Isolation of Regulatory Elements

FasI: Fas ligand

FBS: fetal bovine serum

FDA: food and drug administration

GM-CSF: granulocyte macrophage colony-stimulating factor

GO: gene ontology

H3K9me1: histone H3 lysine 9 monomethylation

H3K4me3: histone H3 lysine 4 trimethylation

H4K16: histone H4 lysine 16

H3K27me3: histone 3 lysine 27 trimethylation

HBSS: hank's balanced salt solution

HDAC: histone deacetylase

HDACi: histone deacetylase inhibitor

HER2: human epidermal growth factor 2 receptor

HLA: human leukocyte antigen

HMGA: high mobility group A

HPSE: heparanase

HS: heparan sulfate

HSPG: heparan sulfate proteoglycan

IFN γ : interferon gamma

IL: interleukin

iNKT: invariant NKT

I.P.: intraperitoneally

ITAM: intracellular immunoreceptor tyrosine-based activatory motif

ITIM: intracellular immunoreceptor tyrosine-based inhibitory motif

KD: knockdown

kDa: kilodalton

KO: knockout

mAb: monoclonal antibody

MAGE: melanoma antigen

MDSC: myeloid derived suppressor cell

MFI: mean fluorescence intensity

MHC: major histocompatibility

MMTV: mouse mammary tumor virus

mTEC: medullary thymic epithelial cells

NCR: natural cytotoxicity receptor

NEAA: nonessential amino acids

NHEJ: non-homologous end joining

NK: natural killer

NOD scid: NOD.CB17-Prkdcscid/J

NSG: NOD/SCID, *Ilfrg2r* -/-

NURF: nucleosome remodeling factor

NY-ESO-1: New York oesophageal squamous cell carcinoma 1

OT1: C57BL/6-Tg(*TcraTcrb*)1100Mjb/J

PBS: phosphate-buffered saline

PD1: programmed death 1

PHD: plant homeodomain

pmel: B6.Cg-*Thy1^a*/Cy Tg(*TcraTcrb*)8Rest/J

PR: progesterone receptor

qRT-PCR: quantitative real-time polymerase chain reaction

RAG: recombination activating gene

RSS: recombination signal

RT: room temperature

S-phase: synthesis phase

S.C.: subcutaneously

shRNA: short hairpin RNA

SP: single positive

TAA: tumor associated antigen

TAP: transporter associated with antigen-processing

TCM: central memory T cells

TCR: T cell receptor

TCR-T: transgenic T

TEM: effector memory T cells

TGF- β : transforming growth factor beta

TLR: toll-like receptor

TME: tumor microenvironment

TNBC: triple negative breast cancer

TNF- α : tumor necrosis factor alpha

Treg: regulatory T cell

TSA: tumor specific antigen

UV: ultraviolet

V(D)J: variable diversity joining

VEGF: vascular endothelial growth factor

WD: tryptophan-aspartic acid

Abstract

THE ROLE OF THE NUCLEOSOME REMODELING FACTOR NURF IN INHIBITING T AND NATURAL KILLER CELL MEDIATED ANTITUMOR IMMUNITY BY SUPPRESSING TUMOR ANTIGENICITY AND NATURAL CYTOTOXICITY RECEPTOR CO-LIGANDS

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A dissertation submitted in partial fulfillment of the requirements for the degree Doctor of
Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2017

Advisor: Dr. Joseph Landry, Assistant Professor in Human and Molecular Genetics

Tumor immunoediting is a dynamic process in which the immune response attacks tumor cells by detecting danger signals and tumor antigens. In order to survive, tumor cells develop mechanisms to avoid detection or destruction by the immune system. To counteract this, several strategies are being developed to enhance the antitumor immune response, including the depletion of immunosuppressive cells, enhancing the activation of antitumor immune cells and increasing tumor cell immunogenicity. These therapies have seen limited success individually, however, and it is likely that combination therapy with novel targets will be necessary to see reproducible beneficial responses. Epigenetic modifications are attractive therapeutic targets because they are reversible and affect gene expression in cancer cells. Within

this framework, this study aimed to elucidate the role of the chromatin remodeling complex nucleosome remodeling factor (NURF) in cancer immunoediting by silencing of bromodomain PHD-finger containing transcription factor (BPTF), the largest and essential subunit of NURF. Using two syngeneic mouse models of cancer, BPTF was found to suppress T cell antitumor activity in the tumor microenvironment. *In vitro*, enhanced cytolytic activity was observed for individual CD8 T cell clones only from mice bearing BPTF-silenced tumors, implicating the involvement of novel antigens. Mechanistic investigations revealed that NURF directly suppresses the expression of genes encoding immunoproteasome subunits *Psmb8* and *Psmb9* and the antigen transporter genes *Tap1* and *Tap2*. PSMB8 inhibition reversed the effects of BPTF ablation, consistent with a critical role for the immunoproteasome in improving tumor immunogenicity. Thus, NURF normally suppresses tumor cell antigenicity and its depletion improves CD8 T cell antitumor immunity. In a concurrent study using different tumor lines, BPTF was also found to suppress natural killer (NK) cell antitumor immunity *in vivo*. Enhanced NK cell cytolytic activity toward BPTF-depleted targets *in vitro* was dependent on the natural cytotoxicity receptors (NCR). Molecular studies revealed that BPTF directly activates heparanase (*Hpse*) expression, resulting in reduced cell surface abundance of the NCR co-ligands: heparan sulfate proteoglycans. Thus, NURF represses NCR co-ligand abundance and its depletion enhances NK cell cytotoxicity. Therefore, NURF emerges as a candidate therapeutic target to enhance CD8 T or NK cell antitumor immunity.

Chapter 1

Introduction

Section 1.1: Cancer

Cancer occurs when normal cells acquire several tumor-promoting characteristics. These characteristics are broadly organized into ten categories that have been widely accepted as hallmarks of cancer. The hallmarks are: resistance to apoptosis, inactivation of growth suppressors, induction of growth signals, unlimited replicative potential, angiogenesis, acquiring invasive and metastatic properties, deregulating energy metabolism, genomic instability, promoting tumor inflammation, and evading immune cell destruction (1). At a fundamental level, acquiring the hallmarks of cancer is a consequence of deregulating any one of a number of basic cellular properties including motility, differentiation, proliferation, and viability. In one theory, this occurs spontaneously as a result of somatic mutation (2). In some cases, these somatic mutations directly cause abnormal gene expression patterns favoring tumor cell growth. A classic example is the t(8;14)(q24; q32) translocation that juxtaposes the immunoglobulin heavy chain locus with the MYC protooncogene, elevating MYC expression (3,4). In other cases, mutations deregulate circuits resulting in the abnormal regulation of a large number of genes to favor tumor cell growth. Activating mutations in Ras (RasG12V), which result in tumor-promoting gene regulation, are a classic example (5,6). Once established, abnormal gene expression profiles maintain the tumor cell phenotype through a process of oncogene addiction (7). A breakdown of the DNA damage repair and maintenance mechanisms (nucleotide-excision repair instability, microsatellite instability and chromosomal instability) contributes to tumor cell genomic

instability and a higher rate of mutation. This instability results in the sporadic rise of clonal populations that have acquired new mutations, and ultimately a tumor comprised of a heterogeneous mixture of cells (1).

In cancer therapy, individual hallmarks are targeted to disrupt tumor growth. For instance, radiation and chemotherapy target rapidly dividing tumor cells, and induce apoptosis through DNA damage. Angiogenesis and metastasis are targeted with drugs that inhibit growth factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (8,9). A number of drugs inhibit cancer metabolism by targeting catabolic pathways, anabolic pathways, and pathways regulating metabolism (10). Additionally, immunotherapy is a promising new approach aimed at enhancing tumor cell immunogenicity and antitumor immunity. Individual immunotherapies have seen great success in treating a subset of patients with specific cancer types. Combination treatment with multiple immunotherapy strategies is expected to see greater benefit (11). Therefore, this study aimed to discover a novel mechanism which could be targeted in cancer immunotherapy.

1.1.1 Melanoma

Melanoma is one of the most immunogenic tumor types, due in part to a high mutation load. A significant initiator of mutations in melanoma is exposure to ultraviolet (UV) light. UV radiation causes two types of DNA damage: 6-4 photoproducts and pyrimidine dimers. Both lesions are formed by covalent linkages of the carbon atoms between two adjacent pyrimidines. When a cell is hit with DNA damage from UV radiation, it initiates G1 cell cycle growth arrest to repair the dimers by nucleotide excision repair (NER). Depending on if the DNA damage is repaired or not, the cell

either re-enters the cell cycle or undergoes apoptosis, respectively. These processes are all regulated by p53, a tumor suppressor which is mutated in approximately 50% of cancers. Thus, cells with mutated p53 are more susceptible to continued cell growth with unrepaired DNA (12). This unrepaired DNA damage is carcinogenic and is the primary source of mutations in melanoma. In fact, compared to other cancer types, melanoma tumors have a higher mutation load due to UV radiation. This high mutation load leads to the production and presentation of more immunogenic neoantigens, and thus a higher degree of immunogenicity (13). It is due to this high level of immunogenicity that makes melanoma tumors so responsive to immunotherapy. Markedly, melanoma is the most responsive solid tumor type, with significant success seen across multiple immunotherapy methods (11).

Briefly, a few well-characterized oncogene driver mutations are key factors in melanoma transformation. First, around 50% of melanoma patients have a specific mutation in the BRAF oncogene- BRAF p.V600E (14). This mutation activates the serine/threonine kinase BRAF, which then stimulates the MAPK pathway. Two BRAF-specific inhibitors (vemurafenib and dabrafenib) and a MEK1/2 inhibitor (trametinib) are used in the clinic to treat melanoma tumors with this mutation and prevent hyperactivated MAPK signaling (15). Around 20% of melanoma tumors have activating mutations in the NRAS oncogene, with the p.Q61R or p.Q61K mutations representing about 90% of NRAS activating mutations in patients (16). Activated NRAS promotes both the MAPK and PI3K signaling pathways. PI3K signaling activates another melanoma oncogene, RAS, whose expression can be necessary for melanoma tumor growth (17). However, no RAS specific inhibitors have been developed. For the

remaining ~30% of melanomas a driver mutation has not been established, though a number of candidates have been suggested based on exome sequencing (15).

1.1.2 Breast Cancer

Unlike melanoma, breast cancer is a poorly immunogenic tumor type. There are three subtypes of breast cancer defined by hormone receptor expression. These subtypes are: estrogen receptor (ER) positive, human epidermal growth factor 2 receptor (HER2) positive, and triple negative breast cancer (TNBC). ER-positive breast cancer tumors express ER and progesterone receptors (PR), which directly contribute to tumorigenesis through ER activation-dependent proliferation. While approximately 65% of breast cancers are ER-positive, the mechanisms by which ER induces cell proliferation are not completely understood. It is known that an ER activated by its ligand homodimerizes and is translocated into the nucleus where it binds estrogen response elements in genes to regulate their activity. Independent of DNA binding, cytosolic ER can activate the ERK1/2 proliferation pathway (18). HER2-positive breast cancer is the second subset which comprises around 25% of breast cancers. These tumors have either HER2 gene amplification or overexpression. HER2 heterodimerizes with another HER family member upon ligand binding and overexpression contributes to tumorigenesis through multiple mechanisms. First, HER2 overexpression increases HER2 dimerization with HER3 and epidermal growth factor receptor (EGFR). HER2-HER3 complexes activate the PI3K/Akt signaling pathway, which promotes cell proliferation and invasion. Second, HER2-EGFR complexes activate the Ras, PI3K and PLC- γ pathways, which induce cell cycle deregulation and invasive properties. Third, HER2 overexpression prolongs the activation of the downstream signaling pathways

MAPK and c-jun. It has also been proposed that an alternatively spliced HER2 transcript that is present in a majority of HER-positive tumors has enhanced tumor promoting abilities (19). There are not many cases in which breast tumors are both ER- and HER2-positive because estrogen represses HER2 expression (20). This occurs through estrogen-mediated downregulation of the transcription factor AP-2 and sequestration of the SRC-1 transcription factor (20). TNBC makes up about 20% of breast cancer tumors and is a highly heterogeneous subtype. TNBCs are more aggressive than hormone receptor-positive tumors and have a lower five year survival rate and a higher rate of relapse, partly due to the absence of hormone receptors, which can be targeted therapeutically (18).

Endocrine therapy targeting hormone receptor positive breast cancer is a mainstay of cancer therapy. ER targeting therapies include the ER antagonist tamoxifen, the aromatase inhibitor anastrozole, the mTOR inhibitor everolimus and the CDK4/6 inhibitor palbociclib. HER2 specific therapies include trastuzumab and lapatinib and pertuzumab. Trastuzumab is a monoclonal antibody which binds to HER2 on cells and induces cell cycle arrest. Lapatinib is a tyrosine kinase inhibitor which disrupts HER2-EGFR signaling and pertuzumab is a monoclonal antibody which binds HER2 and prevents it from dimerizing with HER3. While these targeted treatments drastically improve the survival of patients with hormone receptor positive breast cancer, development of tumor resistance to hormone therapy remains an issue. There are no targeted treatments for TNBC, though unlike hormone receptor positive tumors, TNBCs are responsive to chemotherapy (18).

Because there is no targeted treatment for TNBC, and resistance to hormone therapy is an issue with hormone receptor positive breast cancer, new strategies to treat breast cancer need to be developed. Interestingly, even though breast cancer is poorly immunogenic, tumor infiltration of lymphocytes is correlated with disease-free survival, indicating that the antitumor immune response is relevant to breast tumor growth (21). However, breast tumors are historically only minimally responsive to immunotherapy, due in large part to the lack of immunogenicity. Since tumor infiltrating lymphocytes are associated with positive outcome, methods which enhance the immunogenicity of breast tumor cells are a viable therapeutic strategy to improve tumor regression. Therefore, new methods enhancing tumor immunogenicity in combination with traditional immunotherapy could be the key to enhance the modest results seen with individual immunotherapies.

Section 1.2: The Immune System

The immune system is composed of two arms: innate and adaptive immunity. Innate immunity provides the first wave of defense against infection. Innate immune cells include macrophages/monocytes, dendritic cells (DCs) and natural killer (NK) cells. These cells respond quickly to infection, but most pathogens cannot be cleared by innate immunity alone. Therefore, innate immunity serves two important auxiliary roles: to create an inflammatory environment and to prime immune cells of the adaptive response through immune cell infiltration and cytokine release. Adaptive immunity kicks in 4-7 days after the initial detection of an infection and it mounts a more effective attack against the pathogen. Cells of the adaptive immune response include CD4 T cells, CD8 T cells and B cells. Importantly, adaptive immune cells retain memory of a pathogen, so

that if a second infection occurs by the same pathogen, the adaptive response can clear the re-infecting pathogen rapidly, without the time-consuming need for priming by innate cells (22).

1.2.1 Antigen Presenting Cells

Antigen presenting cells (APC) present antigens resulting from an infection via either major histocompatibility (MHC) class I or II molecules to T cells. Through these functions, APCs prime T cells for T cell activation, expansion, and differentiation. Professional APCs include DCs, macrophages and B cells, DCs being the most physiologically important for T cell activation. DCs uptake antigens from different types of infections through phagocytosis, macropinocytosis, or from infection of the DC itself. Antigens engulfed by phagocytosis or macropinocytosis enter the endocytosis pathway and are presented on MHC II molecules to CD4 T cells, whereas antigens in the cytoplasm resulting from APC infection are presented by MHC I molecules to CD8 T cells. Antigens that are engulfed by DCs can also be cross-presented by MHC I molecules to CD8 T cells. Macrophages and B cells, on the other hand, use antigen presentation as a secondary line of defense. The primary function of macrophages is to engulf microbes and antigens for destruction, though they can present peptides from engulfed microorganisms on MHC II molecules. Antigen-specific B cells can bind antigens via their immunoglobulins, internalize them by receptor-mediated endocytosis and present the processed peptides on MHC II molecules. (22-24).

1.2.2 CD4 T Cells

CD4 T cells, or helper T cells, do not directly kill target cells, but instead promote or inhibit the activities of other effector cells through cytokine secretion. CD4 T cell receptors (TCR) bind to antigens in complex with MHC class II molecules, which are restricted in expression to APCs. MHC II molecules specifically present peptides from the vesicular pathway, thus these antigens are of extracellular origin which have been endocytosed by the APC (22).

Mature naive CD4 T cells are activated in the peripheral lymphoid tissue by interaction with an APC presenting the appropriate antigen. Following activation, and depending on the cytokine environment present, CD4 T cells differentiate into Th1, Th2, Th17, or regulatory T cells (Tregs) (22,25,26). Th1 cell differentiation is induced in the presence of DC and macrophage secreted interferon gamma (IFN γ) and interleukin 12 (IL-12). Th1 cells are the primary CD4 T cell subset implicated in helping CD8 T cell activities through the production of IFN γ and IL-2. Induction of a Th1 response is necessary for the control of both bacterial infections and tumors. Th2 cell differentiation occurs in the presence of IL-4 and this subset promotes B cell activities through IL-4 and IL-13 secretion. Th2 cells are mainly involved in the control of parasitic infections. Th17 cells arise in the presence of IL-6 and transforming growth factor beta (TGF- β) and are characterized by their secretion of the IL-17 family of cytokines. Th17 cells promote inflammation by recruiting innate immune cells, most prominently neutrophils. Treg differentiation can occur in the thymus, but can also be induced in the periphery under conditions of self-antigen recognition or in response to TGF- β . Tregs suppress immunity and maintain tolerance by multiple mechanisms. First, they produce TGF- β

and IL-10, which inhibit T cell proliferation and IL-2 production. IL-10 also suppresses the activity of DCs, which are necessary for T cell activation and Th1 differentiation. Tregs also express high amounts of the T cell co-inhibitory receptor cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), which competitively binds to the available B7-1, reducing the co-stimulatory potential for the surrounding T cells (25).

1.2.3 CD8 T Cells

Cytotoxic CD8 T cells are effector cells of the adaptive immune response which recognize antigens presented on MHC class I molecules. They have direct cytotoxic activities toward antigen expressing cells, typically virus infected cells or tumor cells.

1.2.3.1 CD8 T Cell Maturation

T cell development occurs in the thymus from committed lymphoid progenitor cells, which have migrated from the bone marrow. Committed lymphoid progenitors differentiate into double negative (DN: CD8⁻,CD4⁻) thymocytes, which express neither CD4 nor CD8. At the end of this developmental stage, DN thymocytes express either a $\gamma\delta$ or an $\alpha\beta$ TCR. In a subsequent developmental phase, DN thymocytes begin expressing both CD8 and CD4 to become double positive (DP: CD8⁺,CD4⁺) thymocytes. At this stage, medullary thymic epithelial cells (mTECs) present many self antigens to the DP thymocytes in a process called negative selection (27). In this process, a transcription factor called the autoimmune regulator (Aire) induces the expression of thousands of genes in mTECs whose expression is highly specific to certain tissue(s) (28,29). These tissue specific self antigens are presented on both MHC I and II molecules by mTECs and DCs to the DP thymocytes (30). Approximately 90%

of DP thymocytes do not signal, or signal very weakly, through their TCRs in response to antigen-MHC interaction. These thymocytes undergo death by neglect. Another 5% of DP thymocytes signal strongly in response to an antigen. These T cells are reactive to self antigens and are thus eliminated by negative selection via apoptosis in order to create T cell tolerance to self. The remaining 2-5% of DP thymocytes induce a signal of moderate strength through their TCRs, which stimulates their survival by positive selection. Depending on which antigen-MHC I or antigen-MHC II complex the TCR interacts with, the DP thymocyte will ultimately differentiate into a mature single positive (SP) CD8⁺,CD4⁻ or CD8⁻,CD4⁺ T cell, respectively. SP thymocytes then proliferate in the thymus for about four days before migrating to peripheral lymphoid organs (31).

Unlike innate immune cells which express an array of receptors that can recognize multiple ligands, each CD8 T cell expresses a TCR that is specific to an individual antigen (or, more likely, a set of similar antigens), thus providing a unique level of specificity. This is achieved through TCR V(D)J recombination during the DN and DP stages. Each TCR chain consists of a transmembrane constant (C) region which has signaling functions and an extracellular variable (V) region, which recognizes and binds to antigen. The variable region consists of two to three segments: the variable (V) and joining (J) segments of the TCR α and γ chains, and the V, diversity (D) and J gene segments of the β and δ chains. Each segment exists as multiple copies. The result of V(D)J rearrangement is one V, one D and one J segment forming the β chain variable region and one V and one J segment forming the α chain variable region. Thus, the many possible combinations of individual V, J and/or D segment joining creates a high level of variable region diversity, called combinatorial diversity. Through this

process, the body's T cell repertoire can recognize millions of different antigens, with each processed TCR specific to an array of related peptides (32).

1.2.3.2 CD8 T Cell Priming

The last step of T cell maturation is priming in peripheral lymphoid tissues. DCs that have engulfed antigens migrate to the lymphoid organs near the site of infection, where they present the antigens via MHC class I or II to naive CD8 or CD4 T cells, respectively. T cell TCRs transiently bind DCs in lymphoid organs to sample the presented antigens. Naive T cells which encounter the appropriate antigen are activated by TCR signaling and undergo differentiation and proliferation. Naive T cells which do not encounter the correct antigen exit the lymphoid tissue and circulate via the blood through other lymphoid tissues until they encounter the proper antigen (22).

T cell activation requires three signals: 1) TCR binding to the correct antigen-MHC complex, 2) a co-stimulatory signal and 3) cytokines. These three signals provide vital regulation for the activities of T cells to respond to antigens only in the proper conditions of infection or cellular stress. For example, CD8 T cells which bind to a self antigen in the periphery undergo either apoptosis or anergy because a co-stimulatory signal is not sent in the absence of an inflammatory environment. In an immune response, the two main co-stimulatory ligands B7-1 and B7-2 are secreted by activated APCs. The receptor for B7-1 and B7-2 is CD28, expressed on T cells. This co-stimulatory signal activates T cell cytokine production and proliferation as well as cell survival by inhibiting apoptosis and promoting cellular metabolism. On the flip side, co-inhibitory signals negatively regulate CD8 T cell responses. Activated T cells express three co-inhibitory receptors: CTLA-4, programmed death 1 (PD-1) and B- and T-

lymphocyte associated protein (BTLA). CTLA-4 binds the B7-1 and B7-2 ligands, PD-1 binds to the PD-L1 and PD-L2 ligands, and the BTLA ligand is herpes virus entry mediator (HVEM) (33,34). In addition to antigen-MHC and co-stimulation, cytokines are also necessary for CD8 T cell function, IL-2 being the most important. IL-2 is secreted by activated DCs and CD4 T cells and is necessary for both CD8 T cell proliferation and differentiation following interaction with its antigen. While naive CD8 T cells can be activated by DCs alone, this process is often facilitated by CD4 T cells. CD4 T cells both maximize the DC co-stimulatory signal through CD40L/CD40 interaction and aid in CD8 T cell proliferation and differentiation through IL-2 secretion (22,35).

1.2.3.3 CD8 T Cell Activation

Once the CD8 TCR has made contact with the appropriate antigen-MHC complex on an APC, a signal transduction cascade is activated which prompts the T cell to release the cytotoxic molecules perforin and granzymes or the Fas ligand (FasL). The TCR by itself does not have any signaling capacity; instead, the TCR associates with the co-receptor CD3, which contains ten intracellular immunoreceptor tyrosine-based activator motifs (ITAMs). Each ITAM contains two tyrosine residues that are phosphorylated upon TCR binding to antigen. ITAM phosphorylation is facilitated by CD8, whose extracellular domain interacts with the MHC complex. MHC binding stimulates activation of the tyrosine protein kinase Lck, which is associated with the intracellular domain of CD8, to phosphorylate the CD3 ITAM tyrosine residues. Tyrosine phosphorylation then allows the SH2 domain containing protein Zap-70 to bind. Zap-70 is consequentially activated by phosphorylation by Lck and then activated Zap-70 phosphorylates the adaptor proteins SLP-76 and LAT. The adaptor protein Gads

facilitates the interaction of activated SLP-76 and LAT, and this complex recruits the signaling protein PLC- γ . PLC- γ recruitment and activation is regulated both by the T cell co-stimulatory signal CD28, which is necessary for T cell activation, and by binding to the SLP-76:Gad:LAT complex. Recruited PLC- γ is then activated by tyrosine phosphorylation by Ltk. PLC- γ activation results in the activation of three separate pathways: the release of Ca^{2+} from the endoplasmic reticulum, the activation of the small GTPase Ras, and the activation of PKC- θ . First, activated PLC- γ stimulates the breakdown of PIP_2 into DAG and IP_3 . IP_3 then binds to its receptors on the endoplasmic reticulum membrane. These receptors are Ca^{2+} channels and IP_3 binding results in their opening and Ca^{2+} release into the cytoplasm. Cytoplasmic Ca^{2+} binds to and activates the cytoplasmic protein calmodulin, which then activates calcineurin, which activates the NFAT family of transcription factors. NFAT transcription factors induce the expression of several genes important for T cell activation. Second, membrane bound DAG activates RasGRP, which in turn activates Ras. Ras stimulates a MAPK signaling cascade by phosphorylating and activating Raf. Raf then phosphorylates and activates MEK1, which phosphorylates and activates Erk. Erk activation ultimately stimulates the transcription factor AP-1. Third, DAG also activates PKC- θ , which then phosphorylates and activates the adaptor protein CARMA1. CARMA1 activates TRAF-6, which activates TAK1, which then activates IKK. IKK stimulates I κ B degradation, which releases the NF κ B family of transcription factors. NFAT, AP-1 and NF κ B together directly regulate Il-2 expression (22,36,37).

1.2.3.4 Effector CD8 T Cells

CD8 T cells differentiate into effector cytotoxic T cells (CTLs) and rapidly proliferate in response to IL-2 following activation, resulting in thousands of effector T cells with the same TCR. This is called clonal expansion. Effector CTLs cells then leave the lymphoid tissue and migrate through the blood and into the tissue at the site of infection. Unlike naive CD8 T cells, CTLs can respond to antigen without the need for a secondary co-stimulatory signal. After a CTL has made contact with the appropriate antigen-MHC complex on a target cell, the CTL's local cytoskeleton is rearranged, which results in the excretion of preformed cytotoxic granules or FasL. Activated CTLs also release IFN γ and tumor necrosis factor alpha (TNF- α), cytokines which promote the immune response by helping activate macrophages and by upregulating the expression of MHC I molecules.

In the case of cytotoxic granule release, which is the main mechanism by which CTLs kill targets, perforin and granzymes are secreted from the cytotoxic granules into the immunological synapse between the CTL and target cell. Perforin creates a pore in the target cell membrane, allowing granzymes entry into the cell. Granzymes are serine proteases and granzymes A and B are the most abundant in the cytotoxic granules. Granzyme A degrades the target cell nuclear envelope and induces single stranded nicks in the cellular DNA. Granzyme B cleaves BID and pro-caspase 3 to activate them. Caspase-3 activation triggers a caspase signaling cascade which ultimately results in CAD activation. CAD nuclease activity fragments the cellular DNA, an integral step in the apoptotic pathway. BID activation disrupts the outer mitochondrial membrane,

allowing the release of mitochondrial apoptotic enzymes such as cytochrome c into the cytoplasm (22,38,39).

In Fas mediated apoptosis, FasL binding to its receptor Fas on the target cell induces Fas trimerization. This allows the cytoplasmic death domains of Fas to bind to the death domains of Fas-associated via death domain (FADD), an adaptor protein. Pro-caspase 8 and 10 then bind to the death effector domains of FADD, resulting in their self-cleavage, activation and disassociation from FADD. Caspase 8 activation initiates the apoptosis pathway (40).

1.2.3.5 Memory CD8 T cells

After clearance of an infection, 95% of the clonally expanded effector T cells are eliminated by apoptosis. The remaining CD8 T cells have differentiated into memory T cells that can survive long term in the host, ready to quickly attack if a second infection occurs. Memory T cell differentiation is not fully understood, but it is believed that antigen signal duration, antigen signal strength and the cytokine environment all play important roles in determining the fate of effector T cells. It has been established, however, that memory T cell survival and maintenance depends on the cytokines IL-7, TSLP, and IL-15. Two types of memory T cells have been identified: central memory T cells (TCM) (CD62L+, CCR7+) and effector memory T cells (TEM) (CD62L-, CCR7-). TCM cells are primarily located in peripheral lymphoid tissues while TEM cells are located in nonlymphoid tissues such as the lung, liver and intestine (22,41,42).

1.2.4 $\gamma:\delta$ T Cells

$\gamma:\delta$ T cells make up around 10% of all T cells and much less is known about this subset than the $\alpha:\beta$ CD4 and CD8 T cells. $\gamma:\delta$ T cell development is dependent on whether a DN thymocyte expresses the pre-TCR or the $\gamma:\delta$ TCR first. As the $\gamma:\delta$ TCR requires more rearrangement steps than the pre-TCR, the pre-TCR is almost always expressed first. They are mostly located in mucosal and epithelial sites and they lack CD4 and CD8 molecules. Functions for $\gamma:\delta$ T cells include producing CXCL13 to regulate B organization and presenting antigens to both CD4 and CD8 T cells (43).

1.2.5 MHC I Antigen Processing

All nucleated cells express MHC class I molecules, which present intracellularly derived antigens to CD8 T cells. These antigens are byproducts of normal proteins called “self” antigens, antigens resulting from cell infection or antigens resulting from/associated with transformation.

Proteins are broken down into antigens primarily by the constitutive proteasome. The proteasome is composed of a 20S catalytic domain and two 19S regulatory particles. The 19S regulatory particle recognizes ubiquitinated proteins and unfolds them so they can be fed into the 20S cylindrical core. The 20S core contains four stacked rings. The outer rings are composed of 7 alpha subunits each, which form a physical gate to the two inner rings, which are composed of 7 beta subunits each. The beta subunits create peptides by cleaving the protein at specific sites. Alternatively, three of the proteasome beta subunits can be substituted with PSMB8, PSMB9 and PSMB10, whose expression is upregulated upon type I or II IFN stimulation. The

PSMB8, PSMB9 and PSMB10 beta subunits have different cleavage specificities and cut proteins into more antigenic peptides than those processed by the constitutive proteasome (44). The PSMB8 and PSMB9 genes are located in the MHC region, while PSMB10 is not. This alternate form of the proteasome is called the immunoproteasome (45,46). This specialized proteasome ultimately creates peptides with higher affinity to MHC I molecules, which are therefore more antigenic to CD8 T cells (47,48). Both the proteasome and immunoproteasome cleave ubiquitinated proteins into peptides that are 2-25 amino acid residues in length (49). These peptides are then transported into the endoplasmic ER by the transporter associated with antigen-processing (TAP) complex. The TAP complex is a heterodimer composed of the TAP1 and TAP2 subunits, which contain a hydrophobic transmembrane domain and a nucleotide binding domain (47). The TAP complex transports peptides, preferably between 8-12 residues, into the endoplasmic reticulum in an ATP-dependent manner. Interestingly, changes in expression of the TAP genes result in changes in the antigen repertoire presented at the cell surface (50). In the endoplasmic reticulum, peptides can be further cleaved by enzymes, like ERAP1, to fit into MHC I molecules. Fully processed antigens 8-10 amino acids long are then loaded onto MHC I molecules by the calnexin, calreticulin, ERp57 and tapasin chaperones. ERp57 and tapasin can also remove low affinity peptides from the MHC I molecule and exchange them for higher affinity peptides (51). Peptide loaded MHC I molecules are then transported in vesicles to the cell surface.

MHC I molecule expression is induced by IFN type I and II and consists of an α chain and a β chain. The α chain is composed of three domains (α 1- α 3) and the β domain is encoded by a single gene, β ₂-microglobulin (β 2m). The α 3 transmembrane

domain is bound noncovalently to $\alpha 2m$ near the cell surface and the outer $\alpha 2$ and $\alpha 3$ chains form the peptide binding cleft. MHC I molecules are highly polymorphic at this peptide binding cleft. There are three classical MHC I α chain genes in humans (HLA-A, -B and -C) and in mice (H2-K, H2-D and H2-L), each binding to a different range of peptides. There are approximately 800 alleles in the human population for each gene. In addition, multiple nonclassical MHC I molecules bind specialized peptides. This variability enables the MHC I molecule repertoire to bind to a wide selection of antigenic peptides (47).

1.2.6 Myeloid Derived Suppressor Cells

One immune cell type with remarkable ability to suppress the antitumor immune response in cancer is myeloid derived suppressor cells (MDSC). MDSCs originate from immature myeloid cells and myeloid progenitor cells. During the normal state, these myeloid cells differentiate into granulocytes, macrophages or DCs, thus MDSCs do not represent a significant population of mature immune cells, nor do they have significant roles in immune biology. However, tumor cells can secrete cyclooxygenase 2, prostaglandins, stem-cell factor, IL-6, VEGF, granulocyte macrophage colony-stimulating factor (GM-CSF) and/or macrophage CSF (M-CSF), which induce MDSC differentiation and expansion. In this way MDSCs are significantly amplified and can represent up to 40% of white blood cells in the spleen of tumor bearing mice (22,52). Two distinct subsets of MDSCs have been identified: granulocytic and monocytic MDSCs. Granulocytic MDSCs represent the majority of expanded MDSCs and have different molecular functions than their monocytic counterparts. Monocytic MDSCs have

the unique ability to further differentiate into DCs and tumor associated macrophages (53).

MDSCs are activated by T cell and tumor stromal cell secreted INF γ , IL-4, IL-13 and TGF- β . Activated MDSCs efficiently inhibit the T cell response through a few mechanisms. First, they express high amounts of arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS), which metabolize and thereby deplete the non essential amino acid arginine. Because arginine is essential for T cell proliferation, arginine depletion by MDSCs handicaps T cell expansion. MDSCs also produce nitric oxide (NO) and reactive oxygen species (ROS), which induce T cell apoptosis and produce peroxynitrite. Peroxynitrite in the tumor microenvironment stimulates the nitration of the cysteine, methionine, tryptophan and tyrosine amino acids of the TCR. These post-translational modifications result in the inability of the TCR to respond to antigen (53). It has also been shown that MDSCs can induce the differentiation of tumor infiltrated T cells into Tregs (54). In addition, T cells rely on APCs to secrete cysteine, which they then uptake from the microenvironment for metabolism. MDSCs import and sequester the available cysteine, preventing adequate uptake by T cells (55). MDSCs also produce the immunosuppressive cytokines IL-10, TGF- β and COX2 (52).

Besides T cells, MDSCs also inhibit NK cells and DCs. MDSCs can suppress NK cell cytotoxicity and induce anergy through TGF- β and STAT5 and Arg (55-57). They also suppress NK cell NKG2D expression and IFN γ production (55). Tumor infiltrated DC function is also repressed through several mechanisms. MDSCs prevent DC maturation, which is required for antigen presentation to T cells, they suppress DC

antigen uptake and they inhibit DC migration. Further, MDSCs can also promote the expression of anti-inflammatory cytokines by DCs (58).

1.2.7 Natural Killer Cells

NK cells are cells of the innate immune response that target bacteria infected cells, virus infected cells, or transformed cells without prior sensitization. NK cells develop from common haematopoietic precursor cells predominantly in the bone marrow, but also at peripheral sites including the liver, lymph nodes, thymus and uterus (59). NK cell activity is positively regulated by activating receptors and by dendritic cell or macrophage secreted cytokines, including type I IFN (cytotoxicity), IL-15 (survival and proliferation), IL-12 (IFN- γ production), IL-2 (cytolytic activity) and IL-18 (cytotoxicity) (60). NK inhibitory receptors, on the other hand, provide negative regulation.

Mature NK cells express a variety of activating and inhibitory receptors and the net effect of ligand interaction from a target cell determines whether an inhibitory or activating response is induced. In order to prevent autoimmunity to healthy cells, most inhibitory receptors recognize “self” MHC I molecules, which all nucleated cells express. Downregulation of MHC molecules following viral infection or transformation can induce a “missing-self” NK response. Inhibitory receptors signal through immunoreceptor tyrosine-based inhibitor motifs (ITIMs) in their cytoplasmic tails. To detect pathogen infected or tumor cells, activating receptors recognize pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or viral ligands themselves. These receptors’ ligands are much less well-defined than the inhibitory receptors’ and for multiple activating receptors, their ligands have yet to be discovered

(22). The two main activating receptor families involved in tumor immunoediting are NKG2D and the natural cytotoxicity receptors (NCR). NKG2D, expressed by both mouse and human NK cells, is the best characterized NK activating receptor. NKG2D binds the cellular stress induced nonclassical MHC molecules Rae1 and H-60 in mice, which can result in tumor elimination *in vivo* (61,62). In fact, most tumors express NKG2D ligands (63). NKG2D also binds the human CMV ligand ULBP. Another class of receptors involved in antitumor immunity which are much less characterized are the NCRs. The NCRs bind unknown tumor ligands in combination with the co-ligand heparan sulfate (HS). Generally, activating receptors signal through ITAMs associated with their cytoplasmic domains, which results in NK degranulation and transcription of cytokines. CD16 (FcγRIIIa) is unique activating NK receptor which binds to the Fc portion of immunoglobulins bound to target cells and triggers antibody-dependent cellular cytotoxicity (ADCC) by signaling through its ITAM (64). Like CD8 T cells, while activated NK cells can kill targets through either perforin/granzyme release or through the Fas-FADD pathway, the perforin-dependent pathway is the most physiologically relevant pathway *in vivo* (65). Unlike CD8 T cells, NK cells don't rapidly proliferate following activation.

There are two main NK cell subsets in human: CD56^{dim}CD16+ and CD56^{bright}CD16-. CD56^{dim} NK cells are highly cytotoxic and can secrete IFNγ (66). This subset constitutes the majority of NK cells in humans. Only a small proportion of human NK cells are CD56^{bright} and these poorly cytotoxic NK cells are the source of most NK secreted cytokines, including IFNγ, TNF-α, TNF-β, IL-10, IL-13 and GM-CSF (67). Mouse NK cells also have cytotoxic and cytokine secreting functions, though identifying

two functionally distinct NK cell subsets is less clear because mouse NK cells do not express CD56 (68).

In addition to killing infected and malignant cells, activated NK cells also regulate the activities of DCs, CD4 T cells and CD8 T cells through cytokine secretion. For example, NK cells enhance T cell activity through secretion of IFN γ , which stimulates DC maturation and CD4 T cell differentiation into the Th1 phenotype, further promoting the immune response (69,70). On the other hand, during certain viral infections NK cells secrete IL-10, which suppresses the T cell response (71,72). NK cells can also directly kill Tregs and activated CD8 T cells expressing NKG2D or NCR1 ligands and activated CD4 T cells with downregulated Qa1 (73-75).

1.2.7.1 Natural Cytotoxicity Receptors

One key class of NK cell activating receptors that recognizes tumor ligands is the NCRs (76,77). There are 3 NCRs in humans (NKp30, NKp44, and NKp46) and 1 in mice (NCR1). These receptors have essential functions in NK cell-mediated antitumor immunity, including controlling tumor growth and metastasis *in vivo*, and cytolytic and cytotoxic activity *in vitro* (78-81). These receptors belong to the immunoglobulin superfamily. They have one (NKp30, NKp44) or two (NKp46) extracellular immunoglobulin-like domains which bind ligand and a transmembrane domain which contains a positive amino acid that binds signaling adaptor proteins containing ITAMs (or an ITIM for NKp44) (76). Both human and mouse NCRs recognize N- and O-sulfo modified HS, which is located on the plasma membrane as heparan sulfate proteoglycans (HSPG). NKp30 and NKp46 exhibit similar binding patterns to HS, but

with different affinities, while NKp44 exhibits a completely different binding pattern to HS chains (82).

The NCRs recognize ligands from a variety of sources. NKp44 and NKp46 recognize hemagglutinin (HA) and hemagglutinin neuraminidase (HN) from multiple, but not all, viruses. NCR recognition of HA or HN seems to be based on posttranslational modification of the receptor (76). NKp30 recognizes pp65 from cytomegalovirus, which inhibits NK activity (83). NKp44 can also bind to and is activated by envelope glycoproteins of West Nile virus (84) and vimentin, a ligand expressed by cells infected with *Mycobacterium tuberculosis* (85). NKp46 can be activated by a bacterial ligand of *Fusobacterium nucleatum* and a ligand expressed by pancreatic β Langerhans cells (86,87). B7-H6, expressed by tumor cells and inflammatory innate immune cells is a well-characterized ligand for NKp30 (76,88). For other potential NCR ligands like BAT3, PCNA and a *Plasmodium falciparum* ligand, there are conflicting reports on whether or not they elicit NK responses (76). Thus, NCRs have been implicated in killing virus and bacteria infected cells, as well as play a role in diabetes and antitumor immunity.

Structurally, NKp30 and NKp46 are similar to each other. Both have an arginine amino acid in their transmembrane domains that associates with an FcR γ signaling domain containing intracellular ITAMs. NKp30 and NKp46 engagement results in tyrosine phosphorylation of the associated ITAMs by srk-family kinases. This allows binding of Syk and ZAP70 to the ITAM sequence, where they recruit and phosphorylate the adaptor molecule LAT and NTAL. These molecules then recruit and phosphorylate phospholipase C, Vav2, Vav3 and PI3K. The Vav molecules have guanine exchange factor activity and activate the GTPases Rho and Rac1. These GTPases activate the

MAPK signaling pathway, which is important for NK cell degranulation. Phospholipase C activation stimulates the release of Ca^{2+} into the cytosol, leading to the secretion of cytokines. PI3K activation induces the expression of genes involved in cytoskeletal rearrangement (77,89).

1.2.8 Heparan Sulfate

HSPGs are core proteins with HS glycosaminoglycan side chains covalently attached. Transmembrane HSPGs are called syndecans, GPI linked HSPGs are called glypicans and HSPGs which are shed are called perlecans. HS is a highly structurally diverse molecule which sequesters growth factors, coagulation factors, proteases, morphogens, cytokines, chemokines and cell adhesion molecules at the cell surface to regulate the extracellular matrix (ECM) structure, cell signaling and cell adhesion (90,91). HS synthesis occurs in the Golgi apparatus, is non-template driven and involves multiple enzymes including glycosyltransferases, sulphotransferases and epimerases. HS synthesis is tightly regulated and tissue and cell-type specific. All animal cells have cell surface HS, but HS structure is different between tissue types and between healthy vs tumor cells (92).

In cancer biology, cell surface and soluble HS have multiple functions in DAMP signaling. DAMPs are danger signal molecules released by necrotic cells to promote tissue repair and the inflammatory response. On endothelial cells, HS is necessary for oligomerization of RAGE, the receptor for the DAMP molecules HMGB1 and S100A8/A9. HSPG interactions are also necessary for HMGB1 and S100A8/A9 binding to the endothelial cell surface (93,94). In addition, soluble HS can act as a DAMP molecule itself by binding to the toll-like receptor 4 (TLR-4) on dendritic cells and

causing dendritic cell maturation (95). Soluble HS also acts as a DAMP by stimulating the release of inflammatory cytokines by macrophages *in vitro* (96).

1.2.9 Heparanase

The only known HS modifying enzyme in mammals is heparanase. Heparanase is an endo-B-glucuronidase with demonstrated specificity for multiple HS substrates, though the exact requirements for heparanase activity are not fully understood (97). Two heparanase genes have been identified that are highly homologous to each other, heparanase-1 and heparanase-2. Only heparanase-1, however, has HS-degrading activity. Heparanase-1 and heparanase-2 have distinctly different patterns of expression in tissues and it has been suggested that heparanase-2 acts as an inhibitor of heparanase-1 by binding to HS and heparanase-1 (98,99). Further, the human heparanase-1 gene generates two different mRNA forms by alternative splicing, though both forms have the same open reading frames and produce the same polypeptide amino acid sequence (100,101).

Heparanase-1 (further referred to as heparanase) activity in the ECM is tightly controlled through regulations at three different stages in its life cycle: transcription, cellular uptake and secretion of active heparanase. First, heparanase expression is constitutively suppressed in most cell types by promoter methylation; treatment with the demethylating agent 5-azacytidine stimulates heparanase re-expression (102).

Heparanase expression is also regulated by p53, Sp1, GABP, EGR1 and estrogen (103-107). Heparanase is translated as a 65 kilodalton (kDa) proenzyme and is then secreted from the cell, where it can interact with HSPGs on the cell surface.

Heparanase binding to HS side chains attached to syndecan-1, syndecan-4, lipoprotein

receptor-related proteins or Man-6-P triggers endocytosis of the HSPG and heparanase. This endocytosis step is efficient as latent heparanase does not accumulate in growth medium (108-110). The endosome then transitions into a lysosome, allowing for activation of the enzyme cathepsin L. Activated cathepsin L cleaves 6 kDa from heparanase to create an 8 kDa and 50 kDa heterodimer. This is the active form of heparanase (111,112). Active heparanase is stored in the lysosome until prompted for secretion from the cell. This step is regulated by stimuli like TNF- α , IL-1 β , fatty acids, ATP, ADP, and adenosine (113,114). Active heparanase secreted from the cell cleaves 4-7 kDa HS fragments from cell surface HSPGs (115). Active heparanase can also be translocated to the nucleus, where it possibly binds to nuclear HS and it has been suggested that heparanase in the nucleus can affect gene transcription by increasing histone H3 expression or through indirectly enhancing histone acetyltransferase (HAT) activity (116,117).

Heparanase is frequently upregulated by cancer cells to regulate cell motility, metastasis, angiogenesis and inflammation (118). Heparanase promoter hypomethylation is a common event in cancer cells which upregulates heparanase expression. Additionally, wildtype p53 directly inhibits heparanase at the promoter. p53 is mutated in around 50% of cancers, however, and mutated p53 does not have the ability to bind to the heparanase promoter (103). ETS1 and ETS2 have also been shown to promote heparanase expression in metastatic breast cancer cells through binding to two ETS binding sites in the promoter (119). Because heparanase expression is repressed by promoter methylation, treatment with 5-azacytidine, a DNA methylation inhibitor that is FDA approved for the treatment of AML and chronic myelomonocytic

leukemia, upregulates heparanase expression (120). Enhanced heparanase activity in cancer results in increased cleavage of HS and bound bioactive molecules from the membrane and degraded ECM structure. These soluble molecules contribute to tumor progression by promoting metastasis, angiogenesis and inflammation. One important property of metastatic cells is the ability to degrade cell surface HS to disrupt cell adhesion. Strikingly, ectopic heparanase expression is sufficient to make poorly metastatic T-lymphoma and melanoma cells highly metastatic (121). Heparanase-mediated release of sequestered angiogenic factors like basic fibroblast growth factor (bFGF) and VEGF and promotes angiogenesis (122). High heparanase expression *in vivo* is accompanied by increased vascularization throughout breast tumors and increasing vessel density and maturation (123). Released growth factors also stimulate tumor growth by binding growth receptors on nearby tumor cells. In addition, heparanase regulates inflammation through the release of HS bound cytokines and chemokines and through activation of innate immune cells by interactions of soluble HS with TLRs (124). Heparanase was reported to drive inflammation induced esophageal, pancreatic, hepatocellular and colon carcinoma (125-128). Outside of tumor immunology, heparanase has consequences to autoimmune disorders, diabetes and restenosis. Heparanase-2, on the other hand, is not upregulated in cancer and studies show that expression negatively correlates to disease-free survival and aggressiveness (98,99,129).

Heparanase expression in human tumors is a robust marker for poor prognosis and patients with low heparanase expression live longer than those with high expression (130-134). Because high heparanase expression is common to many cancer

types and promotes tumor cell biology, inhibition of heparanase has been explored as a therapeutic strategy. A multitude of heparanase inhibitors have been developed including heparin analogs (PI-88, Suramin, PG545, PS3, JG3, STMC, SST0001, M402), small molecule inhibitors (2,3-dihydro-1,3-dioxo-1*H*-isoindole-5-carboxylic acids, furanyl-1,3-thiazol-2-yl-acetic acid derivatives, benzoimidazol-2-yl-benzamides, 4-(1*H*-benzoimidazol-2-yl)-phenyl-ureas, Quinolines, DMBO) and compounds from natural products (Trachyspic acid, CRM646, RK-682, Echinocide A). These drugs all demonstrated a degree of anti-tumorigenic, anti-angiogenic or anti-metastatic properties in preclinical studies or clinical trials (135). However, the most promising heparanase inhibitor, PI-88, failed to improve disease free survival in a phase III clinical trial (NCT01402908). Importantly, none of the heparanase inhibitors tested are specific to heparanase. Thus, there is a need for a specific heparanase inhibitor.

Section 1.3: Tumor Immunology

1.3.1 Cancer Immunoediting

Immunoediting describes the process by which the immune system shapes the immunogenicity of the tumor through immune selection and tumor adaptation. There are three phases in immunoediting: elimination, equilibrium and escape.

In the elimination phase, the immune system is able to recognize and kill transformed cells before the tumor can develop resistance. If tumor elimination is successful, the transformed cells are eliminated very early in tumor development, before a clinically diagnosable tumor has formed. Evidence for the immune system quelling spontaneous tumor growth comes from high incidence rates of cancer in immunosuppressed

recipients of transplants compared to the general population. Biologically, the immune response recognizes alarm signals resulting from deregulated genes and pathways in the tumor. For example, tumor cell growth is characterized by stromal remodeling, which produces danger signals and chemokines. These signals recruit innate immune cells such as macrophages, NK cells and DCs to the tumor. Activated macrophages produce $\text{INF}\gamma$, which provides a positive feedback signal for the immune response and NK cells kill tumor cells expressing NK receptor ligands resulting from cellular stress. DCs endocytose peptides from dying tumor cells and present them to CD4 T cells or cross-present them to CD8 T cells. These peptides can include tumor associated antigens and/or tumor specific antigens. Tumor-specific T cells migrate to the tumor where they produce IL-2 and kill antigen positive cells (136).

In the equilibrium stage, tumor variant(s) have developed resistance to total elimination, but suppression of further growth is constantly maintained by the immune system. This phase can last years in humans before the acquired mutation load allows the cells to enter into the escape phase. This phenomenon was evident through transmission of cancer following organ transplantation. In two specific cases, recipients who did not have any history of melanoma received an organ from donors who had been in remission for many years after successful treatment of melanoma. After transplantation, the donors remained cancer free while the recipients developed melanoma (137,138). The combination of a suppressed immune system and the lack of adaptive immunity to the transplanted cancer cells led to escape phase tumor growth in the recipients while an educated and active immune system in the donors continued to contain the tumor cells in an equilibrium.

In the escape phase, tumor variants have developed multiple mechanisms to suppress or avoid the antitumor response. These tumor cells are therefore able to grow virtually unchecked by the immune system. Common escape mechanisms include antigen and MHC loss, NK ligand shedding, recruitment of regulatory immune cells such as Tregs, MDSCs and M2 macrophages (tumor promoting macrophage subset), release of immunosuppressive cytokines such as TGF- β and IL-10, and developing insensitivity to IFN γ . Four of the most frequent escape mechanisms in human tumors are human leukocyte antigen (HLA) class I, TAP1, PSMB8 and PSMB9 loss (139-141). The escape phase of tumor growth is the most clinically relevant as tumors which present in the clinic are in this phase. Thus the goals of immunotherapy are to break the established immunosuppressive mechanisms and re-activate antitumor immunity (136).

1.3.2 Tumor Antigens

There are three types of tumor antigens which CD8 T cells recognize: tumor associated antigens (TAA), tumor specific antigens (TSA) and cancer testis antigens (CTA). TAAs are antigens derived from proteins that are tissue- or development-restricted in expression. While these genes can be expressed by normal tissue, they are overexpressed in tumor cells and may directly promote tumor cell biology. A few examples of melanoma TAAs include gp100 and TRP-1, which are both epitopes from tyrosinase, and MART-1, an antigen from Melan-A (142).

TSAs are antigens derived from proteins that are specifically expressed by tumor cells as a result of mutation, gene fusion from a chromosomal translocation, aberrant posttranslational modification, or from an oncogenic viral protein. Tumor cells usually have a high mutation load, mainly due to carcinogens and genomic instability, and these

mutations can be missense mutations, frameshift mutations, insertions or deletions. Unlike TAAs, TSAs are specific to the individual mutations of a patient, which are highly variable from one individual to the next (143). The best understood aberrant posttranslational modification resulting in TSAs is protein hyperphosphorylation. Tumor cells commonly deregulate protein kinase activity, resulting in an array of hyperphosphorylated peptides which more stably bind MHC I molecules (144). Protein glycosylation is another posttranslational modification that is frequently deregulated in tumor cells and can create TSAs (145).

CTAs are antigens derived from proteins restricted in expression to germline cells- the testis or placenta (143). In somatic cells, their expression is constitutively repressed by methylation and histone posttranslational modifications (146). In tumor cells and during development in germ cells, a wave of global hypomethylation induces the expression of these genes (147-149). These germline gene products, when broken down, create new antigens for T cell recognition. Because CTAs are broadly expressed across many cancer types and not expressed by most normal tissue, they are ideal for immunotherapy (146). The most frequently expressed CTAs include the melanoma antigen (MAGE) family, New York oesophageal squamous cell carcinoma 1 (NY-ESO-1) family and synovial sarcoma/X breakpoint 2 (SSX-2) antigens (150-152).

Section 1.4: Immunotherapy

Ever since the impact of the immune system on tumor growth was understood, there has been great interest in developing strategies to improve the antitumor immune response. Many immunotherapeutic methods targeting different aspects of the antitumor response have been developed. These include approaches to activate

immune cells (cytokine therapy, antigen vaccination), to dampen immunosuppressive mechanisms (checkpoint blockade, TGF- β inhibition) and to expand the infiltration and activities of effector cells (T or NK cell adoptive cell therapy). Unfortunately, there has been limited success with immunotherapy so far, with only a few treatments FDA approved to date (11). There is expectation, however, that with further research and with combination therapy using multiple immunotherapeutic strategies or standard chemotherapy will be necessary to bring about the full potential for individual immunotherapy methods.

1.4.1 Cytokine Therapy

One approach to enhance immune cell activities to tumors is cytokine therapy. Cytokines are integral for immune cell activation and maintenance and for influencing the type of response the immune system initiates. Two cytokine treatments have achieved FDA approval so far- IL-2 and IFN α . IL-2 is the main cytokine necessary for T cell activation and high dose treatment has been approved for melanoma and renal cell carcinoma. However, a broad applicability of high dose IL-2 treatment is limited due to potentially significant toxicities, including capillary leak syndrome. Low dose IL-2, on the other hand, is safe but not as effective at stimulating antitumor T cells (153). Another disadvantage to IL-2 treatment is that it promotes the expansion of Tregs, a T cell subset with inhibitory activities (154). The IFN α cytokine has pleiotropic effects on immune cells. It stimulates MHC I presentation, DC maturation, effector cell activation and it can induce tumor cell apoptosis. IFN α is approved for the treatment of multiple cancer types including leukemia, melanoma, Kaposi's sarcoma and follicular Non-Hodgkin's lymphoma. It is less toxic than IL-2 but efficacy of IFN α treatment is

inconsistent (155). Other cytokines being tested preclinically are IL-12, IL-15, IL-18, IL-7, IL-21, IL-23 and GM-CSF. IL-12 and IL-15 promote NK function, Th1 T cell differentiation and CD8 T cell activities (156-158). Both have shown promise in mouse studies, though IL-12 treatment in a clinical trial only saw one patient partially respond (158-160). IL-18 and IL-21 are two more NK and CD8 T cell promoting cytokines which have seen modest results in clinical trials (161,162). IL-7 is another promising cytokine important for T cell function. Multiple clinical trials determined that IL-7 expands the CD4 and CD8 T cell populations without expanding Tregs and also has less toxicity compared to other cytokines (163). GM-CSF, on the other hand, is a highly pleiotropic cytokine that has proven a good adjuvant for antigen vaccination therapy (164,165).

1.4.2 Checkpoint Blockade

Checkpoint therapy has been the most therapeutically successful immunotherapy method to date. Checkpoint blockade aims to prevent or remove the suppression of T and NK cell activation by blocking co-inhibitory receptors on T and NK cells. The best results have been demonstrated for the blocking of CTLA-4 and PD-1, both co-inhibitory receptors expressed on activated T and NK cells. To date, one anti-CTLA-4 (ipilimumab) and two anti-PD-1 (pembrolizumab and nivolumab) antibodies have been FDA approved (143,166). Interestingly, studies show that treatment with anti-CTLA-4 or anti-PD-1 antibody re-activates T cells and causes tumor regression, and these effects are synergized when patients were treated with both antibodies (167,168). The success of checkpoint blockade is primarily attributed to the reactivation of T cells, but some research shows that NK cell reactivation could contribute to tumor regression. In two studies, anti-PD-1 antibody treatment promotes NK cell cytotoxicity toward tumor

targets *in vitro* and prolongs survival in glioma tumor bearing mice (169,170). Other studies show that while anti-CTLA-4 treatment suppresses NK cell production of IFN γ *in vitro*, it promotes NK cell infiltration into the tumor microenvironment in mice (171,172). TIM-3 is another checkpoint receptor that is expressed on exhausted T cells and all NK cells. TIM-3 blockade on antigen specific CD8 T cells from patients enhances their cytokine secretion and proliferation and synergic effects are seen in combination with anti-PD-1 antibody (173). The function of TIM-3 on NK cells, however, is less clear and conflicting results have been reported on whether it acts in an activating or an inhibitory capacity (174). It is important to note that the success of CTLA-4 and PD-1 blockade is reliant on the immunogenicity of the tumor cells. Reactivated T cells are only cytotoxic to antigen expressing cells. Thus, checkpoint blockade is more effective at treating tumors which have a high mutation load, and thus express more neoantigens (143). The effects of checkpoint therapy, therefore, could be enhanced when used in combination with drugs that stimulate antigen expression or presentation.

1.4.3 T Cell Therapy

Improving T cell function has been the major focus of immunotherapy because T cells, especially CD8 T cells, are major effectors in the antitumor immune response. However, tumor infiltrated T cells are often repressed or unable to target antigen or MHC loss tumor cells. The challenge of enhancing the activity of T cells toward tumor cells has been approached in several ways, including through T cell adoptive cell transfer (ACT) and peptide vaccination. Both therapies utilize strategies which rely on T cells responding to known and unknown antigens.

All three types of tumor antigens are utilized in these therapies, dependent upon the tumor type. The use of TSAs is therapeutically appealing because the restricted nature of their expression prevents any cytotoxicity toward normal tissue. T cells specific for TSAs also have higher binding affinities than those specific for TAAs. TAAs are useful, however, because they are generally expressed by many patients of a single cancer type. The use of CTAs is also appealing due to their broad expression among individuals within a cancer type, or even across several cancer types (143). Autoimmunity is also not a complicating factor with CTAs because besides tumor cells, they are only expressed by the testis, which lacks MHC I expression (175).

1.4.3.1 Adoptive Cell Transfer

One approach to enhancing the T cell response is to treat the patient with activated T cells isolated from either the patient or a donor. The process of autologous T cell ACT consists of isolating T cells from a patient's blood or from their surgically removed primary tumor, expanding and activating the T cells *ex vivo*, and transferring them back into the patient. ACT with allogeneic T cells is performed by isolating T cells from a patient in remission and transferring them into a patient who has the same type of cancer but is not in remission. Autologous ACT is much more commonly performed than allogeneic ACT. Both types of ACT are more effective when performed after radiation or chemotherapy induced lymphodepletion. This is advantageous because lymphodepletion removes the immunosuppressive immune cells so that the T cells are not transferred into an inhibitory environment (176). Tumor-specific T cells can also be selected for based on *in vitro* reactivity to autologous tumor cells. If selecting for a known antigen, antigen-specific T cells can be specifically expanded after repeated

stimulation with peptide pulsed APCs (177). ACT has proven most successful at treating melanoma, with a complete tumor regression rate of 20% (178). However, there are several factors which limit the applicability of T cell ACT. T cell ACT is expensive, difficult to standardize and has limited efficacy in patients. For example, a major limiting factor in ACT use is the difficulty of isolating and expanding antitumor T cells from most tumor types. Melanoma is so far the only tumor type from which T cells can consistently be successfully isolated (176). Another challenge with ACT using antigen-specific T cells is minimizing autoimmunity. This is especially true when using TAA-specific T cells as off tumor side effects are seen toward normal cells expressing the TAA (179). In one study, ACT of T cells enriched for specificity to three different melanoma TAAs was largely unsuccessful at inducing tumor regression and sometimes resulted in autoimmunity (180).

Improving the antitumor cytolytic activity of adoptively transferred T cells was addressed by the development of T cells expressing a transgenic TCR. In this method, autologous T cells are transfected with cDNA encoding a TCR sequence. This sequence is designed to exhibit higher affinity to a known tumor antigen than the corresponding native TCR sequence (176). The main disadvantage to transgenic T (TCR-T) cells is enhanced off target reactivity. While TAA-specific TCR-T cells are more cytolytic toward TAA expressing tumor cells than nontransgenic T cells, they are also more reactive toward TAA expressing normal tissue and can induce off target autoimmunity, as evidenced by clinical trials with MART-1 and gp100 specific TCR-T cells (181). Unintentional cross-reactivity of a TCR-T cell with self antigens poses another source of off-target effects, as was the case with MAGE-A3 TCR-T cells (182).

In addition, the higher affinity of the TCR can cause potentially lethal toxicity (143,181). Like nontransgenic T cells, TCR-T cell recognition of antigen is HLA haplotype-dependent, and thus their use is restricted to HLA-matched patients whose tumor cells also express the antigen of interest. Multiple clinical trials have determined that ACT with TCR-T cells can induce tumor regression and improve survival in the right setting. TCR-T cells against NY-ESO-1, MART-1, MAGE-A3 and gp100 are among those tested (181,183-186).

Chimeric antigen receptor (CAR) T cells are T cells which are engineered to express an immunoglobulin-T cell receptor hybrid. The third generation CAR T cells used today express a fusion of an extracellular antibody domain that is specific to a tumor antigen, an intracellular ITAM signaling domain and a costimulatory molecule domain. The costimulatory domains were added to help promote T cell activation and prolong T cell survival *in vivo*. The major advantages to using CAR T cells are their high cytotoxicity toward targets and that they do not recognize antigen in the context of MHC, and can thus can be transferred into patients regardless of HLA haplotype. However, the downside of the high toxicity of CAR T cells toward cells expressing the target antigen is that lethal toxicity is a risk if the antigen is expressed by nontumor cells (187). Thus, the pool of antigens which can be targeted by CAR T cell therapy is restricted to TSAs. Also, because of their high cytotoxicity, the main toxicity involved in CAR T cell therapy in general is cytokine release syndrome (CRS). In CRS, activated CAR T cells release a high level of the cytokines TNF- α , IFN γ , IL-6, IL-2, IL-1 and IL-8 which can cause a range of symptoms, including vascular leak syndrome and death (188). Many clinical trials are ongoing to test the safety and efficacy of CAR T cells to several tumor

antigens, though it is clear that CAR T cells are much more effective at treating hematological malignancies than solid tumors (189). In fact, CD19 CAR T cell therapy is considered a breakthrough therapy due to its success in treating B cell malignancies (176,189).

1.4.3.2 Vaccination

The second approach to enhancing the antitumor T cell response is antigen or whole cell vaccination. The first step in vaccination is to identify antigens that are expressed by the tumor and elicit a CD8 T cell response. Multiple methods have been developed to identify such antigens, including Serologic Identification by Recombinant Expression Cloning (SEREX), cDNA library screening and sequencing followed by epitope prediction. This last method is the most clinically applicable method to quickly identify neoantigens in individual patients (143). It entails deep sequencing to identify mutations, computationally translating the mutations into proteins and inputting the predicted proteins into MHC I binding algorithms to identify MHC I binding epitopes (190).

Vaccination methods that have been developed for cancer immunotherapy include vaccination with tumor cells, RNA, DNA, peptides plus adjuvants or APCs pulsed with tumor antigens. Vaccination with allogeneic tumor cells relies on the patient's T cells to react to unknown tumor antigens expressed by the tumor cells. Canvaxin and Melcacine are two mixtures of melanoma lines with different HLA haplotypes that are currently being tested in clinical trials for melanoma. Canvaxin is also being tested with colon cancer, with the idea that colon cancer and melanoma share some tumor antigens (175). RNA vaccination is the second method which does

not necessitate the identification of antigens. For this strategy, total tumor RNA or mRNA is pulsed into autologous DCs, which are used for the vaccination. A phase I clinical trial with total renal RNA showed that RNA-transfected DCs stimulate renal tumor antigen specific CD8 T cell cells without inducing autoimmunity (191). DNA vaccination is a method being developed to attempt to elicit an immune response to a known antigen. In this case, cDNA of the gene of interest is cloned into an expression plasmid, which is then used for vaccination. APCs at the vaccination site take up the plasmid and present the epitope(s) to CD8 T cells, while the unmethylated bacterial DNA serves as an adjuvant. GM-CSF treatment has been shown to improve the efficacy of DNA vaccinations through promoting DC activity (192). However, DNA vaccination has failed to prove therapeutically beneficial so far. Peptide and protein can also be used for vaccination, either alone or pulsed into APCs. Peptide vaccination not only requires the identification of the antigen, but also that patients express the appropriate HLA allele on which the peptide is presented, which limits the applicability of this method. Protein vaccination, on the other hand, also requires the identification of the antigen, but is not restricted by HLA type. APCs can be pulsed with an individual protein/peptide that is known to form a tumor antigen, or with a cocktail of peptides/proteins that are specific to an individual patient's tumor mutations. This strategy is the only vaccination method which has been FDA approved; sipuleucel-T is the use of autologous peripheral blood mononuclear cells (PBMC) pulsed with a recombinant fusion protein of prostatic acid phosphatase and GM-CSF to treat prostate cancer (193). Additionally, prolonged survival and expansion of TSA specific T cells have been reported in clinical trials using individual or pooled TSAs (194,195).

Vaccination with TAAs has, however, yielded disappointing results due to the limitations mentioned above (143). Peptides and proteins can also be directly vaccinated.

However, since direct peptide and protein vaccination methods utilize an antigen(s) from a single protein, tumor resistance can be achieved by antigen loss tumor variants.

Peptides/proteins tested in clinical trials include gp100, MAGE-A3 and NY-ESO-1 (192).

In general, while vaccination as a monotherapy can stimulate an antigen-specific T cell response, the overall immune response is too weak to result in therapeutic benefit.

There is hope that combination therapy as well as the identification of more immunogenic antigens will improve therapeutic responses to vaccination.

1.4.4 NK Cell Therapy

Historically, the immune system was thought to play no role in influencing cancer growth because tumor studies using immunodeficient mouse models did not develop spontaneous tumors more frequently or exhibit reduced tumor growth over time compared to wildtype mice. These models, however, were not completely immunodeficient, and had functional NK cells, whose activity was providing that antitumor selection. Today, we know that NK cells are most effective against targeting hematological malignancies and metastases, and less efficient at infiltrating solid tumors (174).

Several different therapeutic strategies have been developed in order to improve NK cell cytolytic activity toward tumors. One mechanism by which tumor cells suppress NK cell function is through the secretion of TGF- β , which is the main immunosuppressive cytokine that negatively regulates NK cell function. To prevent tumor secreted TGF- β from suppressing tumor infiltrating NK cells, multiple TGF- β

inhibitors have been developed with one, galunisertib, currently being tested in a clinical trial (196). However, as mentioned above, cytokines have pleiotropic effects and thus TGF- β inhibition has a high degree of toxicity (174). Another strategy to remove NK cell inhibition is to block the inhibitory NK receptors, and two monoclonal antibodies (mAb) are being tested in clinical trials that bind to and block signaling through the KIR2DL1-3 (IPH2101) and NKG2A-CD94 (monalizumab) inhibitory receptors. Multiple approaches are also being developed to improve NK cell detection of tumor cells by the NK activating receptor CD16. First, mAb to tumor specific antigens (α -CD20, α -GD2, α -Her2, α -EGFR) are FDA approved and commonly used in the clinic to promote tumor cell apoptosis. They bridge tumor cells and NK cells together by concurrently binding to tumor cell signaling receptors and to CD16 on NK cells, thereby inducing ADCC of tumor cells (174). Bispecific and trispecific killer cell engagers are being developed pre-clinically as an alternative method to bridge together tumor antigens to CD16 on NK cells. Bispecific killer cell engagers are formed by fusing an Fv domain that binds to a tumor antigen with an Fv domain that binds CD16. Trispecific killer cell engagers contain a third Fv domain that binds to a different tumor antigen, the same tumor antigen or the IL-15 receptor (196). Two other NK activating receptors, NKG2D and NKp30, are promising targets in immunotherapy. In phase I and II clinical trials, treating melanoma and non-small-cell lung cancer patients with exosomes containing IL-15R α and NKG2D or NKp30 ligands prolongs progression-free survival (197,198).

Compared to CD8 T cells, NK cell ACT is in the early stages of development and clinical testing. NK ACT methods include adoptive transfer of autologous (from the patient) or allogeneic (from a donor) NK cells with none, short term or long term ex vivo

expansion and either IL-2, IL-15 or IL-18 treatment to maintain NK cell activation. The NK cell adoptive transfer method most tested in clinical trials is the use of short term ex vivo expanded allogeneic NK cells with systemic IL-2 treatment. The use of allogeneic NK cells is advantageous to autologous NK cells because cancer patients often have impaired NK cell functions which cannot be overcome by ex vivo expansion and activation (199). Autologous NK cells are also more inhibited by MHC expressing tumor cells due to inhibitory receptor matching. Indeed, adoptive transfer of autologous NK cells does not affect tumor growth in patients with metastatic melanoma or renal cell carcinoma (200). Adoptive transfer with haploidentical allogeneic NK cells, however, induces tumor regression and remission in patients with acute myeloid leukemia by targeting MHC-loss tumor cells (201,202). Haploidentical allogeneic NK cells are able to kill MHC mismatched tumor cells, and are therefore not dependent on tumor MHC loss for activity. As for cytokine treatment, IL-2 was found to activate Tregs as well as NK cells, making it less than ideal for use with NK ACT. Other cytokines that have been explored to replace IL-2 are IL-12, IL-15 and IL-21. IL-15 has emerged as the best cytokine for specifically improving NK cell function *in vivo* (174). Like with T cells, genetic modification of adoptively transferred NK cells is being explored to improve antitumor NK cell cytotoxicity. CD19-specific CAR NK cells show promise by enhancing adoptively transferred NK cell cytotoxicity against B cell malignancies in mice and clinical trials are currently underway to determine if there is therapeutic advantage with CAR NK cell treatment (203). The human NK cell line NK-92 is another source of donor NK cells. NK-92 cells lack expression of the inhibitory receptors and are highly cytotoxic to many tumor types *in vitro*, but not to healthy allogeneic cells (204). The use of NK-92

cells is advantageous to primary NK cells because they are immortalized, and therefore have unlimited expansion potential. The use of a cell line also eliminates all sources of variability originating from isolating and expanding NK cells from individual donors, thus yielding more reproducible results (205). Importantly, phase I clinical trials determined that transferred irradiated NK-92 cells do not develop into a tumor themselves and the body does not develop HLA antibodies in response to injected NK-92 cells (204,206). Overall, while several completed phase I clinical trials show that the discussed methods of NK cell adoptive therapy are safe and do not induce graft vs. host disease, it remains to be seen whether adoptive therapy of NK cells has therapeutic benefit.

1.4.5 Invariant NKT Cell Therapy

Invariant NKT (iNKT) cells are a small population of DN T cells that express an antigen-specific TCR, but instead recognize targets expressing lipid antigens presented by the nonclassical MHC molecule CD1d (207). In tumor immunity, iNKT mainly function by influencing other immune cells through cytokine secretion. iNKT cells produce IFN γ and IL-12, which promote Th1 differentiation and NK cell activation. iNKT knockout (KO) and adoptive transfer experiments show that iNKT cells are important for tumor protection (208,209). iNKT cells can also directly kill tumor cells expressing CD1d, though most tumor cells do not express this molecule (210). α GalCer, an iNKT agonist which activates iNKT cells, is the only NKT targeting immunotherapy. Multiple studies show that α GalCer treatment effectively protects against tumors and metastases in mice (211,212). Clinical trials have shown α GalCer treatment to be safe, though whether or not improving iNKT activity will be therapeutically beneficial still remains to be determined (207,213).

1.4.6 Radiotherapy and Chemotherapy

Radiotherapy and chemotherapies are often used in combination therapy with immunotherapies. This is because they not only directly kill cancer cells, but can also enhance tumor cell immunogenicity or improve the activity of immune cells. In fact, radiotherapy depends on the immune system for efficacy in treating tumors; mouse studies revealed that radiotherapy induces tumor regression in immunocompetent mice, but not in mice lacking T cells (214). There are two mechanisms by which radiotherapy and chemotherapies enhance the immune response to tumors: through immunogenic cell death or through the regulation of specific genes involved in tumor cell immunogenicity.

Radiotherapy or chemotherapy induced immunogenic cell death is the process by which inflammatory molecules like cytokines, chemokines, antigens and DAMPs are released upon cell death. The cytokines and chemokines attract immune cells like DCs, CD8 T cells, macrophages, Tregs and MDSCs to the tumor. The released tumor antigens and DAMPs activate the infiltrated DCs and NK cells. A DNA damage response is induced following irradiation or chemotherapy (cytarabine, cisplatin, 5-fluorouracil or bortezomib) treatment, which upregulates the expression of NKG2D receptor ligands on tumor cells, further promoting NK cell activation (215,216). Activated DCs migrate to the draining lymph node, where they prime T cells and promote CD8 T cell infiltration into the tumor microenvironment (217). Radiotherapy also activates type I IFN expression, which promotes DC activation and further attracts CD8 T cells to the tumor. However, prolonged type I IFN signaling promotes tumor cell survival mechanisms (217). In addition, radiotherapy recruits two pro-tumor immune cell types-

Tregs and MDSCs. Radiotherapy induced recruitment of MDSCs, however, is temporary and ultimately reversed (218). Tregs are not only recruited as a result of radiotherapy induced immunogenic cell death, but radiotherapy also promotes Treg expansion and function in the tumor microenvironment (219).

In addition to immunogenic cell death, there are a number of mechanisms by which radiotherapy and chemotherapy promote tumor cell immunogenicity. First, radiotherapy and high dose cyclophosphamide (CPA) temporarily induce lymphopaenia. Temporary lymphopaenia ultimately enhances the antitumor response because all the immunosuppressive immune cells are depleted. It is then possible for the reconstituted lymphoid cells to overcome tumor induced immunosuppression (220). Radiotherapy and 5-aza-2'-deoxycytidine (DAC) specifically enhance CD8 T cell activity by upregulating MHC molecule or CTA expression, which expand the repertoire of antigens in tumor cells (221,222). Irradiation also increases the pool of antigens by promoting protein degradation, by stimulating mTOR-dependent protein production, and by inducing the expression of previously unexpressed proteins (223). Paclitaxel, doxorubicin and methotrexate can stimulate DC expression of antigen processing genes and antigen presentation to T cells (224). In addition, paclitaxel, cisplatin and doxorubicin stimulate Man-6-P upregulation, rendering tumor cells more sensitive to Granzyme B (215). Radiotherapy upregulates FAS expression and DAC treatment restores sensitivity to apoptosis by reversing death-associated protein kinase (DAPK) hypermethylation (223,225). 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has several effects on the antitumor immune response, including enhancing CD4 T cell, macrophage and NK cell activities and promoting CD8 T cell infiltration into the tumor microenvironment (223).

Doxorubicin treatment enriches tumor associated macrophage populations and stimulates tumor infiltrated T cell proliferation (226,227). Paclitaxel promotes Th1 responses and suppresses Treg cytokine production (215). Drugs which enhance NK antitumor activities include lenalidomide, which increases the number of NK cells in the periphery and imatinib mesylate, which promotes DC-dependent NK cell activation and IFN γ production (174,228). Interestingly, imatinib mesylate also induces the expansion of IFN-producing killer DCs (IKDCs). IKDCs have properties of NK cells and DCs but can also lyse target cells and secrete IFN γ and have been shown to have antitumor functions *in vivo* (229). Immunosuppressive immune cells can be depleted by multiple chemotherapies. Gemcitabine and ranitidine deplete MDSCs, and T cell reactivation following MDSC depletion can effectively induce tumor regression (230,231). Gemcitabine also depletes Tregs and B cells and suppresses antibody production, though B cell depletion does not affect tumor growth (230,232). Low dose CPA depletes and inhibits Tregs by suppressing FOXP3 and GITR expression (233).

Because radiotherapy and chemotherapy stimulate an antitumor immune response, these treatments are ideal for combination therapy with immunotherapies. Indeed, radiotherapy combined with PD-1 or CTLA-4 checkpoint blockade have had major successes in the clinic (217). Lymphopaenia combined with CD8 T cell adoptive cell therapy has also shown great promise in a clinical trial (234). Gemcitabine in combination with cytokine treatment or vaccination increased the number of tumor antigen-specific CD8 T cells in tumors in other trials (223).

1.4.7 Conclusion

While immunotherapy has had major success at treating a subset of patients with specific cancer types, most solid tumor types are poorly responsive to immunotherapy treatment due to difficulty of tumor infiltration and poor tumor cell immunogenicity. Additionally, while these methods show promising results in mice, few of them have reproducibly induced therapeutic benefit in phase II clinical trials. Like with any cancer treatment, combination therapy targeting complementary aspects of the tumor immunoediting process is likely to enhance the antitumor effects of individual immunotherapies. New targets must be identified, therefore, in order to maximize the promising potential of combinatorial immunotherapy treatment.

Section 1.5: Epigenetics as a Target in Cancer Therapy

Tumor cells frequently downregulate MHC molecules, antigen processing genes and NK receptor ligands to avoid detection by immune cells. In order to enhance tumor immunogenicity, these changes in gene expression must be corrected. Several approaches have been proposed to correct abnormal gene expression in tumors. First, by changing the DNA sequence, one can attempt to correct abnormal gene expression (235). Correcting DNA sequences in a patient's tumor cells is certainly plausible but is not yet a practical approach to correcting abnormal gene expression. Alternatively, abnormal gene expression can be corrected by altering any one of several posttranslational modifications found on the chromatin of tumor cells that are important for gene expression (236). In contrast to genetic regulatory mechanisms that are stable, posttranslational modifications on chromatin are dynamic, and in theory, reversible. Because posttranslational modifications on chromatin are required for regulating pro-cancer gene expression in tumor cells, do not change the DNA sequence, are in theory

reversible, and are established by the actions of enzymes, this makes them attractive targets to correct abnormal gene expression as a means of therapy.

Targeting epigenetic regulators for the treatment of human cancers has proved successful for histone deacetylases (HDAC) and DNA methyltransferases (DNMT). In each case, the therapeutics developed are approved for difficult to treat cancers, providing valuable assets to oncologists (237,238). Examples include: vorinostat and romidepsin, FDA-approved HDAC inhibitors (HDACi) used to treat cutaneous T cell lymphoma, and azacitidine and decitabine, FDA-approved DNMT inhibitors (DNMTi) for the treatment of myelodysplastic syndromes (239,240). Next generation, more specific HDAC and DNMT inhibitors are currently being developed to improve on these successes. Because of these successes, many other small molecules are actively being pursued which target posttranslational modifications on chromatin for therapeutic benefit. These drugs primarily target a large number of histone modifying enzymes and small RNA regulatory pathways. Histone modifying enzymes currently being targeted include deacetylases, acetyltransferases and demethylases. In some cases, small molecules targeting these regulators have made it into Phase II clinical trials (236,237,239,240). These molecules are tested alone and in combination chemotherapy and include HDAC inhibitors (entinostat, mocetinostat, panobinostat, bellinostat, valproic acid, givinostat, CHR-3996, CHR-2845, and SB939), the KDM1A demethylase inhibitor tranilcypromine, and the HAT inhibitor curcumin (236,237,239,240). Phase II trials revealed promising results for mocetinostat and bellinostat.

Section 1.6: Epigenetics in Cancer Biology

Childhood cancers have the highest mutation load in epigenetic machinery. However, mutations or changes in expression of epigenetic modifiers occur in a broad range of tumor types. Alterations in DNA methylation is most common in haematological malignancies and is due to frequent mutations in DNA methyltransferases and methylcytosine dioxygenase enzymes like DNMT3A, DNMT1, TET1, TET2, TET3, MBD1 and MBD3 (241). DNMT3A and TET2 mutations are common in lymphomas and leukemias and are correlated with poor prognosis (241-244). Changes in DNA methylation patterns induced by DNMT3A and TET2 mutations promote the biology of cancer stem cells (241). Additionally, a common phenomenon in cancer is global DNA hypomethylation and promoter CpG hypermethylation. Alterations in histone modifications are also typical in many tumor types, hematological and solid. For example, EZH2, a histone methyltransferase enzyme, exhibits both gain of function and loss of function mutations, depending on cancer type, which leads to either hyper- or hypo- histone methylation, respectively. Mutations in another histone methyltransferase MLL2 causes elevated levels of repressive H3K27me3 in non-Hodgkin lymphomas (245,246). SETD2 mutation is correlated with poor prognosis in renal cell carcinoma (247). Mutations in the histone acetyltransferase CBP/p300 affect tumor cell proliferation and DNA stability (248). Other histone modifiers mutated in cancer include MLL, MLL3, MLL4, SETD1A, PRDM9, NSD1, NSD2, KDM5C, KDM6A, KDM2B, EP300, HDAC2, HDAC4, and HDAC9 (241).

Section 1.7: Epigenetics

Chromatin is composed of nucleosomes, which in turn are composed of two copies of each of the canonical histones H2A, H2B, H3, and H4 wrapped by ~150 bp of

DNA (249). Nucleosomes are positioned along DNA in a “beads on a string” configuration to create a 10-nm fiber that through internucleosome contacts creates a variety of higher ordered chromatin structures, including a molten globule with short stretches of a 30-nm fiber (250,251).

In most cases, the highly compacted chromatin structure limits DNA accessibility, and therefore must be modified to promote essential nuclear processes like transcription, DNA replication, and DNA repair (252). In higher eukaryotes, posttranslational modification regulates the function of chromatin. These diverse post-translational modifications are commonly, but not accurately, referred to as epigenetic regulatory mechanisms (253). Posttranslational modifications to chromatin do not change the DNA sequence but rather are deposited onto chromatin by enzyme catalyzed reactions (254). These modifications include histone posttranslational modifications, DNA methylation, incorporation of histone variants, and chromatin remodeling (254). These alterations arise and are subsequently maintained on chromatin through interactions between the enzymes that deposit them and sequence-specific DNA-binding factors (255).

1.7.1 Histone Modifications

The posttranslational modification of histones occurs by histone modifying enzymes. And just as histones can be modified, the modifications can be removed by de-modifying enzymes. Histones are primarily modified on the N-terminal and C-terminal ends, but can also be modified throughout their sequence (255). Histone modifications alter chromatin structure or recruit additional chromatin-modifying complexes, which in turn regulate nuclear processes like transcription. Common

modifications include acetylation, methylation, and phosphorylation. Histone modifications are usually found in combinations in chromatin (e.g., H3K9me1 with H3K4me3) so as to provide redundancy in their ability to direct nuclear processes (256).

1.7.2 Histone Variants

To similar effect, the incorporation of histone variants serves to alter chromatin structure and stability, and it provides unique contact surfaces for the recruitment of chromatin-modifying complexes (257). The incorporation of histone variants can occur through both ATP-dependent and ATP-independent mechanisms. ATP-independent mechanisms include histone chaperones dedicated to the deposition of specific histone variants. Histone chaperones support replication dependent (coupled with DNA replication during S-phase) and independent (outside S-phase) histone deposition pathways. Examples include the ATRX/DAXX pathway for the deposition of histone H3.3 and the HJURP pathway for the deposition of CENP-A (257).

1.7.3 DNA Methylation

Just like histones, DNA can be modified to provide a stable method of regulating gene expression. In mammals, modifications to DNA usually occur on CpG dinucleotides, but they have also been observed outside of this context (258,259). The most common modification to CpG dinucleotides is the methylation of the fifth carbon of cytosine (5mC), catalyzed by the DNMT family of enzymes. 5mC is removed by both passive and active means, the latter including the TET family of enzymes and to a lesser extent APOBEC (260). DNA methylation largely represses gene expression by

altering nucleosome positioning and stability and by recruiting chromatin-modifying complexes containing subunits that bind methylated DNA (261,262).

1.7.4 Chromatin Remodeling Complexes

Chromatin remodeling is the process whereby the position, occupancy, or the histone composition of a nucleosome is altered in the chromatin. These activities occur by both ATP-dependent and ATP-independent mechanisms (263). ATP-independent mechanisms can occur from transcription factors shifting the position of nucleosomes as a consequence of DNA binding, or by the action of histone chaperones, depositing or removing histones from chromatin (264,265). In contrast to ATP-independent mechanisms, ATP-dependent mechanisms are enzymatic and constitute the majority of the remodeling activity in the cell (266). These activities are usually catalyzed by multisubunit ATP-dependent chromatin remodeling complexes which are organized into SWI/SNF, ISWI, CHD, and INO80 families based on the sequence homology of the incorporated ATPase (267). Each family of ATPase catalyzes distinct remodeling activities: incremental nucleosome sliding on DNA in cis, the creation of DNA loops on the surface of the nucleosome, eviction of histone H2A/H2B dimers, eviction of the histone octamer, or the exchange of histone octamer subunits within the nucleosome to change its composition. Each of these activities alters the accessibility of DNA in the chromatin to DNA-binding factors, which in turn regulates essential nuclear processes like transcription, DNA replication, and DNA repair.

Complexes in the SWI/SNF family include the large multisubunit BAF, PBAF, and WINAC complexes, which function as coregulators of transcription and in aiding of the repair of DNA damage (268). Remodeling reactions catalyzed by SWI/SNF family

members include simple nucleosome sliding reactions, but they can catalyze more dramatic reactions, like creating DNA loops on the surface of nucleosomes or the eviction of H2A/H2B dimers from the nucleosome structure (269).

Like SWI/SNF, the members of the INO80 family of complexes are also large multisubunit complexes which regulate transcription, but they have more prominent functions in DNA damage repair (270). These complexes, including SCRAP, Tip60/p400 and INO80, are unique among ATP-dependent remodeling complexes because they can catalyze the exchange of histones in the nucleosome (271-275).

The CHD family contains nine different ATPases, the most numerous of the remodeling families (276). The best characterized complex in this family is NURD, which contains both ATP-dependent chromatin remodeling and histone deacetylase activities (277). The MBD2-NURD complex can bind 5mC DNA and promote the repression of genes through its remodeling and histone deacetylase activities and the MBD3-NURD complex functions as an activator of transcription (278,279). In addition, a NURD-like complex containing CHD5 has been identified in the brain and acts as a coregulator of transcription (280). However, most of the complexes containing other CHD ATPases await characterization.

As opposed to the large SWI/SNF, INO80, and CHD complexes, most ISWI complexes are comparatively small, consisting of only two or three subunits (281). At a minimum, each of these complexes contains a single large subunit with several histone-binding domains (including PHD and bromodomains) and an ISWI family ATPase. ISWI family chromatin remodeling complexes catalyze the sliding of nucleosomes in short increments without DNA looping, histone exchange, or nucleosome displacement.

These complexes have diverse functions including the spacing of nucleosomes after DNA replication (CHRAC, ACF), RNA polymerase elongation (RSF), serving as coregulators of transcription (CERF, NURF, NoRC, b-WICH), and regulating DNA damage repair (WICH) (282-289).

Recruitment of nucleosome remodeling complexes commonly occurs through direct interactions with transcription factors, histone modifications, and DNA sequences (290). Domains found in nucleosome remodeling complex subunits that recognize specific histone modifications include a variety of well-characterized plant homeodomain (PHD), chromo, and bromodomains, among others. The incorporation of individual subunits into chromatin remodeling complexes varies based on cell type expression, thus providing a means to generate distinct complexes with unique interaction surfaces (291).

The biological functions of chromatin remodeling complexes during normal development have been extensively studied using mouse models (291). As expected, the subunits of many chromatin remodeling complexes have essential functions during mammalian development. However, because subunits of chromatin remodeling complexes are often found in multiple chromatin-associated complexes, understanding how specific chromatin remodeling complexes function during development has remained a challenge. As such, the deletion of a single subunit likely compromises several complexes resulting in pleiotropic effects. In somewhat of a surprise, several ATPases are not essential for the cell: SWI/SNF family members BRG1 and BRM, CHD family member CHD4, and ISWI family member SNF2L (292-295). The importance of chromatin remodeling complexes for development, but not necessarily for cell viability,

suggests that their mutation can result in a viable cell with abnormal developmental pathways. Because deregulated developmental pathways are known to contribute to the transition to cancer, it is likely that the mutation of subunits in chromatin remodeling complexes could contribute to cancer-related phenotypes (1,236).

1.7.4.1 Chromatin Remodeling Complexes in Cancer Biology

Chromatin remodeling complexes are known to be mutated and deregulated in expression in a variety of cancer types. As an exercise to quantify the mutation frequency for each chromatin remodeling complex subunit from a wide array of tumors, we mined the COSMIC database of somatic mutations in human cancers (296).

Consistent with what was published by others, we observed that subunits of the SWI/SNF family of remodeling complexes are the most prominently mutated of the chromatin remodeling complex families (Fig. 1.1)(Table 1.1) (297,298). Mutations in the INI1 (also known as SNF5) and ARID1A (also known as BAF250A) components are frequently observed in tumors from diverse tissues including central nervous system, stomach, large intestine, bone, endometrium, liver, soft tissue, ovary, and urinary tract. Additional SWI/SNF subunits include the BRG1 ATPase, which is frequently mutated in tumors from ganglia and to a lesser extent the esophagus, large intestine, lung, and urinary tract. The PBAF-specific BAF180 (also known as PBRM1) and ARID2 subunits are frequently mutated in kidney cancer and to a lesser extent in a variety of tumors from the skin, esophagus, large intestine, cervix, liver, and lung. Interestingly, several of the relatively uncharacterized CHD ATPases were found modestly mutated in a variety of cancers including the stomach, large intestine, cervix, endometrium, lung, and urinary tract.

Figure 1.1

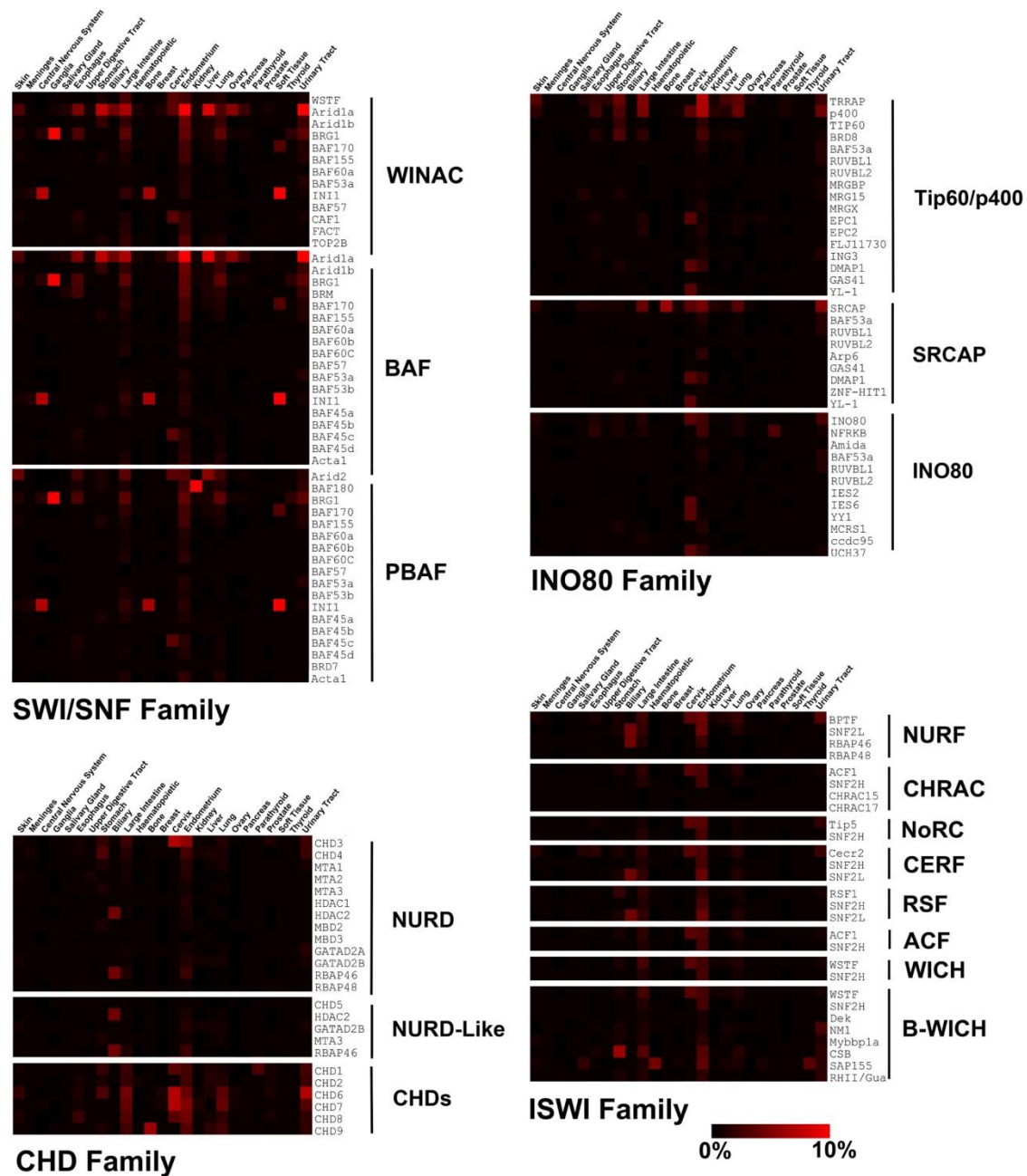


Figure 1.1: Heat map representation of somatic mutation rate for subunits of chromatin remodeling complexes in human cancers. The COSMIC database was queried using each of the subunits found in the chromatin remodeling complexes. The frequency of somatic mutation is expressed as a percentage of sequenced tumors from each tissue type. Percentages are not corrected for background mutation rates which vary with individual tumors and tumors from tissue types. The heat map range is shown on the lower right and varies from 0% to 10% of sequenced tumors in the COSMIC database.

Table 1.1

Remodeling family	Subunit	Mutated cancer tissue types	Complexes
SWI/SNF family	WSTF	Large intestine, cervix, endometrium	WINAC, b-WICH, WICH
	ARID1A	Skin, esophagus, stomach, biliary tract, large intestine, cervix, endometrium, liver, ovary, urinary tract, lung	WINAC, BAF
	BRG1	Autonomic ganglia, esophagus, lung, urinary tract, large intestine, endometrium	WINAC, PBAF, BAF
	ARID2	Skin, esophagus, large intestine, cervix, endometrium, liver, lung	PBAF
	BAF180	Endometrium, kidney	PBAF
INO80 family	SRCAP	Large intestine, cervix, bone, endometrium, lung, urinary tract	SRCAP
CHD family	CHD3	Cervix, endometrium	NURD
	CHD4	Stomach, endometrium	NURD
	CHD7	Large intestine, cervix, endometrium, lung, urinary tract	CHD7
	CHD8	Large intestine, cervix, endometrium	CHD8
ISWI family	Tip5	Cervix, endometrium	NoRC
	SAP55	Hematopoietic and lymphoid tissue, endometrium, thyroid	b-WICH

Table 1.1: Summary of chromatin remodeling subunits identified with probable or potential driver somatic mutations in human tumors. Subunits of chromatin remodeling complexes whose somatic mutation was identified as a probable or potential driver of human cancer are shown. The tissues in which the gene encoding the subunit was mutated in greater than 5% of the sequenced tumors from the COSMIC database are shown. Known chromatin remodeling complexes that each subunit can assemble into are also listed.

In addition to somatic mutation rates, the abnormal expression of genes in cancers can indicate roles in tumor development. Toward this end, we surveyed genomewide expression datasets from primary tumors using ONCOMINE to determine the frequency of deregulation for each subunit for chromatin remodeling complexes (Fig. 1.2; Table 1.2) (299). Consistent with their frequent somatic mutation in tumor samples, SWI/SNF family members are frequently deregulated in expression in tumors, thus underscoring their likely importance to cancer biology. The BRG1 ATPase, BAF155, BAF53A, and INI1 are overexpressed in a variety of tumor types including bladder, liver, ovarian, melanoma, leukemia, and myeloma cancers. In addition to the prominent representation of SWI/SNF subunits, subunits from the ISWI, CHD, and INO80 complexes are also deregulated. Subunits of the INO80 family of chromatin remodeling complexes including BAF53A, RUVBL1, and RUVBL2 subunits are overexpressed in bladder, cervix, myeloma, colon, and ovarian cancers. The NURD subunits MTA1 (but not MTA2 or 3), HDAC 1 and 2, and pRBAP48 are modestly deregulated in cervix, pancreatic, leukemia, colon, and esophagus cancers. Compared to the other families of remodeling complexes, the ISWI families are only modestly deregulated in cancers including overexpression in head, cervix, and kidney cancers. In support of these genome-wide analyses, independent reports in the literature have accumulated for several decades and have been used to establish correlative (germline and somatic mutation, deregulated expression, copy number changes), causative (mouse models), and mechanistic (physical and functional connections to cancer relevant pathways) connections between chromatin remodeling complexes and cancers.

Figure 1.2

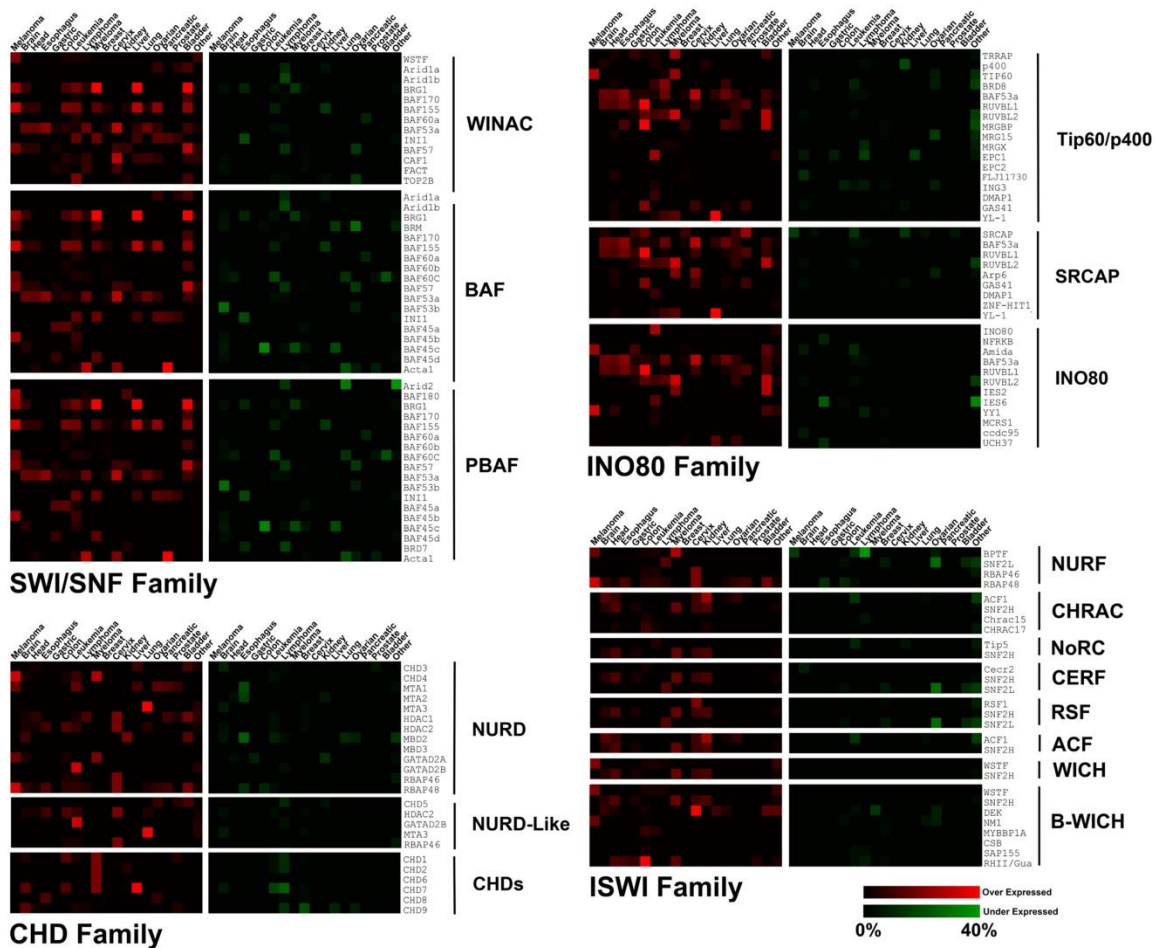


Figure 1.2: Heat map representation of gene expression changes for subunits of chromatin remodeling complexes in human cancers. The ONCOMINE database was queried using each of the subunits found in the chromatin remodeling complexes. Data is expressed as a percentage of unique analyses for which transcript levels of the subunit differ by greater than twofold between tumor and control normal tissue ($P \text{ value} \geq 1.0 \times 10^{-4}$). Transcripts for which overexpression was observed in tumor tissue are colored in red, and those where an underexpression is observed are shown in green. The heat map range is shown on the lower right and varies from 0% to 40% of unique analyses found in the ONCOMINE database.

Table 1.2

Remodeling family	Subunit	Overexpressed	Underexpressed	Complexes
SWI/SNF family	CAF1	Cervix		WINAC
	BAF180	Melanoma		PBAF
	Arid2		Lung	PBAF
	Acta1	Lymphoma, pancreatic		PBAF, BAF
	BAF45b	Melanoma		PBAF, BAF
	BAF53b		Brain	PBAF, BAF
	BAF45c		Colon	PBAF, BAF
	BAF155	Melanoma, liver		WINAC, PBAF, BAF
	BAF57	Bladder		WINAC, PBAF, BAF
	BRG1	Melanoma, myeloma, liver, bladder		WINAC, PBAF, BAF
	BRM		Ovarian	WINAC, PBAF, BAF
	BAF53a	Cervix		WINAC, PBAF, BAF, Tip60/p400, SRCAP INO80
INO80 family	SRCAP	Pancreatic		SRCAP
	Amida	Melanoma		INO80
	IES2	Bladder		INO80
	IES6		Esophagus	INO80
	INO80	Leukemia		INO80
	YY1	Melanoma		INO80
	EPC1	Leukemia		Tip60/p400
	MRGBP	Colon, bladder		Tip60/p400
	TIP60	Melanoma, myeloma		Tip60/p400
	TRRAP	Myeloma		Tip60/p400
	YL-1	Liver		Tip60/p400, SRCAP

Table 1.2: Summary of chromatin remodeling subunits identified with frequent deregulated expression in human tumors. Subunits of chromatin remodeling complexes whose expression is deregulated in at least 20% of the unique analyses from each tissue type from Fig. 1.2 are listed. Known chromatin remodeling complexes that each subunit can assemble into are also listed.

Table 1.2

Remodeling family	Subunit	Overexpressed	Underexpressed	Complexes
INO80 family	RUVBL2	Myeloma, bladder		INO80, Tip60/p400, SRCAP
	RUVBL1	Colon		INO80, Tip60/p400, SRCAP
CHD family	CHD4	Melanoma, myeloma		NURD
	MTA3	Lung		NURD
	MBD2		Esophagus	NURD
	GATAD2B	Leukemia		NURD-Like
	CHD7	Liver	Lymphoma	CHD7
ISWI family	BPTF	Myeloma	Lymphoma	NURF
	DEK	Cervix		b-WICH
	DDX21	Colon		b-WICH
	ACF1	Kidney		CHRAC, ACF
	RBAP48	Melanoma		NURF, NURD
	SNF2L		Ovarian	NURF, RSF, CERF

Table 1.2: Summary of chromatin remodeling subunits identified with frequent deregulated expression in human tumors. Subunits of chromatin remodeling complexes whose expression is deregulated in at least 20% of the unique analyses from each tissue type from Fig. 1.2 are listed. Known chromatin remodeling complexes that each subunit can assemble into are also listed.

BRG1, a catalytic ATPase of the SWI/SNF family, has well-characterized roles in regulating several tumor suppressor and oncogene pathways, including regulating cell cycle arrest, metastasis and p53-dependent regulation of DNA damage (300-304). Further, mouse models have demonstrated that tumor incidences are increased in heterozygous BRG1 KO mice (305). The use of mouse models has presented strong evidence that INI1 is a tumor suppressor. As in humans, mice heterozygous for INI1 develop sarcomas closely resembling the human malignant rhabdoid tumors (306,307). The SWI/SNF subunit ARID1A (also known as BAF250A) is frequently mutated in several types of human cancer. In some tumor types, ARID1A is the most frequently mutated gene (308,309). BAF180 has been shown to act as a tumor suppressor through functioning to inhibit the cell cycle and promote senescence (310,311). Possibly as a result of its interactions with p53, BAF180 has also been shown to regulate genome stability through functions in regulating DNA damage repair (312). BAF57 truncation mutations found in human cancers were shown to lead to artificially elevated ER- and androgen receptor (AR)-regulated gene expression and provide a plausible molecular mechanism for their functions in regulating prostate and breast cancer cell biology (313).

The Tip60/p400 subunit TRRAP of the INO80 family is an essential factor for MYC transformation activity and through interaction with acetylated p53, it is essential for p53 regulation of gene expression (314-316). Another INO80 subunit, RUVBL1, is also necessary for transformation by E1A, β -catenin, and MYC (317-319).

The CHD4 subunit interacts with EGR1, which regulates cell growth, differentiation and apoptosis in prostate cancer and with ZIP, which is a transcriptional

repressor of genes involved in cell proliferation, survival, and migration (320,321). Similar to the INO80 complex subunits, MTA1 is required for MYC-induced transformation (322). MTA1 also interacts with the BCL11B repressor in T cell leukemia cells and ER α in breast cancer cells to regulate gene expression (323,324). Genes important to metastasis are also known targets of MTA1 regulatory activity. MTA1, recruited to the E-cadherin and BRCA1 promoters, silences the genes and in doing so promotes metastasis (325,326). Knockdown (KD) of HDAC1 and HDAC2 in several human cancer cell lines results in upregulation of p21 followed by cell cycle arrest both in tissue culture and in xenograft tumor models (327). MBD2 has important functions in the development of cancers through its ability to recruit the coregulatory activities of the NURD complex to the hypermethylated promoters of tumor suppressor genes (328-333). When the NURD and NURF subunit RBAP46 is overexpressed in breast cancer cell lines both colony formation *in vitro* and tumor growth in mice is suppressed (334). CHD5 was found to be one among several tumor suppressor genes found on chromosome 1q36, which is the most common deletion found in a variety of human cancers, most prominently cancers of the CNS, hematopoietic system, and epithelium (thyroid, colon, cervix, and breast) (335).

RSF1 of the ISWI family is located on chromosome 11q13.5, which is frequently duplicated in human tumors. In oral squamous cell cancers, RSF1 was found to be overexpressed relative to normal tissue, which correlates with decreased survival (336). In addition, a short hairpin RNA (shRNA) KD screen showed that the RSF1 gene located in the duplicated region is essential for paclitaxel resistance (337).

1.7.4.2 Chromatin Remodeling Complexes as a Target in Cancer Therapy

The discovery that chromatin remodeling complexes regulate gene expression important for cancer progression has stimulated interest in screening them for small molecule regulators. Using completely different approaches, two successful screens have been performed for the BRG1 and RUVBL1 ATPases (338,339). To discover small molecule regulators of BRG1, a live cell screen was performed by measuring the expression of Bmi, a known target of BRG1 in embryonic stem cells, by high-throughput qRT-PCR. Follow-up assays were performed using embryonic stem cell lines with a knock-in Bmi-luciferase reporter allele. Several compounds were discovered with this approach and are being investigated further. In contrast to the live cell approach used for BRG1, a structure-based biochemical screen was performed to discover small molecule regulators for the RUVBL1 ATPase. To identify inhibitors of the RUVBL1 ATPase activity, the authors used its known structure to model molecules from several libraries to the ATP-binding site. Promising compounds that could bind the ATP-binding site were assayed for the ability to inhibit a DNA-dependent ATPase activity for recombinant RUVBL1. This approach yielded several compounds, some of which inhibited the proliferation of cancer cells.

In addition to discovering small molecules targeting the ATPase activity, as was performed for RUVBL1, resources can be invested to discover molecules to inhibit key protein–protein interactions essential for the function of chromatin remodeling complexes. The inhibition of the BAF57–AR interaction is a successful example of this strategy. BAF57 is known to physically interact with AR, and is required for AR binding to chromatin and AR-regulated gene expression in prostate cancer cells. The ectopic

expression of a peptide mimic to the sequence on BAF57 that interacts with AR resulted in inhibited AR binding to chromatin, reduced AR-stimulated transcription, and inhibited AR-dependent prostate cancer cell proliferation (340). These results are consistent with the possibility that the peptide is inhibiting the BAF57–AR interaction surface and preventing BAF57-dependent AR binding and transactivation. Additional means to inhibit chromatin remodeling complexes with this approach can also include targeting essential subunit interactions within a remodeling complex. While this approach benefits from the focus of a structure-based design, it will suffer from the difficulty of delivering peptides to intracellular targets in a patient's tumor.

Another strategy to therapeutically regulate chromatin remodeling is to identify small molecules that can regulate the expression of genes encoding essential subunits of chromatin remodeling complexes. This has successfully been done to re-express the BRM ATPase in cancer cell lines that have silenced its expression without a deletion (341,342). This screen used a glucocorticoid receptor-regulated luciferase reporter that is induced in the presence of BRM. As a result of this screen, two compounds were identified which increased the expression of BRM, inducing the expression of BRM target genes, and decreased the growth of cancer cell lines. Each of these compounds regulates BRM expression through an unknown mechanism. Additionally, ¹⁹fluorine nuclear magnetic resonance (¹⁹F NMR) small molecule screening identified an inhibitor to the NURF subunit BPTF, AU1 (343).

1.7.4.3 The NURF Complex

The nucleosome remodeling factor (NURF) is the founding member of ISWI family of chromatin remodelers. It was first identified in *Drosophila melanogaster* and

subsequent purification human cells revealed that NURF is conserved across species (286,344). Mammalian NURF is composed of three subunits: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SNF2L) or member 5 (SNF2H), RB binding protein 4, chromatin remodeling factor (pRBAP46/48) and bromodomain containing phd finger transcription factor (BPTF) (Fig. 1.3). SNF2L/H is the catalytic ATPase domain, which is also incorporated into the RSF1 and CERF complexes. It contains the C-terminal HAND, SANT and SLIDE domains, which interact with unmodified histone H4 N-terminal tails and linker DNA (345). Mammalian NURF preferentially incorporates SNF2L over SNF2H. The pRBAP46/48 proteins are members of a family of WD repeat proteins, proteins which have a highly conserved propeller-like structure. Besides NURF, the pRBAP46/48 subunit is also incorporated into the NURD and NURD-like complexes (346). BPTF is the largest subunit in the NURF complex and is both unique to the NURF complex and essential for NURF remodeling activity (286). BPTF contains several domains including a bromodomain, two PHD domains, a high mobility group (HMGA) domain, three LXXLL putative nuclear receptor-binding motifs, a DNA binding homeobox and different transcription factors (DDT) domain and a polyglutamate region (Fig 1.4). The functions of most of these domains have been characterized, and thus aid in our understanding of NURF recruitment to and interaction with chromatin. The HMGA domain contains an acidic patch which associates with nucleosomes and the C-terminal PHD2 and bromodomain motifs recognize the histone modifications H3K4me2/3 and H4K16, respectively (347-349). These modifications are enriched at the transcription start site of transcriptionally active genes. The LXXLL motifs are important for interacting with other proteins and the

Figure 1.3

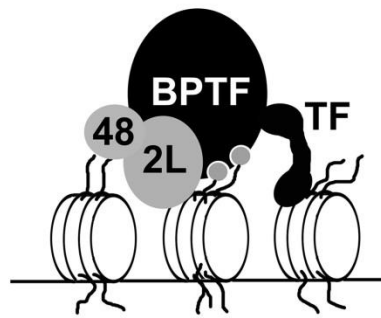


Figure 1.3: Cartoon of NURF. Subunits of NURF (BPTF, 2L, 48) bound to chromatin. Gray circles, histone modifications; TF, transcription factor.

Figure 1.4



Figure 1.4: BPTF is highly conserved between mouse and human. Red: acidic patch, orange: DDT domain, yellow: PHD finger, green: glutamine-rich domain, purple: bromodomain

DDT domain likely interacts with DNA (345). In the mouse, BPTF is most highly expressed in the testis, spleen, brain and kidney tissues, with expression significantly declining after birth (286,350). NURF's remodeling activities are restricted to sliding nucleosomes bi-directionally in 10 bp increments with minimal unwrapping of DNA from the nucleosome.

NURF is recruited to chromatin by a combination of histone modifications, histone variants and transcription factors. While several remodeling complexes have conserved functions across cell types, NURF is a cell type-specific regulator of chromatin, likely through interaction with transcription factors that are cell type-restricted in expression (351). NURF's interaction partners have been best characterized in *Drosophila melanogaster*, where it is known to interact with a variety of transcription factors including heat shock factor (HSF), GAGA, VP16, TRF2, TAF4b and PR (352,353). Mammalian NURF interacts with the SMAD2, SRF, AP-1, PR, USF1, MAZ and KEAP1 transcription factors, among others (345). NURF also interacts with another broad regulator of chromatin structure, CCCTC-binding factor (CTCF). CTCF interacts with cohesin to create loops in chromatin, allowing distal regulatory elements to regulate their target genes. NURF was shown to both localize to CTCF binding sites in chromatin and to interact with CTCF itself (351). NURF also preferentially remodels nucleosomes incorporating the H2A.Z histone variant, which is enriched at regulatory elements and transcription start sites of active genes (354). It is also possible that NURF binds to specific DNA sequences, though a consensus sequence has yet to be identified.

NURF has essential functions in mouse embryogenesis but is not essential for adult cell viability (350,355). BPTF KO mice are embryonically lethal due to crucial

functions for BPTF in regulating the formation of the distal visceral endoderm through coregulation of Smad signaling (350). Critical functions for BPTF have also been shown in the ectoplacental cone during trophoblast differentiation and BPTF KO mice fail to gastrulate (356). In addition, several groups found that NURF regulates homeobox gene expression in embryonic stem cells (286,350,357). Multiple functions for BPTF have been identified in the adult mouse, including in thymocyte, erythrocyte and melanocyte differentiation, and oocyte development. A conditional BPTF KO study discovered that thymocytes fail to differentiate beyond the SP state into DP thymocytes without NURF activity. The thymocyte differentiation genes *Egr1*, *AP-1* and *SRF* were identified as a BPTF regulated by either gene expression or protein function (355). In erythroid cells NURF is enriched at promoters of erythroid genes and NURF binding is associated with open chromatin and gene expression during erythroid differentiation (358). In adult melanocyte stem cells, BPTF and microphthalmia-associated transcription factor (*MITF*) coregulate genes important for melanocyte differentiation and BPTF KD results in transcriptional repression of these genes and defects in differentiation into melanocytes (359). In addition, NURF interaction with PR recruits it to hormone response elements in the MMTV promoter. NURF remodeling activity at the MMTV promoter creates a nucleosome free region, allowing the transcription factor NF1 to bind and stimulate transcription (360). SNF2L also interacts with PR to regulate *StAR* expression in granulosa cells, indicating a role for NURF in oocyte development (361).

1.7.4.3.1 The NURF Complex in Cancer Biology

Two reports document that SNF2L expression is important for the growth of human cancer cell lines. In these studies, SNF2L was found to be expressed in a wide

variety of normal tissues, tumors, and cancer cell lines. KD of SNF2L in several cancer cell lines resulted in reduced cell proliferation, arrest in G2/M, increased DNA damage and activation of DNA damage repair pathways (phosphorylation of CHK1/2, ATM, and p53), and apoptosis (362). Follow-up studies identified SNF2LT as a splice variant of SNF2L that lacks its HAND–SANT–SLIDE domains. In these follow-up studies, they observed that KD of SNF2LT or the full-length SNF2L gene resulted in similar cancer cell-specific phenotypes (363). Similar KD studies have shown that SNF2L is an essential regulator of the Wnt signaling pathway (364). In these experiments, contrary to previous SNF2L studies, SNF2L depletion increased proliferation and chemotaxis. This study used genome-wide gene expression profiling to discover that many targets of the Wnt signaling pathway are upregulated with SNF2L KD.

BPTF resides on 17q and is frequently duplicated in several cancer types, with the greatest frequency in neuroblastoma (365). A nonreciprocal translocation of BPTF on 17q was characterized in a human lung cell line following continuous culturing (366). This translocation resulted in increased BPTF expression and correlated with increased cell proliferation and KD of BPTF in these cells reduced proliferation. Consistent with the frequent duplication of 17q in human tumors, the BPTF gene was found to be amplified in various human tumors, especially neuroblastomas and lung cancers and many human cancer cell lines. Whether BPTF duplication provides an advantage to cancer cells, or is just a benign consequence of the 17q duplication, needs to be specifically addressed. High expression of BPTF in colon cancer and hepatocellular carcinoma is associated with high expression of epithelial-to-mesenchymal (EMT) markers and poor prognosis (367,368). Several genome-wide and exome studies have

identified mutations in BPTF to be associated with lung, bladder and BRCA1+ breast cancer (369-371). In these studies, KD of BPTF in bladder cancer cells reduced colony formation *in vitro*. In another bladder cancer study, H2A.Z overexpression and elevated incorporation into nucleosomes recruits BPTF, which promotes the expression of normally repressed genes. This contributes to the genomic instability of bladder cancer cells and contributes to the expression of oncogenes, like cyclin D1 (CCND1) (372). In pancreatic cancer, BPTF interacts with the oncogene c-MYC and helps recruit it to its gene targets. This interaction is necessary for c-MYC dependent proliferation and replication stress. The authors further showed that BPTF KO reduced pancreatic tumor growth in mice and prolonged survival (373). BPTF regulates many cell cycle genes in lung adenocarcinoma and BPTF KD suppresses proliferation (374). In melanoma, BPTF promotes both metastasis and proliferation and high BPTF expression correlates with poor prognosis in a study of melanoma patients. In this study, BPTF was found to promote the MAPK pathway, which regulates the cell cycle genes BCL2, BCL-XL and CCND2. Additionally, BPTF expression lends resistance to BRAF inhibition therapy (375). Other BPTF regulated genes involved in tumor cell biology include twist, N-cadherin, E-cadherin and MHC class I and II genes (350,351,355,367). Because NURF regulates genes involved in multiple hallmarks of cancer, including tumor cell growth, invasion and immunogenicity, we sought to understand the role of NURF in breast cancer and melanoma using mouse models.

Section 1.8: Mouse Tumor Models

Three mouse mammary carcinoma cell lines are used in this study: 4T1, 66cl4 and 67NR. These three lines were isolated by sequential dissemination from a single

spontaneous tumor harvested from a BALB/cfC₃H mouse (376). The 4T1 subpopulation is highly metastatic through the blood to the liver, lung, bone, lymph nodes and brain, the 66cl4 subpopulation is metastatic through the draining lymph node to the liver and lung and the 67NR subpopulation is nonmetastatic (376,377). All three lines are triple negative for hormone receptor expression and the 4T1 model is frequently used in breast cancer research as it is a good model for advanced stage human breast cancer (378,379). The fourth tumor line used in this study is the mouse melanoma B16F10 cell line. It is a highly metastatic subpopulation of B16, which was isolated from a spontaneously arising tumor in a C57BL/6 mouse (380). B16F10 metastasizes through the draining lymph node to the lungs (381).

The immunogenicities of 4T1 and B16 have been well studied *in vivo*. The 4T1 tumor model is highly immunogenic, meaning it expresses high levels of MHC molecules and has high levels of infiltration by both antitumor and immunosuppressive immune cells, including M1 and M2 macrophages, DCs, Tregs, MDSCs, T cells and B cells. The B16 model, on the other hand, is poorly immunogenic and contains low numbers of both antitumor and immunoregulatory immune cells in the tumor microenvironment. Interestingly, qRT-PCR of B16 tumors revealed high levels of CTLA-4 and low expression of MHC I (382). Consequently, these two models have adapted two different mechanisms to avoid destruction by the immune system: 4T1 tumor cells escape destruction from infiltrating effector cells by recruiting immunosuppressive cells and B16 tumor cells avoid detection from immune cells by loss of antigenicity. Because the 66cl4 and 67NR are much less well studied, their immunogenicities are unknown. It

is known, however, that 67NR cells express MHC I, and not II, molecules and lack expression of the costimulatory molecules B7-1 and B7-2 (383).

Chapter 2

Materials and Methods

2.1: Mice

BALB/cJ, C57BL/6J, NOD/SCID, Ifrg2r -/- (NSG), NOD.CB17-Prkdcscid/J, (NOD scid) B6.Cg-*Thy1^a*/Cy Tg(TcraTcrb)8Rest/J (Pmel) (Jackson Laboratories) and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT1) (Gift from Dr. Shawn Wang, VCU) female mice 6-8 weeks of age were housed under aseptic conditions. These studies have been approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

2.2: Cell Culture

4T1, 67NR, 66cl4 (Fred Miller, Wayne State University), B16F10 and HEK 293T (ATCC) cells were cultured in complete media (CM) (Dulbecco's Modified Eagle Medium [DMEM] [Thermo-Fisher], 10% fetal bovine serum [FBS] [Hyclone], 1% nonessential amino acids [NEAA] [Thermo-Fisher], 2 mM glutamine [Thermo-Fisher] and 1% Pen/Strep [Thermo-Fisher]). The 67NR and 66cl4 lines were isolated from the same tumor as 4T1. 66cl4 is metastatic through the lymph and 67NR is nonmetastatic (376). T cells were cultured in CM with 10 mM HEPES (Thermo-Fisher), 5×10^{-5} M β -mercaptoethanol (Sigma) and 50 U/ml recombinant mouse IL-2 (R&D Systems). Hybridoma lines were cultured in Roswell Park Memorial Institute medium (RPMI-1640) (Life Sciences), 10% FBS, 1% NEAA, 2 mM glutamine and 1% Pen/Strep. Cell lines were authenticated by ATCC prior to shipment by short tandem repeat profiling.

Mycoplasma contamination was tested and confirmed to be negative every 2 years using Universal Mycoplasma Detection Kit (ATCC).

Short-hairpin RNAs (shRNA) were introduced into 4T1, B16F10, 67NR and 66cl4 cells using Moloney murine leukemia virus (MMLV) using the pSIREN-RetroQ (Clontech) system. pSIREN plasmids Ctrl-sh1, Ctrl-sh2, Bptf-sh1 (4T1), Bptf-sh2 (4T1), Bptf-sh1 (B16F10, 67NR, 66cl4), Bptf-sh2 (B16F10) and Bptf-sh2 (67NR, 66cl4) are available at Addgene as stock numbers 73665, 73666, 73669, 73668, 73667, 73669 and 83045, respectively. Transduced cells were selected with 0.5 µg/ml puromycin (Life Technologies) after 48 hours (hr) and maintained in CM containing puromycin thereafter.

2.3: Population Doubling Time

1×10^4 4T1, B16F10, 67NR or 66cl4 cells were plated in triplicates and counted using a hemocytometer every 24 hours for 4 consecutive days. 5 counts were taken per sample. Population doubling times were calculated using <http://www.doubling-time.com>. These measurements were repeated for 3 independent cell line pools.

2.4: Tumor Studies

1×10^4 4T1 cells, 1×10^5 67NR cells or 1×10^4 66cl4 cells were injected subcutaneously (S.C.) into the fourth mammary fat pad of BALB/c or NSG mice. 5×10^4 B16F10 cells were injected S.C. into the flank of C57BL/6 mice. Viable tumor cells were assessed with 0.2% trypan blue using a Cellometer Auto T4 cell counter (Nexcelom). Cells were resuspended in DMEM and 50 µl single cell suspension was injected into

mice. Tumors were analyzed at 21 days (4T1, 67NR), 28 days (66cl4) or 18 days (C57BL/6). For gemcitabine treated mice, 1×10^5 4T1 or 5×10^5 B16F10 cells were injected into BALB/c, NSG or C57BL/6 mice and 1.2 mg/mouse gemcitabine (Hospira) was injected intraperitoneously (I.P.) on day 5 and every 7 days following until tumors were analyzed on day 37 (4T1) or day 18 (B16F10).

2.5: Monoclonal Antibody Depletions

To generate ascites fluid, NOD scid mice were injected I.P. with 200 μ l pristane (Sigma) 14 days prior to I.P. injection of 5×10^6 GK1.5 (anti-CD8), 2.43 (anti-CD4) (Gift from Dr. Mosoud Manjili, VCU) or SH-34 (anti-asialo GM1) (ATCC) hybridoma cells in 100 μ l. Once abdominal distension was noticeable, animals were tapped by inserting a sterile 22 gauge needle into the abdominal cavity and withdrawing fluid with a 5 ml syringe. Animals were tapped three times each. Ascites fluid for each antibody was pooled and centrifuged at 10,000 x rcf for 30 min at 4°C. The supernatant was transferred to a new tube and saturated ammonium sulfate was added drop wise to reach a final concentration of 25%. The sample was incubated overnight at 4°C, centrifuged, and the supernatant was transferred to a new tube. Saturated ammonium sulfate was added drop wise to reach a final concentration of 50% and the sample was incubated overnight at 4°C. The sample was then centrifuged and the pellet was resuspended in 1/5 the starting volume of phosphate-buffered saline (PBS). The sample was then dialyzed against a 3,500 KDa tube with 5 changes of PBS. Protein concentration was then measured using the DC Protein Assay and samples were run on a 10% SDS-PAGE gel to verify antibody presence in the sample.

225 µg/mouse in 100 µl mAb was injected I.P. into BALB/c or C57BL/6 mice on day -2 and day -1 and mice were inoculated with 4T1, B16F10, 67NR or 66cl4 tumor cells on day 0. mAb was injected once every 5 days following tumor inoculation and 4T1 tumor bearing mice were treated with gemcitabine as described. Efficient depletion of CD8+, CD4+ and NK cells was confirmed by flow cytometry analysis of splenocytes.

2.6: Metastasis Assay

Lungs from 4T1 tumor bearing BALB/c mice were minced and digested in 5 ml of 1 mg/ml collagenase type IV (Sigma) in hank's balanced salt solution (HBSS) for 75 min at 4°C. The digested lungs were filtered through a mesh 40 µm filter and centrifuged at 400 x rcf for 8 min. The supernatant was removed and cells were washed with 10 ml HBSS, followed by a 10 ml CM wash. Cells were then resuspended in 10 ml CM containing puromycin and 60 µM 6-thioguanine and plated on a 10 cm dish. After 14 days, colonies were washed with 5 ml PBS and stained with 5 ml 2% methylene blue in 50% methanol for 10 min. Plates were washed with water and colonies were counted.

2.7: Protein Extraction

For total cell lysates, cells were washed in PBS and lysed with trizol (Sigma) for 10 min at RT. For tumor extracts, tumors were minced and homogenized using a Polytron homogenizer (IKA) and lysed in 1 ml trizol per 100 mg tissue. 200 µl chloroform per ml trizol was added and samples were vortexed and incubated for 15 min at RT. Extracts were centrifuged at 12,000 x rcf for 15 min at 4°C and the organic phase was moved to a new tube. 1 ml isopropanol per ml trizol was added to the organic phase and samples were mixed and incubated for 10 min at RT. Extracts were centrifuged at 12,000 x rcf

for 10 min at 4°C and the supernatant was removed. 1.5 ml 0.3 M guanidine in 95% ethanol per ml trizol was added to the samples and incubated overnight at 4°C.

Samples were centrifuged at 12,000 x rcf for 10 min at 4°C and the supernatant was removed. 1.5 ml 100% ethanol per ml trizol was added, samples incubated for 8 hrs at 4°C and centrifuged at 12,000 x rcf for 10 min at 4°C. The supernatant was removed and extracts were dissolved overnight at 65°C in 200 µl 8M urea in 1% SDS per ml trizol.

For cell surface extracts, cells were washed with PBS and removed from the dish using a cell scraper. Cells were then lysed in lysis buffer (20 mM phosphate buffer, pH 7.4, 350 mM NaCl, 10 mM KCl, 1 mM EDTA, 1% Triton X-100, 20% glycerol) supplemented with EDTA-free protease inhibitor cocktail (Roche) for 3 hrs at 4°C. Samples were centrifuged at 10,000 x rcf for 30 min at 4°C to remove large aggregates and the supernatant was transferred to a new tube.

Protein was quantified using the DC Protein Assay (Bio-Rad) according to the manufacturer's instructions with a Ultrospec III spectrophotometer (Pharmacia LKP).

2.8: Western Blot

40 µg protein was loaded into SDS-PAGE gels and electrophoresed for 1 hr at 200 V and 300 mA. Protein was transferred to a PVDF membrane (Bio-Rad) for 1-1.5 hrs at 200 V and 200 mA in transfer buffer (10 mM CAPS-NaOH pH 10.5). Membranes were blocked with 5% nonfat dry milk in PBST (PBS 0.1% Tween-20) for 1 hr at RT. Membranes were incubated with primary antibodies overnight at 4°C: 1:5000 anti-BPTF, 1:10,000 anti-HS (Millipore), 1:1000 anti-OVALBUMIN, 1:1000 anti-PMEL17, 1:10,000

anti-HPSE (Santa Cruz Biotechnology), 1:10,000 anti-CYCLOPHILIN B (Abcam), anti-PSMB9, anti-TAP2 (Thermo Scientific), anti-PSMB8 and anti-TAP1 (Cell Signaling). Membranes were washed 3 times with PBST for 5 min at RT and incubated with secondary antibody for 1 hr at RT: 1:10,000 horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (Cell Signaling). Membranes were then washed 3 times with PBST for 5 min each and exposed with electrochemiluminescence. Loading was determined by cyclophilin B.

2.9: RNA Extraction

Cultured cells and tumors were prepared and treated with trizol as described in the Protein Extraction section. 500 μ l isopropanol per ml trizol was added to the aqueous phase containing the RNA, samples were mixed, incubated for 10 min at RT and centrifuged at 12,000 x rcf for 10 min at 4°C. The RNA pellet was washed with 1 ml 70% ethanol per ml trizol, dried for 10 min at RT and resuspended in 100 μ l molecular grade water. RNA quality was verified by nanodrop and degradation was assessed by a 0.8% agarose gel.

2.10: qRT-PCR

5 μ g RNA was reverse transcribed to cDNA using Superscript II First-strand kit (Invitrogen) according to the manufacturer's instructions. qPCR was performed using SYBR green PCR master mix (Biorad) with a 7900 HT fast real-time qPCR system (Applied Biosystems). 5 μ l cDNA, 5 μ l 280 nM forward and reverse primer mix and 10 μ l 2X SYBR green ROX mix were mixed together for each reaction. qRT-PCR conditions:

95°C for 15 min, 50 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a dissociation curve cycle. The ddCt method was performed using normalization to *Gapdh*. Primer pairs are found in a Table 2.1.

Table 2.1: Primers used for qRT-PCR

qRT-PCR Primers	
Mouse Primers	
Gapdh Forward	TGGCAAAGTGGAGATTGTTGCC
Gapdh Reverse	AAGATGGTGATGGGCTTCCCG
Psmb9 Forward	CTCTGCTGAGATGCTGCGGGC
Psmb9 Reverse	CCACTGCTGTTCCCGCTGACAC
Psmb8 Forward	TTGGCCAAGGAGTGCAGGTTGTAT
Psmb8 Reverse	GTCCCGAGAGCCGAGTCCCAT
Tap1 Forward	TCGGGAATGCTGCTGAAGCTTC
Tap1 Reverse	GGGTGCCAACGAGCCACTGA
Tap2 Forward	CGCCTTTGCAAGCGCCATCTTT
Tap2 Reverse	TCGAGTTCAGCTCCCCGTGCTT
B2m Forward	GTCGCTTCAGTCGTCAGCAT
B2m Reverse	GATCACAGTGCTCGATCCAG
H2K1 Forward	CAGAACTCAGAAGTCGCGAATCGCC
H2K1 Reverse	ACGAACTCCGTGTCTGTCACG
H2D1 Forward	GAGCCTCCTCCGTCCACTGACTC
H2D1 Reverse	CCAGGCAGCTGTCTTCACGCTTTA
Oas1a Forward	CATTTTCAGCTAGGCTGGGGAGACCC
Oas1a Reverse	GAGCCACCCTTCACCACCTTGGA
Oas2 Forward	CCTGGCTGTACTCTCCCAGCCT
Oas2 Reverse	TGGCAGTGGATCCTCCCTTGACG
Hpse Forward (67NR)	GAGCGGAGCAAACCTCCGAGTGTATC
Hpse Reverse (67NR)	GATCCAGAATTTGACCGTTCAGTT
Hpse Forward (66cl4)	GGAAATCTCAAGTCAACCATGATAT
Hpse Reverse (66cl4)	ATCTCCACTGAGCTTCTTGAGTAG
Human Primers	
Gapdh Forward	CTCTGCTCCTCCTGTTTCGAC
Gapdh Reverse	GCGCCCAATACGACCAAATC
Hpse Forward	GGCAAGTATTCTTTGGAGCA
Hpse Reverse	TGGATTGTCAGTGTTTGTGC

2.11: Flow Cytometry

Cultured tumor cells were washed with PBS and collected with Cellstripper (Corning). Cells were washed and resuspended in FACS buffer (2% FBS in HBSS) and stained with 0.5 μ l H2K, H2D, H2A, Qa1, OVA antibodies or Annexin V for 20 min at 4°C. Cells were then washed twice with FACS buffer and suspended in 500 μ l FACS buffer with 1 μ l 7AAD viability dye (Sigma).

To stain infiltrating lymphocytes, tumors were minced and digested in HBSS with 0.04% DNase I (Sigma) and 0.1% collagenase type IV for 90 min at 37°C. Tumor digests were filtered through a 40 μ M nylon filter and centrifuged at 500 x rcf for 5 min at RT. Percoll was thawed to RT and 9 volumes percoll were mixed with 1 volume 10x PBS to obtain the working concentration. Cells were resuspended in 40% percoll (Sigma), layered on top of 70% percoll and centrifuged at 3000 x rcf for 30 min at RT with 0 acceleration and 0 brake. Lymphocytes at the interphase were removed, diluted in PBS, and centrifuged at 500 x rcf for 5 min at RT. Purified lymphocytes were subsequently stained with 0.5 μ l CD8, CD69, TCRb, CD44, or BTLA antibodies and 7AAD viability dye.

For NK receptor binding, 1×10^5 67NR or 66cl4 cells were washed with PBS, collected with trypsin and incubated with 1 μ g *Ncr1*-Ig (gift from Ofer Mandelboim from The Hebrew University of Jerusalem) for 1 hr at 4°C. Cells were washed twice with FACS buffer and stained with 0.5 μ l PE- anti-human-IgG Fc (Biolegend) for 20 min at 4°C. For heparinase treatment, 67NR or 66cl4 cells were treated with 10 U/ml bacterial heparinase I/III (Sigma) in DMEM 1% BSA for 1 hr at 37°C. 1×10^5 cells were then

collected with trypsin and incubated with 1 μ g *Ncr1*-Ig, washed, stained with PE- anti-human-IgG Fc and analyzed by flow cytometry.

All flow cytometry data collection was performed using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using Cyflogic software. All antibodies were purchased from BD Pharmingen.

2.12: T Cell Cytotoxicity Assay

Spleens from 4T1 or B16F10 tumor bearing mice were collected and cut lengthwise. Splenocytes were extracted by gentle massaging and suspended in MACS buffer (0.5% BSA, 2 mM EDTA in PBS). Splenocytes were filtered through a mesh 0.40 μ m filter and counted with a hemocytometer. MDSCs were removed by positive selection using the Myeloid-Derived Suppressor Cell Isolation Kit, mouse according to the manufacturer's instructions (Miltenyl). CD8a⁺ cells were then purified by negative selection using the CD8a⁺ T Cell Isolation Kit mouse (Miltenyl) according to the manufacturer's instructions. For CTL hyperactivation assays, purified CD8⁺ T cells were directly treated with 0.8 μ M PMA, 0.35 μ M Ionomycin and placed on mitomycin C-treated tumor cells. For polyclonal CTL assays, the purified CD8⁺ T cells were cocultured with 5×10^4 mitomycin C-treated 4T1 or B16F10 tumor cells in one well of a 24 well plate for 1-3 weeks of *in vitro* expansion in T cell media with 500 U/ml IL-2. The CD8⁺ T cells were then harvested and purified by percoll gradient a mini percoll gradient: 2 ml 40% percoll layered on top of 1 ml 70% percoll. Lymphocytes recovered from the interphase were cocultured with 5×10^3 mitomycin C-treated tumor cells for assay.

For total splenocyte and OVA analysis, splenocytes from tumor bearing mice or naïve OT1 mice were collected and red blood cells were removed by 5 min incubation at RT with red blood cell lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA). Splenocytes were then restimulated *in vitro* for 1-3 weeks before coculture with targets for assay. For ONX-0914 treatment, ONX-0914 was added to mitomycin C treated targets for 24 hours. Cells were then placed in fresh media and incubated with splenocytes at a 50:1 effector:target ratio.

For all assays, CD8+ T cells/splenocytes were cocultured with 5x10³ targets in V-bottom 96 well plates for 48 hours in LDH media (DMEM, 5% heat inactivated FBS (Serum Source), 1% NEAA, 2 mM glutamine and 1% Pen/Strep) with 500 U/ml IL-2. Cell death was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega). Percent cytotoxicity was calculated using (Experimental – Effector Spontaneous – Target Spontaneous)/(Target Maximum – Target Spontaneous) x 100.

2.13: NK Cell Cytotoxicity Assay

Mouse NK cells were purified from the spleen of naïve BALB/c mice by negative selection using the NK Cell Isolation Kit II mouse (Miltenyl) and placed on mitomycin C-treated targets for assay. For antibody blocking experiments, NK cells were pre-incubated for 1 hour with 10 µg/ml blocking antibody in 100 µl to mNcr1 (NK1.15 from Stipan Jonjic, University of Rijeka). For hyperactivation, purified mouse NK cells were treated with 0.8 µM PMA, 0.35 µM Ionomycin in cocultures. For media preconditioning, mitomycin C treated targets were incubated in 6 well plates for 24 hours. The media

was then removed and incubated with freshly purified NK cells for 24 hours. NK cells were then plated on mitomycin C treated targets for assay. For heparin competition experiments, heparin (Sigma) was added to the coculture experiment to 100 µg/ml final. For heparinase treatment, mitomycin C treated 66cl4 cells were pretreated with 1 U/ml bacterial heparinase I/III (Sigma) for one hour at 37°C, washed, and cocultured with NK cells for assay.

For all NK cytotoxicity assays, mouse NK cells were cocultured with 5×10^3 targets in LDH media with 50 U/ml IL-2 for 24 hours and cell death was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay.

2.14: Enzyme-Linked ImmunoSpot (ELISPOT)

Coat MultiScreen-IP plates (Millipore) were coated with 10 µg/ml rat anti-mouse IFN-γ (clone R4-6A2)(BD Pharmingen) at overnight 4°C. Plates were washed twice and blocked with CM containing 10% FBS for 2 hrs at 37°C. Splenocytes from naïve mice and B16F10 tumor infiltrating lymphocytes were obtained as described above and 2×10^6 lymphocytes were cocultured with 1×10^6 splenocytes. Cocultures were stimulated with 1 µg/ml Gp100₂₅₋₃₃ (KVPRNQDWL), TRP2₁₈₀₋₁₈₈ (SVYDFVWL) or p15E₆₀₄₋₆₁₁ (KSPWFCTL) and 50 U/ml IL-2 for 96 hours at 37°C. Plates were then washed once with distilled water, 6 times with PBS 0.05% Tween-20, and incubated with 5 µg/ml biotinylated IFN-γ antibody (clone R4-6A2)(BD Pharmingen) in PBS 1% BSA for 2 hrs at 37°C. Plates were then washed 6 times with PBS 0.05% Tween-20 and incubated with 0.2 unit/ml avidin-alkaline phosphatase D (Vector) for 2 hrs at RT. 5-bromo-4-chloro-3-

indolyl phosphate/nitroblue tetrazolium (Vector) was added and spot development was monitored for 20 min at RT.

2.15: Limited Dilution

CD8⁺ T cell clones were purified and expanded as described above. After one week of *in vitro* expansion, clones were diluted in media to ~ 1 cell/ 100 μ l and 100 μ l was plated to each well of a V-bottom 96 well plate on 1×10^4 mitomycin C treated 4T1 targets. Media was changed approximately three times a week and clones were placed on new targets approximately once every 2-3 weeks. After ~3 months, wells were screened for live T cells by enzyme-linked immunosorbent assay (ELISA) using the Ready Set Go Interferon Gamma ELISA kit (eBioscience) according to the manufacturer's instructions. After ~6 months, clones identified by ELISA were divided into 2 wells each, and after ~1 year, clones were moved to a single well of a 24 well plate on 5×10^4 targets. Clones were assayed for activity against control and BPTF KD targets as described above, with 50 U/ml IL2.

2.16: Chromatin Immunoprecipitation (ChIP)

Cultured cells of equal confluency were fixed in 10 ml 1% paraformaldehyde in PBS for 15 min at RT. Cells were washed 3 times with PBS and removed from the plate using a cell scraper. Cells were transferred to a slick 1.5 ml Eppendorf tube and centrifuged at max speed for 1 min at RT. The supernatant was removed and cells were resuspended in 1 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) supplemented with protease cocktail inhibitor. Chromatin was sheared to an average

size of 300 bp by 3 rounds of 15 min alternating 30 sec on, 30 sec off Bioruptor sonication (Diagenode). Samples were then centrifuged at max speed for 15 min at RT. The supernatant was divided and transferred to a slick tube with either 0.5 µg anti-BPTF (Millipore) or control rabbit IgG (Cell Signaling) bound to Protein G Dyna beads (Life Technologies) diluted in 1.6 ml dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCL pH 8.0, 167 mM NaCl) supplemented with protease cocktail inhibitor. 40 µl of the supernatant was removed for 10% input. Samples were bound to antibodies overnight at 4°C. Samples were subsequently washed with 500 µl low salt buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl), high salt buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris HCl pH 8.0) and two washes with TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA) for 5 min on ice each. Antibody was then eluted twice with elution buffer (250 µl 0.1 M NaHCO₃ in 1% SDS) for 30 min at RT. 460 µl elution buffer was added to inputs and all samples and inputs were incubated with 20 µl 5 M NaCl at 65°C overnight. Samples/inputs were subsequently incubated with 10 µl 0.5 M EDTA, 20 µl 1 M Tris and 2 µl 10 mg/ml proteinase K at 45°C for 1 hour to reverse the crosslinks. 2 µl glycogen and 500 µl basic phenol/CHCl₃ were added to samples/inputs, which were then vortexed for 5 min. Samples/inputs were centrifuged at max speed for 15 min at RT and the aqueous phase was transferred to new slick tubes. DNA was precipitated with 2.5x volume of 100% ethanol overnight at -20°C and samples/inputs were centrifuged at 15000 x rpm for 30 min at 4°C. The supernatant was removed and pellets were washed with 500 µl 70% ethanol, dried by Savant SpeedVac sc100 and resuspended in 50 µl molecular grade

water. qRT-PCR was performed as described above. Primer pairs are found in Table 2.2.

Table 2.2: Primers used for ChIP

ChIP qRT-PCR Primers	
Site 1 Forward	AGAGCTGTGGAGTTGAAGCT
Site 1 Reverse	CCACCTTACCTCCCCACAAT
Site 2 Forward	CAGGGGAGGTGGAGTCACTA
Site 2 Reverse	TGGTTCTTCCTTACAGAACAGCA
Site 3 Forward (4T1)	CAGGCTAGGACCAGACTCTG
Site 3 Reverse (4T1)	GAGAAGATTCCCGGGAGCAT
Site 3 Forward (B16F10)	GATCACTCCACCCGCTGAC
Site 3 Reverse (B16F10)	TTTGCTCTGGGCGCCAAAT
Site 4 Forward	AGCTCTAGGGGTTCTGATGC
Site 4 Reverse	CCATGTCCTCCTCCTCATCG
Site 5 Forward	ACGCCTAGGTGGATCTGCT
Site 5 Reverse	TTGGGGACGAGTTTTCTGC
Site 6 Forward	GGCAGTGAAGTGAAAGCGAA
Site 6 Reverse	ATGATGACGCCAAGGGTTTG
Site 7 Forward	CCCAACCCTAACTCCACCAT
Site 7 Reverse	GTGTGGGGTAGGGTTCTGTT
Site 8 Forward	CTCACACCCATCCACCTCTT
Site 8 Reverse	TGGAAGAGCAGCCTTAACCA
Site 9 Forward	CCCAAACCAAAACCCTCCAG
Site 9 Reverse	AACTGGCGCGGAAATTACAG
Enhancer Forward	GAGGAACCGGCTACTCTCCT
Enhancer Reverse	GTGTGGGCCAAGATGGAAGT
Promoter Forward	GGTGGCCAGAATCCAAGATCC
Promoter Reverse	AAAAACAGGGTCCCCACCAC
Intragenic Forward	ACCTCCACACTGGGTTTGAC
Intragenic Reverse	TGTGTCTGGTCCTCGTTTGG

2.17: Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE)

Cultured cells of equal confluency were fixed with 10 ml 1% paraformaldehyde in PBS for 15 min at RT. Cells were washed 3 times with PBS and removed from the plate using a cell scraper. Cells were transferred to a slick 1.5 ml Eppendorf tube and centrifuged at max speed for 1 min at RT. The supernatant was removed and cell pellets were resuspended in 500 µl FAIRE lysis buffer (10 mM Tris HCl pH 8.0, 2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail. Chromatin was sheared to an average size of 300 bp by 3 rounds of 15 min alternating 30 sec on, 30 sec off Bioruptor sonication and samples were centrifuged at max speed for 15 min at RT. 450 µl (sample) and 50 µl (input) were transferred to new tubes. 1 µl DNase-free RNase A was added to the input and incubated for 30 min at 37°C. 1 µl proteinase K was then added and inputs were incubated for 1 hr at 55°C and overnight at 65°C to reverse the crosslinks. 100 µl 10 mM Tris HCl pH 7.4 was then added to inputs. 1 volume phenol/chloroform/isoamyl alcohol solution was added to both inputs and samples, vortexed and centrifuged at 12,000 x rcf for 5 min at RT. The aqueous phase was transferred to a new tube and 150 µl 10 mM Tris-HCl pH 7.4 was added to the interphase/organic phase. The interphase/organic phase samples were then vortexed and centrifuged and the aqueous layer was pooled with the previous aqueous extraction layer. One more round of phenol/chloroform/isoamyl alcohol extraction was performed and the aqueous phase was transferred to a new tube. 200 µl chloroform/isoamyl alcohol was added to the aqueous phase, vortexed, and centrifuged. The aqueous phase was transferred to a new tube and incubated with 1/10 volume 3 M sodium acetate pH 5.2, 2 volumes 95%

ethanol and 1 μ l linear acrylamide at -80°C overnight. Samples/inputs were precipitated by centrifugation at 12,000 x rcf for 15 min at 4°C, washed with 500 μ l 70% ethanol and dried by SpeedVac. Pellets were resuspended in 20 μ l (inputs) or 50 μ l (samples) 10 mM Tris HCl pH 7.4. Samples/inputs were then purified using Zymo-I spin columns (Zymo Research): DNA binding buffer was mixed with the DNA at a 2:1 ratio and passed through the column via high speed centrifugation for 1 min. The column was washed twice with 200 μ l wash buffer and DNA was eluted with TE buffer.

qRT-PCR was performed as described and FAIRE results were normalized to a control locus which does not have BPTF dependent FAIRE when normalized to equal DNA mass. Primers are found in Table 2.3.

Table 2.3: Primers used for FAIRE

FAIRE qRT-PCR Primers	
Site 1 Forward	CGCAGTCTAGAGTGAAAGCGA
Site 1 Reverse	CATGCGAAGCTGGTGAGAAG
Site 2 Forward	AGGCAACTTGCAGACTGAGG
Site 2 Reverse	AGATTTGCCCTGCTCAGGTT
Site 3 Forward	TTCCTCTAAACGCCAGCACT
Site 3 Reverse	GAAGGAAGAAGGGCGGGTC
Control Site Forward	ATACAACCAAACAGACACACAACC
Control Site Reverse	CTACTGGCTGCCATGGCTTA

2.18: Microarray

RNA extraction, microarray analysis and statistical analysis were performed as described (384,385). Tumor tissues were subjected to gross histological analysis using hematoxylin and eosin staining to determine percentages of tumor, necrotic and stromal cells before tissues were isolated for RNA extraction. If present, necrotic tissue was selected out by macrodissection. Total RNA was then extracted from frozen tumor tissues with TRI reagent and the MagMAX-96 Total RNA Isolation Kit in a MagMAX Express Magnetic Particle Processor (Life Technologies). RNA quality was assessed with an Agilent 2100 Bioanalyzer and NanoDrop 8000 spectrophotometer. 5 µg of total RNA was reverse transcribed and the GeneChip 3' IVT Express kit (Affymetrix) was used to generate biotinylated cRNA by *in vitro* transcription. 10 µl of fragmented cRNA was applied to the GeneChip® Mouse Genome 430A 2.0 Array (hybridization conditions: 60 x rpm for 16 hrs at 45°C)(Affymetrix). Microarrays were then washed and stained with streptavidin phycoerythrin (Molecular Probes) using an Affymetrix Fluidics work station. Microarrays were scanned as previously described using the Affymetrix GeneChip Scanner 3000 and data was saved as .dat and .cel files. Array quality was accepted if the 3'/5' ratio of the housekeeping gene GAPDH is less than three and the present gene percentage is more than 40%.

Microarray statistical analysis was performed as previously described (384,385). Log-scale robust multiarray analysis (RMA) was used for noise correction, normalization and estimation for probe expression. Relative differences between control and KD samples were analyzed using two-sample-t-test for each pairwise comparison with α -level equal to 0.01 to determine differentially expressed probes at the univariate level.

Each p-value was corrected for multiple testing using FDR false discovery rate < 15%.

Data are available at GEO accession GSE71864 and GSE785756.

2.19: Statistical Analysis

All variation shown represent standard deviation from the mean and all tests of significance were tested with a student's unpaired two-tailed t-test.

Chapter 3

BPTF Depletion Enhances T Cell Mediated Antitumor Immunity

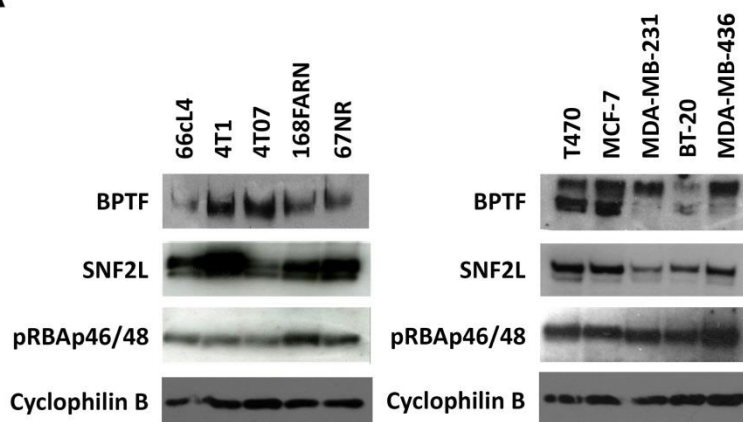
3.1 BPTF Depletion Increases T Cell Antitumor Immunity to 4T1 Tumors

Consistent with previous work showing that the subunits of the NURF complex are frequently overexpressed in cancer cells (346), we found each of the NURF subunits is expressed in a variety of mouse tumor cell lines (Fig. 3.1A). To discover functions of NURF in cancer cell biology, we repressed BPTF expression in mouse 4T1 cells by retroviral introduced shRNA KD (Fig. 3.1B). BPTF KD was used to deplete NURF because it is unique and essential to the complex (286,352). Surprisingly, BPTF KD cells showed no significant differences in doubling time, considering previous studies have shown decreases in proliferation with BPTF KD (Fig. 3.1C) (366,374,375). Additionally, we observed no differences in *in vitro* cellular morphology or levels of apoptosis (Fig. 3.1D and E).

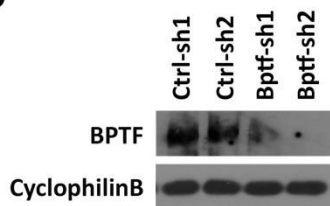
To uncover functions for BPTF in tumor biology, we introduced BPTF KD 4T1 cells into the 4th mammary fat pad of syngeneic BALB/c mice. As part of our studies, we inoculated the cells into both untreated and gemcitabine treated mice. Gemcitabine is a routinely used chemotherapeutic for treating breast cancer which inhibits DNA replication and induces apoptosis in tumors (386). We observed significantly reduced weights for BPTF KD tumors only in gemcitabine treated mice (Fig. 3.2A). We chose weight and not volume to monitor tumor growth because BPTF KD tumors are

Figure 3.1

A



B



C

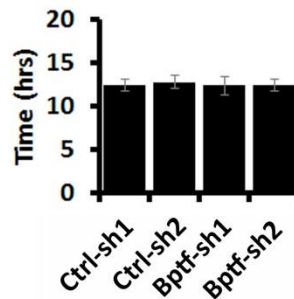


Figure 3.1: BPTF does not affect 4T1 cellular morphology or proliferation in vitro. A. Western blot analysis of the three subunits of NURF: BPTF, SNF2L and pRBAP46/48 in total cell extracts of several mouse (left) and human (right) mammary tumor cell lines. **B.** BPTF Western blot analysis of control (Ctrl-sh1, Ctrl-sh2) and BPTF KD (Bptf-sh1, Bptf-sh2) B16F10 total cell extracts. Cyclophilin B, loading control. **C.** Population doubling time of control and BPTF KD 4T1 cells (n = 3 biological replicates).

Figure 3.1

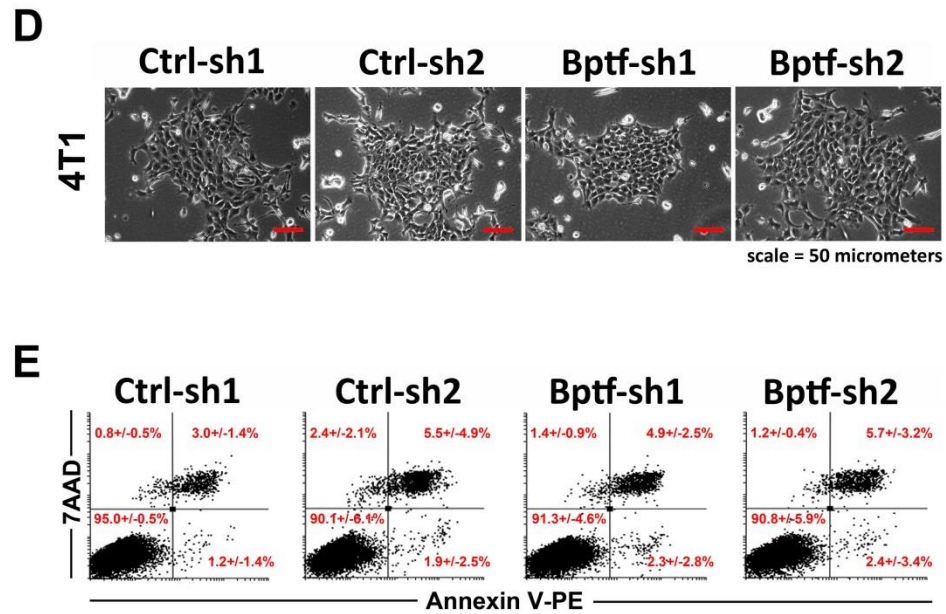


Figure 3.1: BPTF does not affect 4T1 cellular morphology or proliferation in vitro.
D. Images of 4T1 cells in culture. Scale = 50 μ m. **E.** Flow cytometry analysis of cultured 4T1 cells stained with Annexin-V and 7AAD. The percentages of positive cells are indicated in each quadrant (n = 3 biological replicates).

Figure 3.2

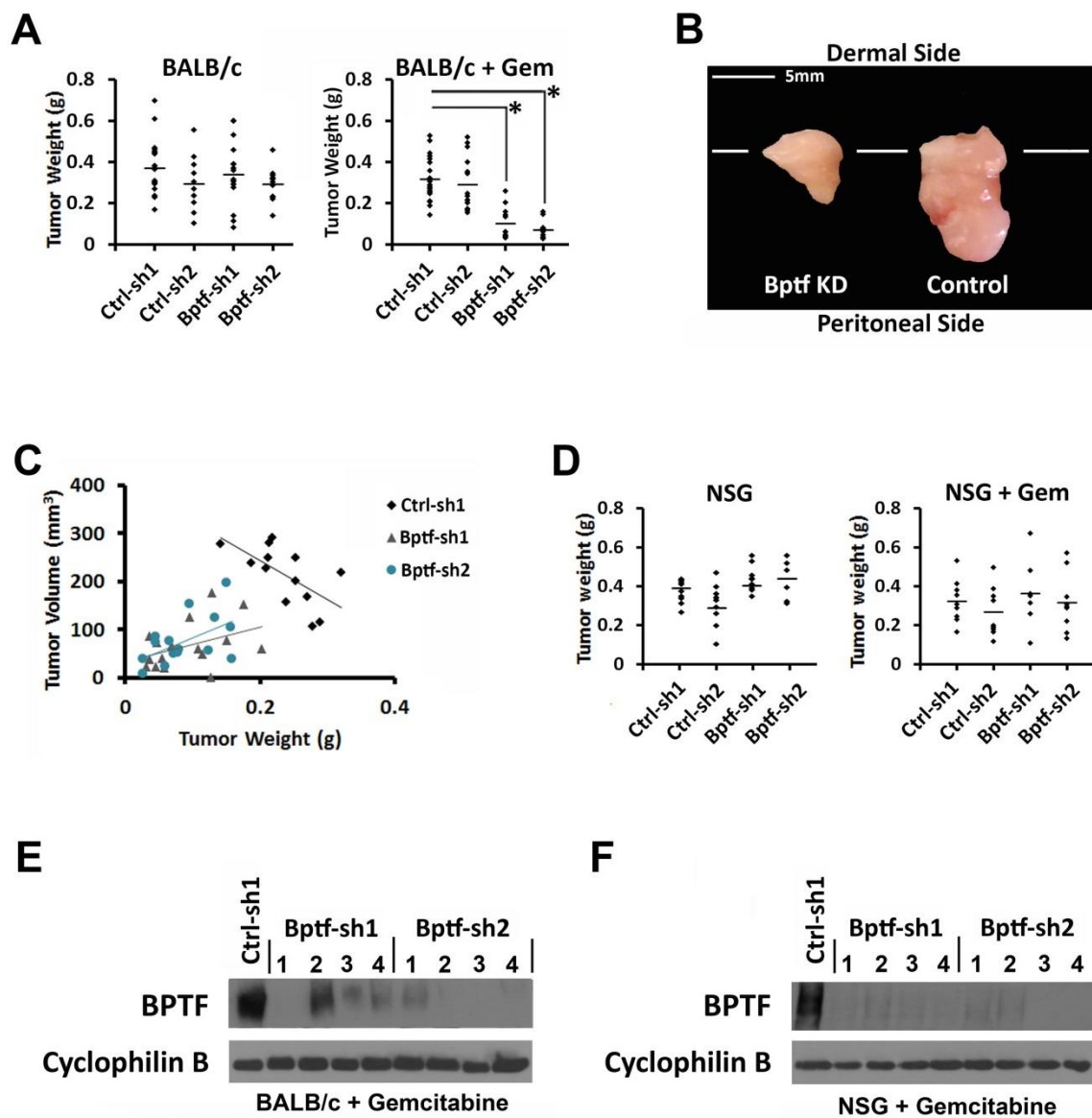


Figure 3.2: Depletion of BPTF reduces 4T1 tumor weights in mice with CD8+ and CD4+ T cells. A. Weights of primary control and BPTF KD 4T1 tumors after growth in BALB/c mice ($n \geq 12$; *, t test, $P < 9.6 \times 10^{-7}$). **B.** Image of a representative 4T1 control and BPTF KD tumors from BALB/c mice. **C.** Plot of 4T1 tumor weight vs. volume at sacrifice is shown with the best fit line for each hairpin ($n \geq 13$). Mice in B and C were treated with gemcitabine. **D.** Weights of primary control and BPTF KD 4T1 tumors after growth in NSG mice ($n = 9$). **E-F.** BPTF Western blot analysis of representative 4T1 tumors (1-4) harvested from E, BALB/c and F, NSG mice.

morphologically flat compared to controls, confounding a volume based measurement (Fig. 3.2B and C).

In addition to inducing cancer cell apoptosis, gemcitabine also inhibits the proliferation of several cells of the immune system, most prominently MDSCs (57,386-389). MDSCs are dramatically amplified in mice bearing 4T1 tumors, which suppresses the antitumor immune response (389). To determine if the effect of gemcitabine on BPTF KD tumors is a result of enhanced antitumor immunity, we repeated our tumor studies in immune compromised NOD/SCID, *lfrg2r*^{-/-} (NSG) mice (390). In NSG mice, we observed similar growth of control and BPTF KD tumors with gemcitabine treatment (Fig. 3.2D), demonstrating that the reduced tumor growth in BALB/c mice is not due to gemcitabine alone, but rather in combination with antitumor immunity.

To determine if BPTF KD cells are eliminated from tumors grown in immune competent mice, we measured BPTF levels in the primary tumors. We show that BPTF expression increases in tumors from BALB/c (Fig. 3.2E) but not NSG mice (Fig. 3.2F), suggesting that a functional immune system either directly or indirectly selects for cells which re-express BPTF.

To understand the immune response to gemcitabine treated BPTF KD 4T1 tumors, we selectively depleted CD8⁺ T cells, CD4⁺ T cells or NK cells by ADCC. We observed a rescue of BPTF KD tumor growth when CD8⁺ or CD4⁺ cells were depleted (Fig. 3.2G), demonstrating that the antitumor response to BPTF KD 4T1 tumors requires T cells.

Figure 3.2

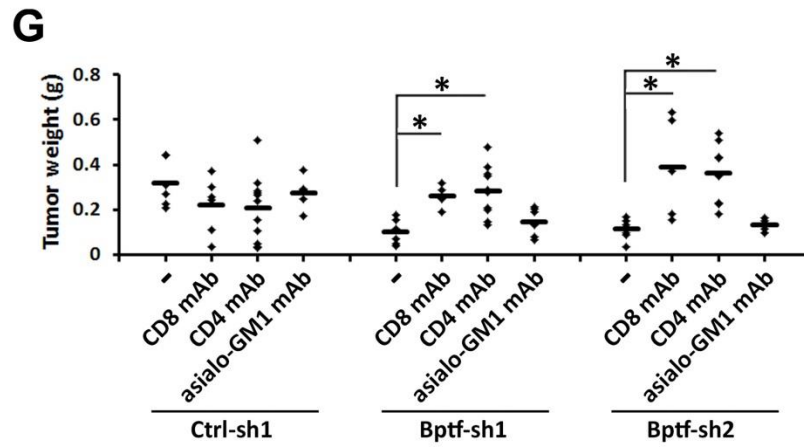


Figure 3.2: Depletion of BPTF reduces 4T1 tumor weights in mice with CD8+ and CD4+ T cells. G. Weights of 4T1 tumors after growth in undepleted, CD8+, CD4+, or asialo-GM1+ mAb-depleted BALB/c mice ($n \geq 5$; *, t test, $P < 0.02$).

3.2 MDSC Amplification and Tumor Metastasis are BPTF-Independent

4T1 tumors dramatically amplify MDSCs, resulting in splenomegaly with spleen size proportional to the degree of MDSC amplification (389). As previously reported, we observed a significant increase in spleen weight with introduction of 4T1 tumors into BALB/c mice (Fig. 3.3A). The observed increase in spleen weight was proportional to tumor weight and was not affected by BPTF KD, demonstrating that splenomegaly is BPTF-independent (Fig. 3.3B).

The 4T1 model is also widely used to study breast cancer cell metastasis (376). To determine if BPTF regulates metastasis, we analyzed metastases to the lung of both untreated and gemcitabine treated BALB/c mice using a colony formation assay (376). We show that tumor size, but not BPTF KD, was significantly correlated with the number of colonies, suggesting that metastasis of 4T1 is BPTF-independent (Fig. 3.3C and D) (see discussion).

3.3 BPTF Depletion Increases T Cell Antitumor Immunity to B16F10 Tumors

To further verify our findings, we used the B16F10 melanoma model which is syngeneic to C57BL/6 (391). First, we confirmed that each NURF subunit is expressed in B16F10 cells (Fig. 3.4A). Additionally, BPTF KD (Fig. 3.4B) had no significant effect on doubling time, cellular morphology or apoptosis of B16F10 cells (Fig. 3.4C-E).

Consistent with 4T1, B16F10 KD tumors had a flattened morphology, preventing caliper measurements from accurately comparing tumor growth (Fig. 3.5A and B). As with 4T1, we observed significant reductions in BPTF KD B16F10 tumor weights (Fig.

Figure 3.3

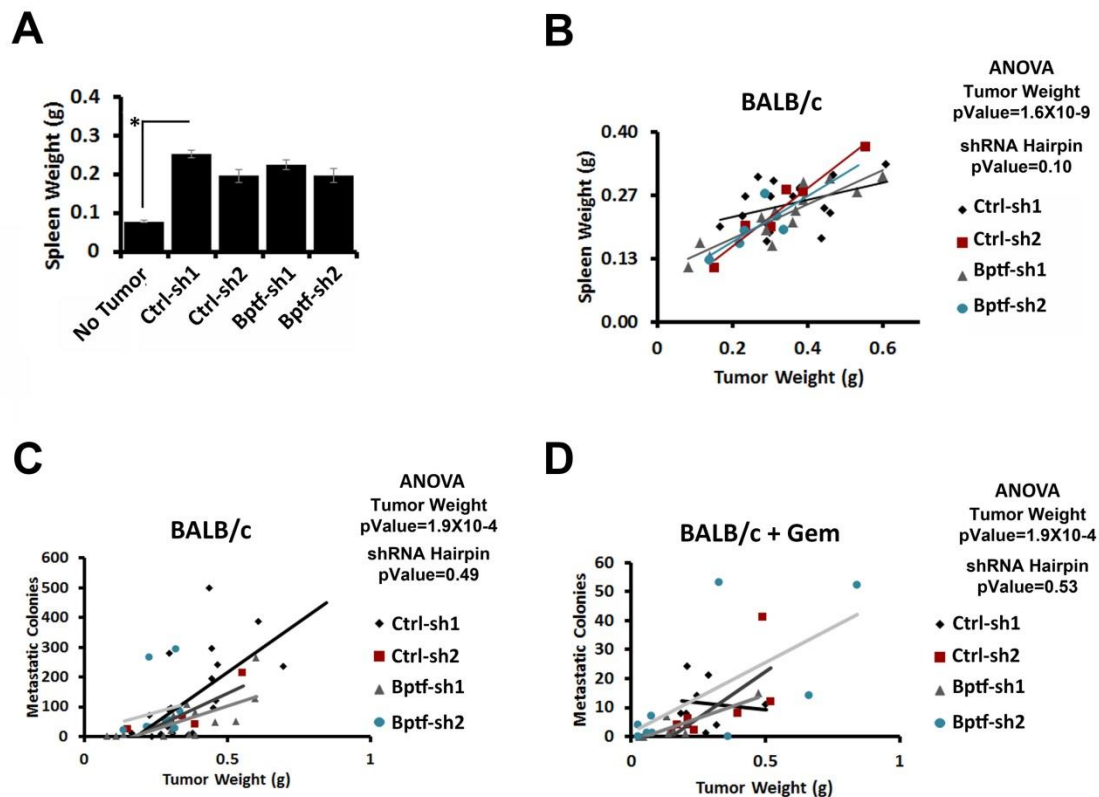


Figure 3.3: BPTF does not affect 4T1 MDSC amplification or metastasis to the lung. **A.** Spleen weights from 4T1 tumor bearing BALB/c mice treated with gemcitabine ($n \geq 12$) and naïve BALB/c mice ($n = 5$, * = ttest pvalue < 3.9×10^{-8}). **B.** Plot of BALB/c 4T1 tumor weights vs corresponding spleen weights with the best fit line for each hairpin ($n \geq 12$). **C-D,** Plot of 4T1 primary tumor weight vs. meta-static colonies in the lung with the best fit line for each hairpin. Tumors and lungs were harvested from 4T1 tumor bearing mice with C, no gemcitabine treatment ($n \geq 5$) and D, gemcitabine treatment ($n \geq 8$).

Figure 3.4

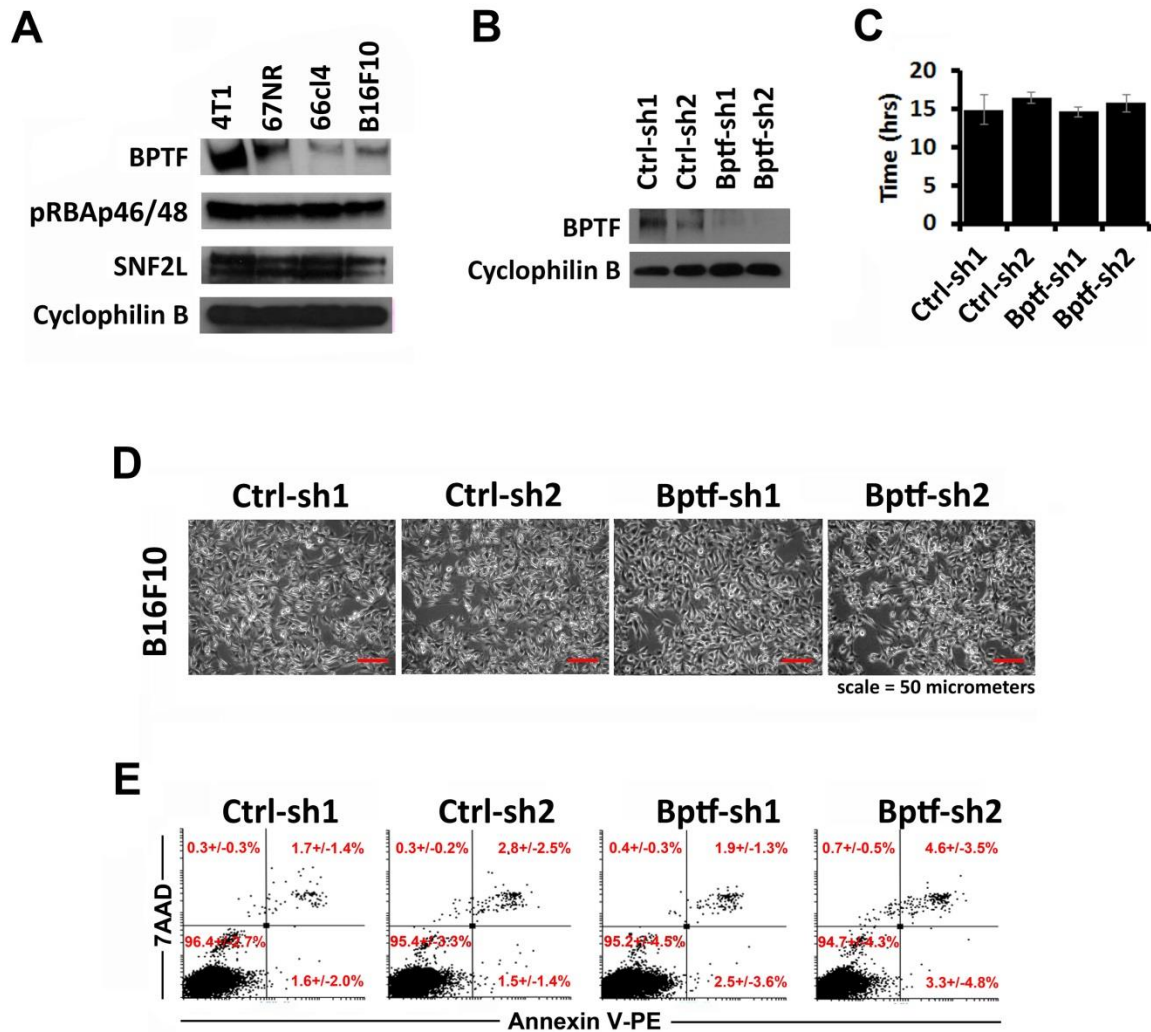


Figure 3.4: BPTF does not affect B16F10 cellular morphology or proliferation in vitro. **A.** Western blot analysis of the three subunits of NURF in total cell extracts of several mouse mammary tumor cell lines and the mouse melanoma cell line B16F10. **B.** BPTF Western blot analysis of control (Ctrl-sh1, Ctrl-sh2) and BPTF KD (Bptf-sh1, Bptf-sh2) B16F10 total cell extracts. Cyclophilin B, loading control. **C.** Population doubling time of control and BPTF KD B16F10 cells (n = 3 biological replicates). **D.** Images of B16F10 cells in culture. Scale = 50 μ m. **E.** Representative flow cytometry analysis of cultured B16F10 cells stained with Annexin-V and 7AAD. Percentages are from 3 biological replicates.

Figure 3.5

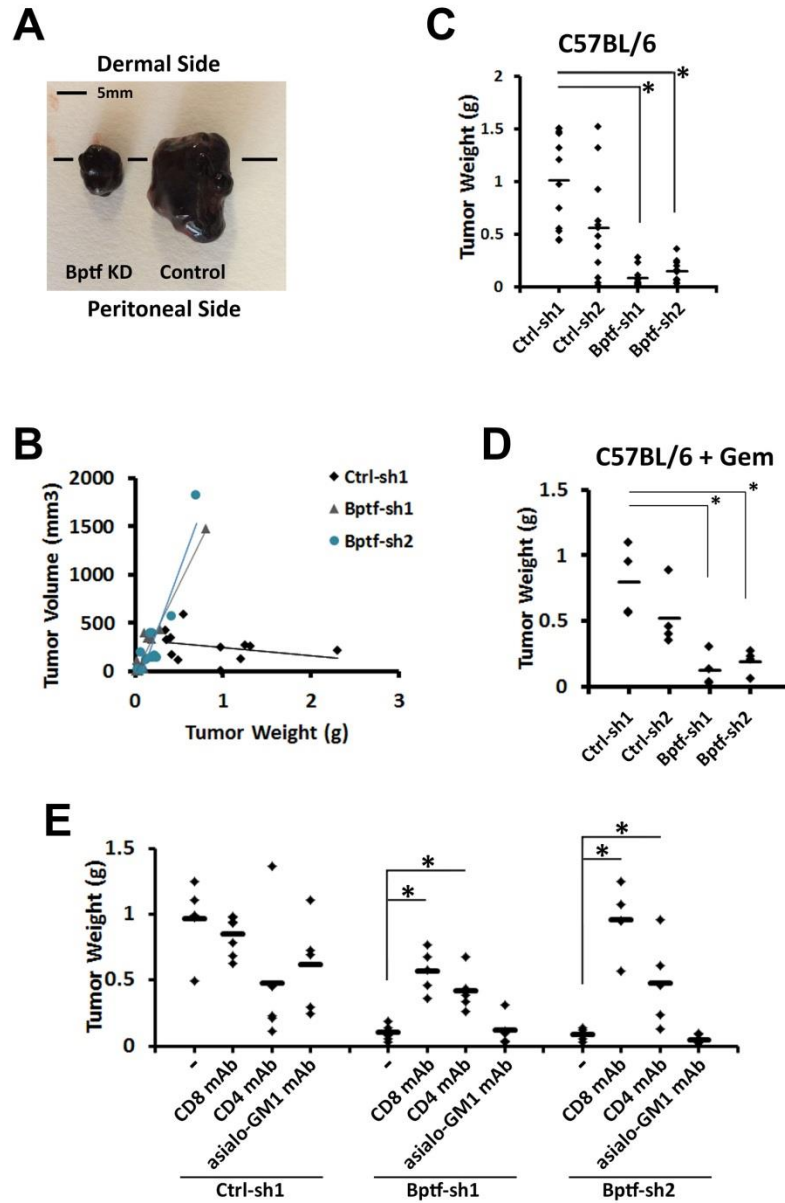


Figure 3.5: BPTF depletion reduces B16F10 tumor weights in mice with CD8⁺ and CD4⁺ T cells. **A.** Image of a representative B16F10 control and BPTF KD tumors. **B.** B16F10 tumor weight vs. volume at sacrifice is shown with the best fit line for each hairpin ($n \geq 13$). **C.** Weights of primary control and BPTF KD B16F10 tumors after growth in C57BL/6 mice ($n \geq 9$; *, t test, $P < 1.4 \times 10^{-7}$). **D.** Primary B16F10 tumor weights in C57BL/6 mice treated with gemcitabine ($n \geq 4$, * = ttest pvalue < 0.05). **E.** Weights of B16F10 tumors after growth in undepleted, CD8⁺, CD4⁺, or asialo-GM1⁺ mAb-depleted C57BL/6 mice ($n \geq 5$; *, t test, $P < 0.02$).

3.5C). However, unlike in the 4T1 model, gemcitabine treatment did not selectively reduce BPTF KD B16F10 tumor growth (Fig. 3.5D).

To determine if CD4 and CD8 T cells are required for reduced B16F10 BPTF KD tumor growth, we repeated our mAb depletion experiments. Consistent with an enhanced T cell response, we observed a rescue of BPTF KD tumor growth with CD4+ and CD8+ cell

depletion, but not NK cell depletion (Fig. 3.5E). These results in combination support a model of an enhanced CD8 T cell cytotoxic response to BPTF KD tumors, which requires CD4 T helper cell activity.

3.4 CD8 T Cells are More Abundant and Activated in BPTF KD Tumors

Because a T cell-mediated antitumor response was required for a reduction of BPTF KD tumor growth, we then measured the infiltration and activation status of intratumoral CD8 T cells by flow cytometry. As expected, we observed a greater number of CD8 T cells (CD8+, TCRb+) in the BPTF KD tumor microenvironment (Fig. 3.6A and B). We next measured the abundance of activation markers CD69 and CD44 and the anergy marker BTLA on intratumoral CD8 T cells. We observed a greater percentage of CD69^{high} CD8+ cells in both 4T1 and B16F10 BPTF KD tumors (Fig. 3.6C and D). In contrast, CD44 and BTLA expression differed significantly on CD8 T cells between control and BPTF KD B16F10, but not 4T1, tumors (Fig. 3.6E-H). This could be the result of differences in the tumor microenvironment between 4T1 and B16F10 (see Discussion). Together, these results indicate that the BPTF KD tumor microenvironment has a greater number of active CD8 T cells.

Figure 3.6

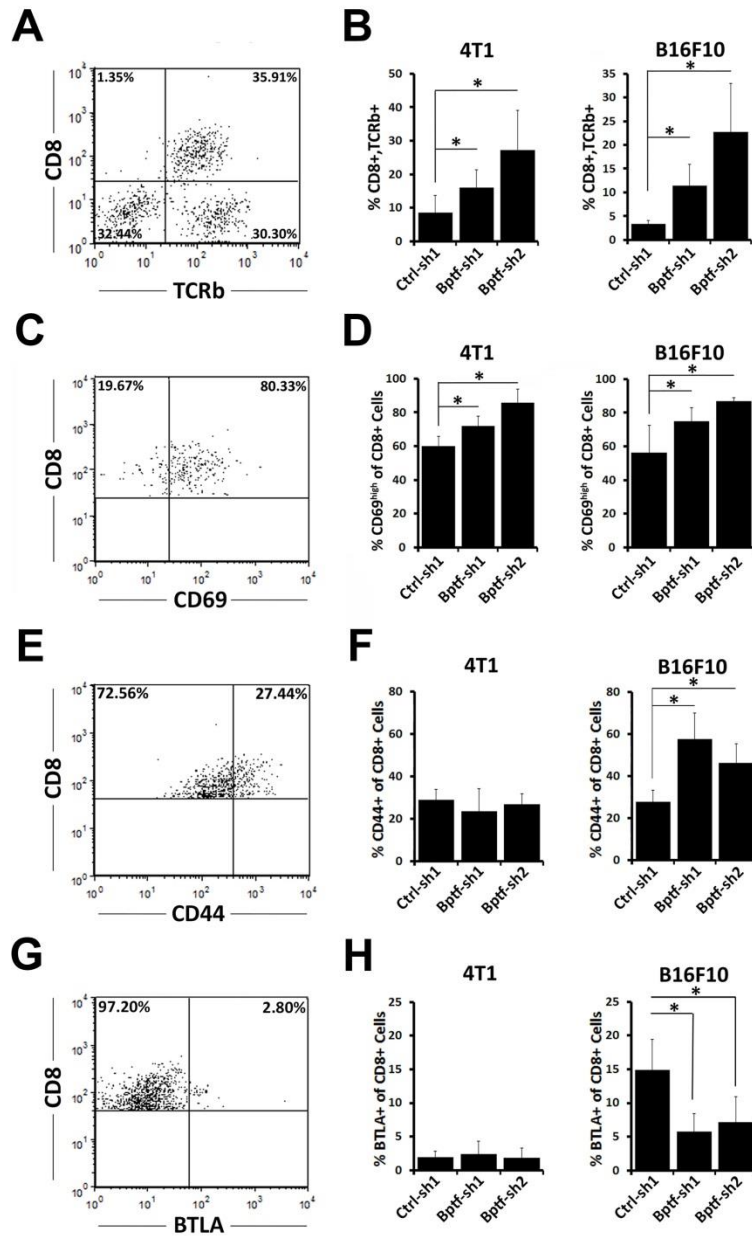


Figure 3.6: Enhanced presence and activity of CD8 T cells in BPTF KD tumors. A, C, E, and G. Representative dot plots of live 4T1 tumor-infiltrating lymphocytes stained for CD8 and TCRb, CD69, CD44, or BTLA, respectively. **B.** Percentages of live intratumor CD8⁺, TCRb⁺ lymphocytes as a percent of all live lymphocytes from 4T1 and B16F10 tumors (n ≥ 7; *, t test, P < 0.05). **D, F, and H.** Percentages of intratumor CD8⁺ lymphocytes that are CD69^{high}, CD44⁺, or BTLA⁺ from 4T1 and B16F10 tumors, respectively (n = 6; *, t test, P < 0.05).

3.5 CD8 T Cells have Enhanced Cytotoxic Activity Toward BPTF KD Cells *In Vitro*

To investigate if BPTF KD 4T1 and B16F10 tumor cells are more efficiently targeted by CD8 T cells, we performed *in vitro* cytotoxicity assays. Coculture of purified splenic CD8 T cells isolated from BPTF KD tumor bearing mice with BPTF KD targets results in enhanced cytolytic activity compared to similar experiments using controls (Fig. 3.7A).

We next determined if BPTF KD cells are more susceptible to T cell induced cell death. Toward this end, we used PMA + ionomycin activated naïve CD8 T cells and observed similar cytolytic activity between control and BPTF KD targets (Fig. 3.7B). These results demonstrate that enhanced CD8 T cell killing of BPTF KD target cells was not due to increased sensitivity to CD8 T cell mediated cell death.

To elucidate if BPTF KD cells are more antigenic than control cells, we used the OT1 and pmel CD8 T cell TCR transgenic models (392,393). The pmel TCR recognizes peptides from the endogenously expressed Pmel, whereas the OT1 TCR recognizes peptides from chicken Ovalbumin (OVA) presented by H2-Kb, which is expressed on B16F10, but not 4T1 (392,393).

Coculture experiments show that neither pmel nor OT1 CD8 T cells had enhanced reactivity to BPTF KD B16F10 cells (Fig. 3.7C and D) even though they expressed equivalent OVA and increased PMEL protein (Fig. 3.7E and F). Consistent with BPTF-independent OVA antigen presentation, an antibody which recognizes the OVA peptide (SIINFEKL) in context with H2-Kb equivalently stains control and BPTF KD B16F10 cells (Fig. 3.7G) (394). To further characterize the CD8 T cell response to

Figure 3.7

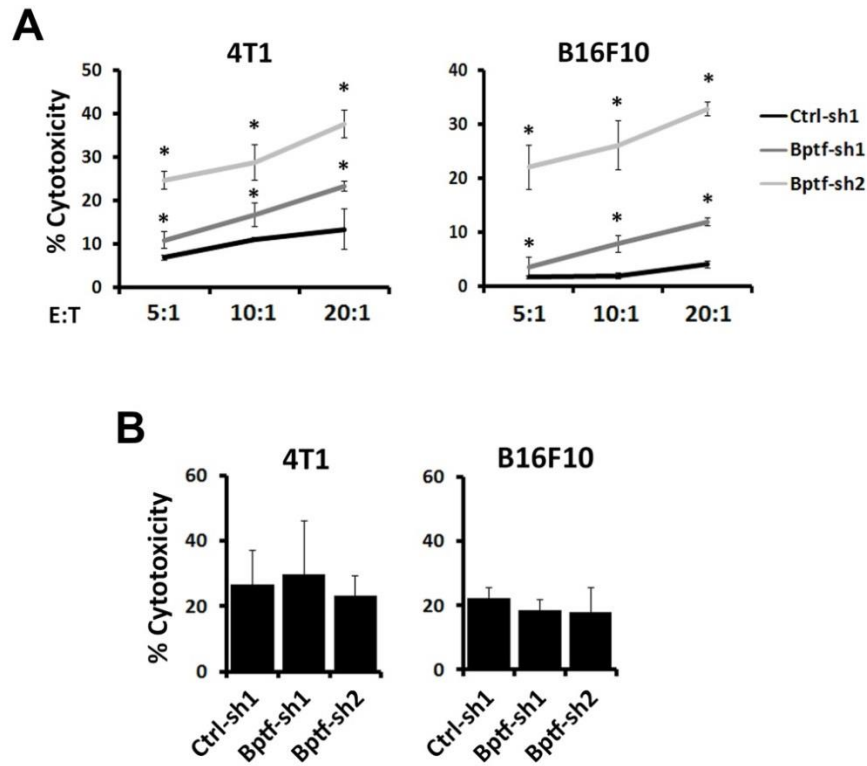


Figure 3.7: BPTF depletion sensitizes tumor cells to CD8 T-cell cytotoxicity in vitro. A-B. Percent target cell cytotoxicity determined by LDH release. **A.** Purified splenic CD8 T cells from control or BPTF KD tumor-bearing mice were cocultured on control or BPTF KD targets, respectively, at the indicated E:T ratios ($n = 3$; *, t test $P < 0.05$). **B.** Coculture of naïve purified CD8 T cells treated with PMA + ionomycin with 4T1 or B16F10 targets at a 10:1 E:T ratio.

Figure 3.7

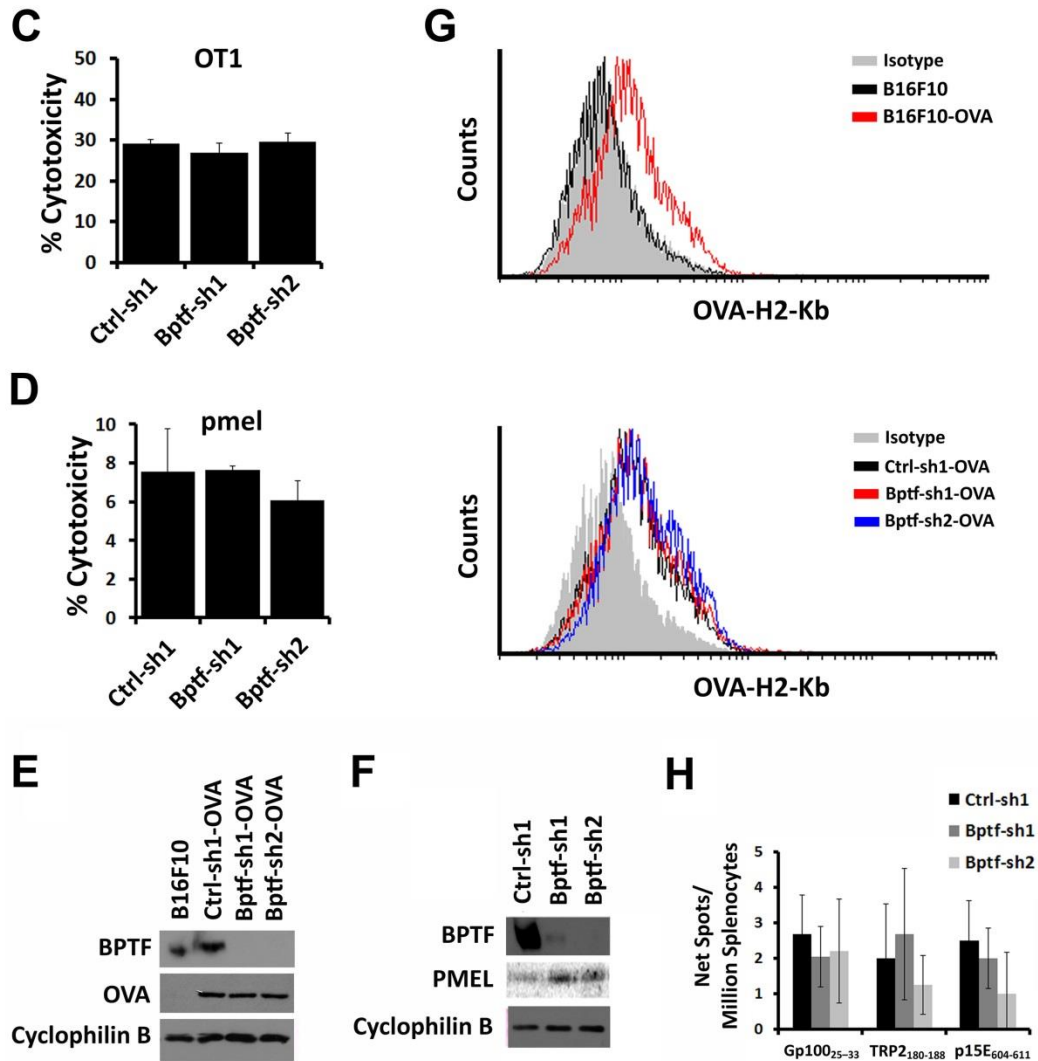


Figure 3.7: BPTF depletion sensitizes tumor cells to CD8 T-cell cytotoxicity in vitro. C-D, Percent target cell cytotoxicity determined by LDH release into the media. C. OT1 transgenic CD8 T cells were cocultured with B16F10-OVA targets at a 5:1 E:T ratio. D. pmel transgenic CD8 T cells were cocultured with B16F10 targets at a 20:1 E:T ratio. E. PMEL Western blot analysis of B16F10 total cell extracts. F. OVA Western blot analysis of B16F10-OVA expressing cell lines from total cell extracts. G. Flow cytometry analysis of OVA-H2-Kb presentation on B16F10-OVA cells. H. Peptide specific response of tumor infiltrating T cells determined by IFN γ ELISPOT (n = 3 biological replicates).

known antigens, we quantified gp100, TRP2, and p15E reactive intratumoral CD8 T cells by ELISPOT. From this we observed similar numbers of peptide reactive CD8 T cells between control and BPTF KD tumors (Fig. 3.7H). Together, these results suggest that enhanced CD8 T cell activity to BPTF KD tumors occurs due to novel antigens.

To ascertain if BPTF KD cells present novel antigens, we cloned CD8 T cells from both BPTF KD and control tumor bearing mice. Coculture assays showed enhanced activity of CD8 T cell clones from BPTF KD tumor bearing mice to BPTF KD target cells compared to similar experiments using controls (Fig. 3.7I). Furthermore, this enhanced activity usually occurs only when cocultured with BPTF KD, but not control, target cells (5 of 9 assayed clones)(Fig. 3.7J). In contrast, we do not observe enhanced cytolytic activity when CD8 T cells isolated from control tumor bearing mice are cocultured with BPTF KD target cells (0 of 4 assayed clones)(Fig. 3.7J). These results support the hypothesis that BPTF KD tumors express novel antigens.

3.6 BPTF Directly Regulates Antigen Processing

To identify BPTF-dependent genes which could alter tumor cell antigenicity, we performed genome wide expression arrays on 4T1 tumors harvested from NSG mice (Fig. 3.8A). We observed 115 upregulated genes and 199 downregulated genes (>1.5 fold change in expression, $FDR < 0.05$)(Fig. 3.8A). Of genes identified, we focused on *Psmb9* because it regulates antigenicity as a subunit of the immunoproteasome (395). We confirmed *Psmb9* upregulation *in vitro*, and observed elevated expression of the neighboring antigen processing genes *Psmb8*, *Tap1* and *Tap2* in both tumor models, though these genes were not significantly upregulated in our microarrays (Fig. 3.8B and C). The upregulation of these genes correlates with equivalent to slight increases in cell

Figure 3.7

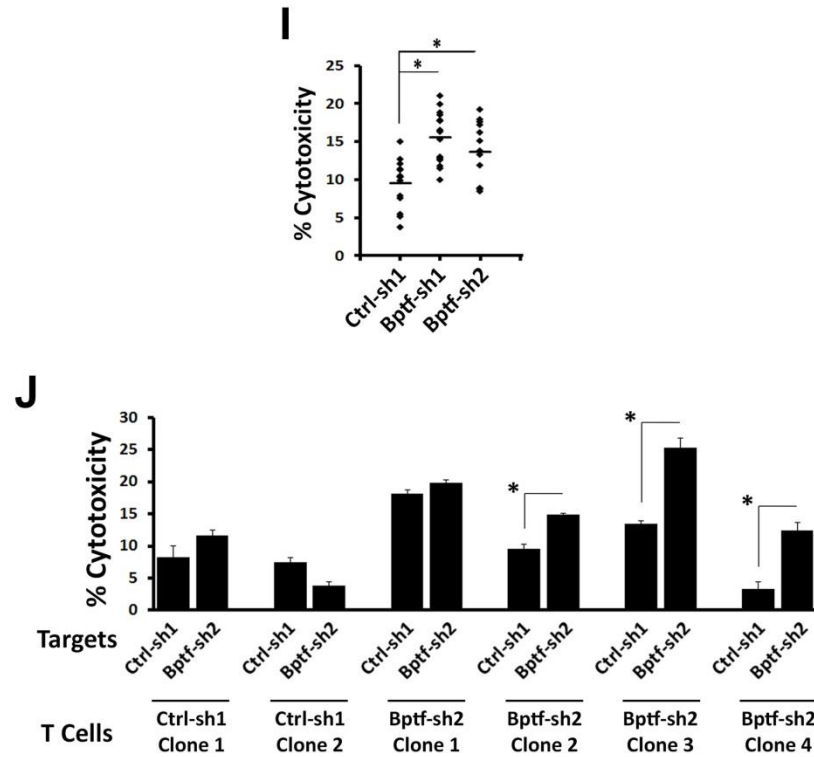


Figure 3.7: BPTF depletion sensitizes tumor cells to CD8 T-cell cytotoxicity in vitro. I-J, Percent target cell cytotoxicity determined by LDH release into the media. I, CD8 T-cell clones isolated from spleens of 4T1 control or BPTF KD tumor-bearing mice were cocultured with 4T1 control or BPTF KD targets, respectively, at a 10:1 E:T ratio. Each dot represents one clone and is an average of three biological replicates (*, t test, $P < 5.0 \times 10^{-3}$). J, Six representative CD8 T-cell clones from C were cocultured with either control or BPTF KD 4T1 targets at a 10:1 E:T ratio. Results are representative of three biological replicates for each clone (*, t test, $P < 0.04$).

Figure 3.8

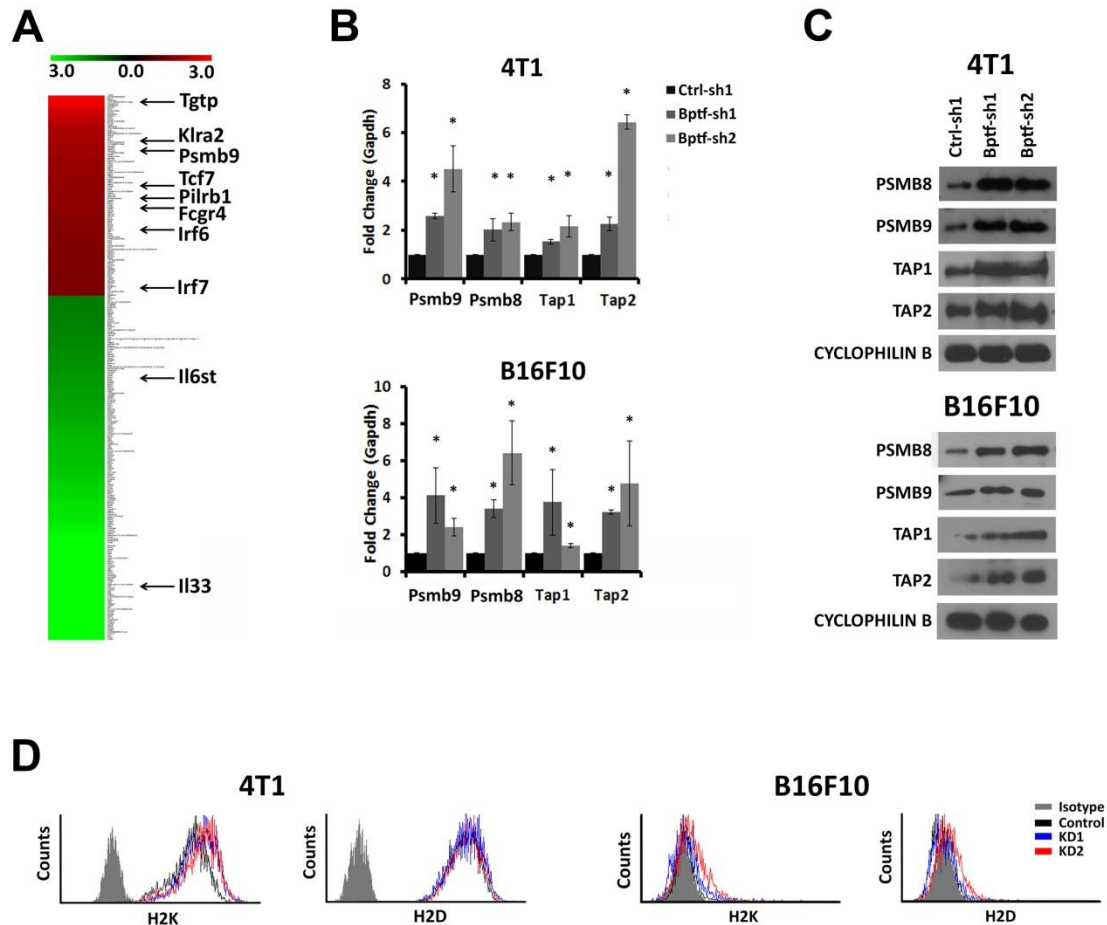


Figure 3.8: BPTF regulates immunoproteasome and TAP subunit expression. **A.** Microarray heat-map of genes significantly deregulated in BPTF KD 4T1 tumors from NSG mice ($n = 3$). Scale represents ± 3 -fold expression change. Genes related to the immune response are highlighted. **B.** qRT-PCR analysis of Psmb8, Psmb9, Tap1, and Tap2 expression from control or BPTF KD 4T1 and B16F10 cells ($n = 3$ biological replicates; *, t test, $P < 0.05$). **C.** PSMB8, PSMB9, TAP1, and TAP2 Western blot analysis of 4T1 (top) and B16F10 (bottom) total cell extracts. **D.** Representative flow cytometry histograms of 4T1 and B16F10 cells stained for the MHC class I molecules H2K and H2D.

surface expression of H2K or H2D in BPTF KD cells as measured by mean fluorescence intensity (MFI), which were not the result of increased gene expression (Fig. 3.8D-F). In addition, the expression of the interferon inducible genes *Oas1a* and *Oas2* were not upregulated with BPTF KD, indicating that upregulation of *Psmb8*, *Psmb9*, *Tap1* and *Tap2* are not a result of a general interferon response (Fig. 3.8G).

To determine if BPTF directly regulates the expression of *Psmb8*, *Psmb9*, *Tap1* and *Tap2*, we measured BPTF occupancy by chromatin immunoprecipitation (ChIP) (396). Sites chosen for ChIP were guided by previously identified DNaseI hypersensitivity hotspots, and therefore possible regulatory elements, from genome wide studies done in the 3134 mouse mammary epithelial cell line (Fig. 3.9A) (397). The most consistent enrichment of BPTF between 4T1 and B16F10 was detected at the promoters, consistent with BPTF directly repressing these genes (Fig. 3.9B and C). To determine if BPTF chromatin remodeling activity could be relevant to changes in gene expression, we focused on the well characterized *Psmb9-Tap1* divergent promoter (398), using formaldehyde assisted isolation of regulatory elements (FAIRE) (399). With this technique, open chromatin is isolated from fixed cells using phenol extractions and quantified by qPCR relative to a BPTF independent reference site. FAIRE shows that BPTF maintains chromatin structure of the *Psmb9-Tap1* promoter in both cell lines (Fig. 3.9D).

To verify that increased expression of the immunoproteasome subunits is responsible for the enhanced antigenicity of BPTF KD cells, we utilized the PSMB8 selective inhibitor ONX-0914 (400). Cytotoxicity assays show that enhanced CD8 T cell cytotoxicity to BPTF KD targets is ablated after treatment with ONX-0914 (Fig. 3.9E).

Figure 3.8

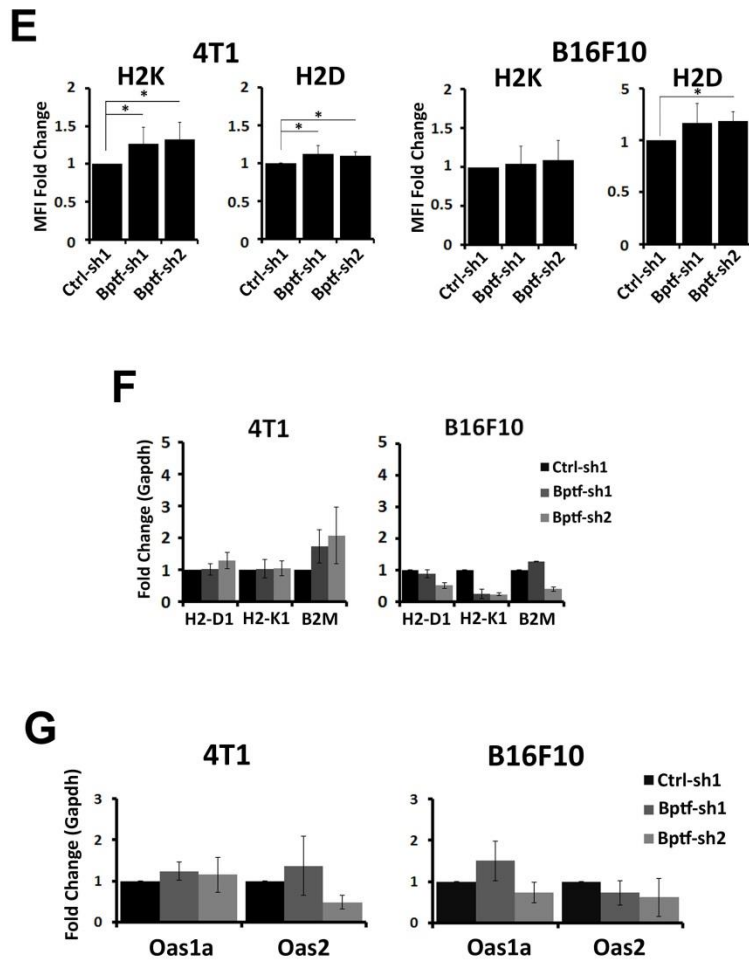


Figure 3.8: BPTF regulates immunoproteasome and TAP subunit expression. E. Fold change in MFI of H2K and H2D ($n \geq 3$ biological replicates; *, t test, $P < 0.05$). **F.** qRT-PCR analysis of MHC class I gene expression from 4T1 and B16F10 cells ($n = 3$ biological replicates). **G.** qRT-PCR analysis of Oas1a and Oas2 interferon type I induced genes in 4T1 and B16F10 cells ($n = 3$ biological replicates).

Figure 3.9

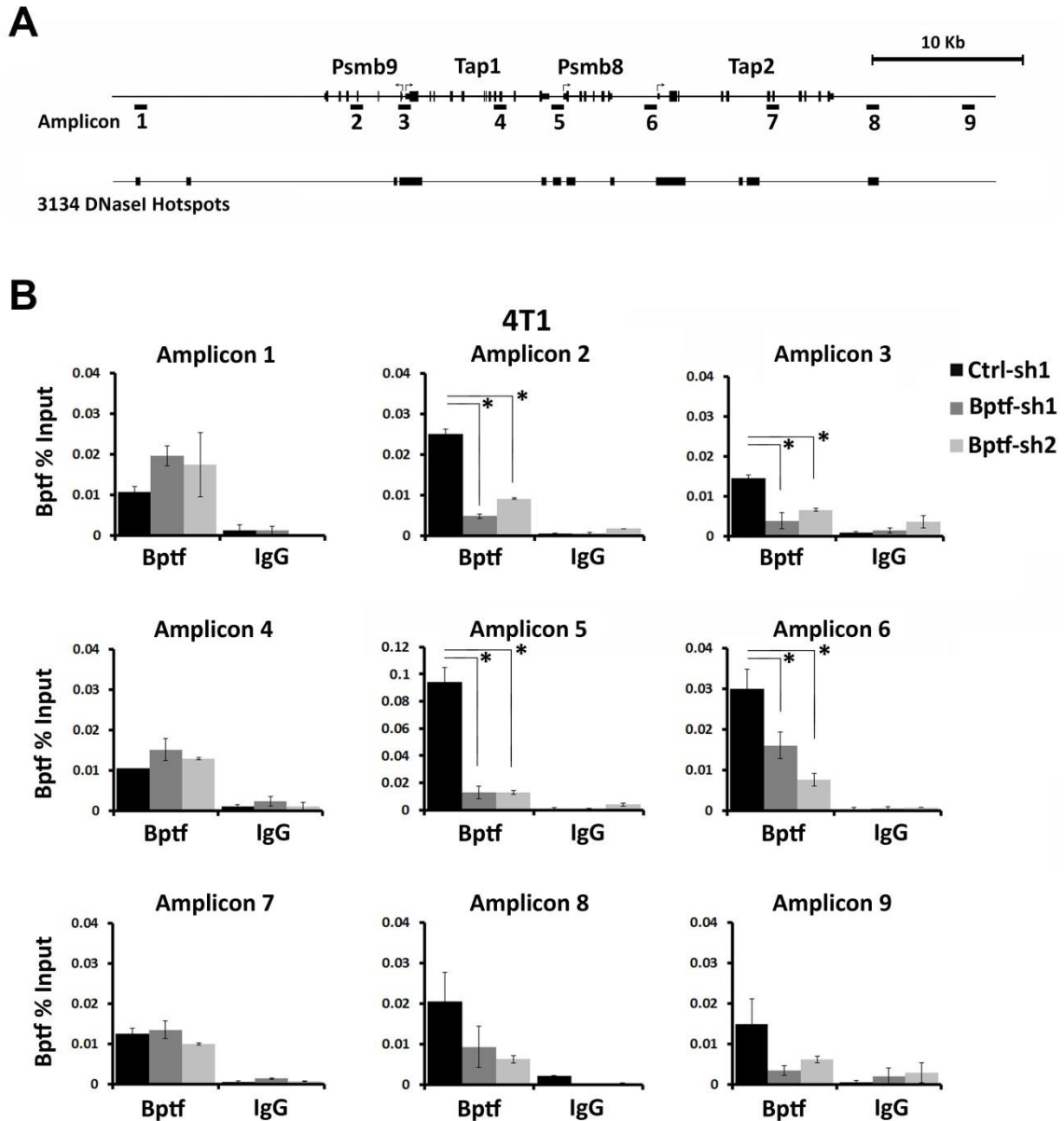


Figure 3.9: BPTF occupies and regulates chromatin structure at Psmb and TAP genes.
A. Schematic showing the position of PCR amplicons used for ChIP analysis and DNaseI hotspots from the mouse mammary epithelial line 3134. **B.** BPTF ChIP at nine sites surrounding Psmb9, Psmb8, Tap1, and Tap2 in 4T1 cells (n = 3 biological replicates; *, t test, $P < 0.05$).

Figure 3.9

C

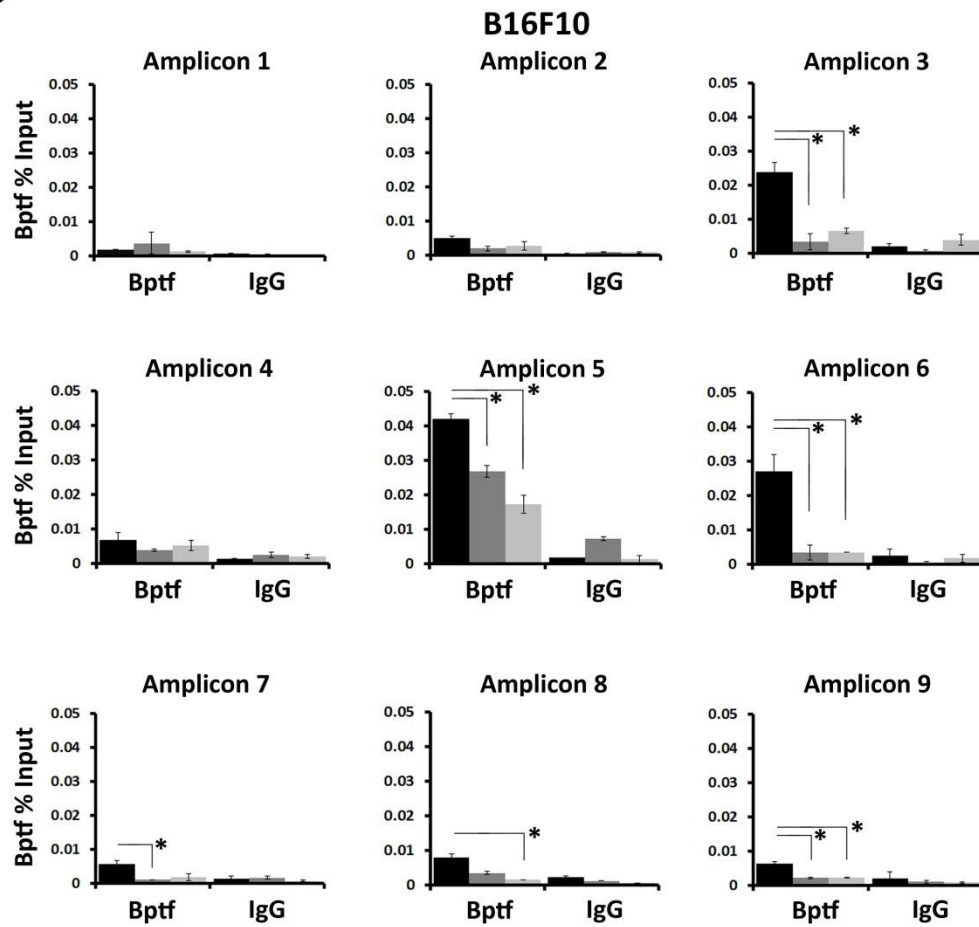


Figure 3.9: BPTF occupies and regulates chromatin structure at Psmb and TAP genes. C. BPTF ChIP at nine sites surrounding Psmb9, Psmb8, Tap1, and Tap2 in B16F10 cells (n = 3 biological replicates; *, t test, $P < 0.05$).

Figure 3.9

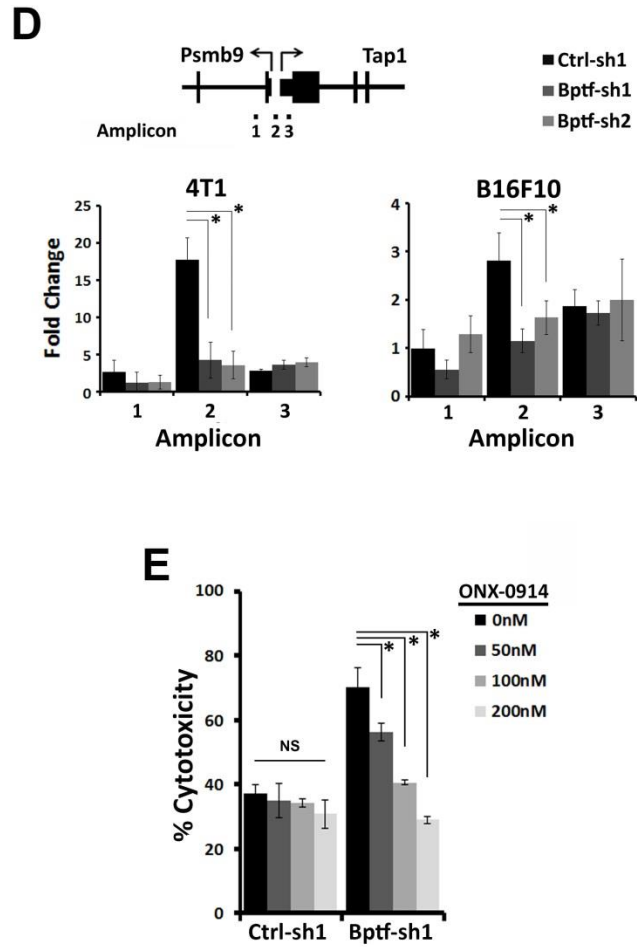


Figure 3.9: BPTF occupies and regulates chromatin structure at Psmb and TAP genes. D. FAIRE at the divergent Psmb9-Tap1 promoter. Values are normalized to a BPTF-independent control site (n = 3 biological replicates; *, t test, $P < 0.05$). **E.** Percent target cell cytotoxicity determined by LDH release for 4T1 splenocytes from control or BPTF KD tumor-bearing mice stimulated at a 50:1 E:T ratio on control or BPTF KD targets, respectively, after treatment with 50 to 200 nmol/L ONX-0914 (n = 3 biological replicates; *, t test, $P < 0.04$).

These results allow us to propose a model where BPTF depletion upregulates the antigen processing genes *Psmb8*, *Psmb9*, *Tap1* and *Tap2*, which results in enhanced antigenicity and improved T cell antitumor immunity. As a corollary, we propose that BPTF normally suppresses antitumor immunity by repressing antigen processing in cancer cells (Fig. 3.10).

Figure 3.10

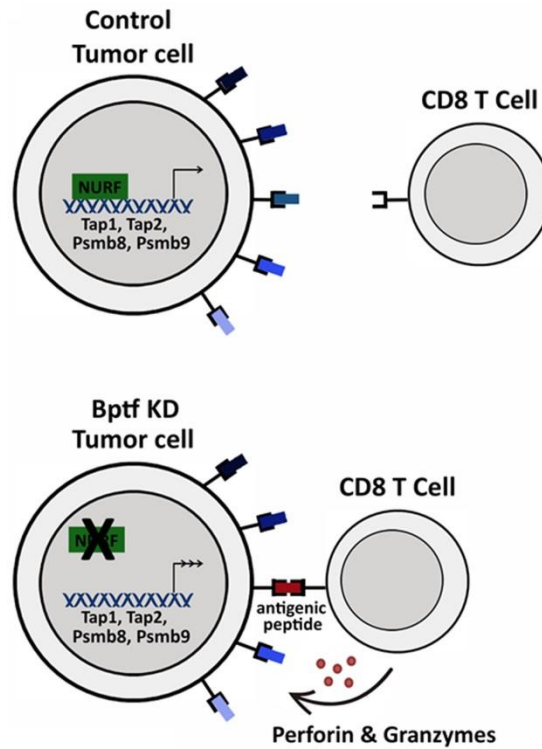


Figure 3.10: Model for NURF regulation of tumor cell antigenicity. In tumor cells, NURF represses the expression of the immunoproteasome subunits Psmb8 and Psmb9 and the TAP complex subunits Tap1 and Tap2. This results in the processing and presentation of poorly antigenic peptides. When NURF is depleted, expression of these genes is increased, leading to the processing of novel antigenic peptides by the immunoproteasome, thereby enhancing CD8 T cell-mediated antitumor immunity

Chapter 4

BPTF Inhibits the NK Cell Antitumor Response by Suppressing Natural Cytotoxicity Receptor Co-ligands

4.1 NK Cell-Mediated Antitumor Immunity is Enhanced to BPTF-Depleted Breast Tumors

To further investigate roles for NURF in breast cancer cell biology, we transduced the well-established 67NR and 66cl4 mouse breast cancer cell lines with retroviruses expressing control or BPTF shRNAs (Fig. 4.1A) (376). In culture we observed equivalent doubling times, cellular morphology, and levels of apoptosis for BPTF KD cells compared to controls (Fig. 4.1B-D). Control and BPTF KD 66cl4 or 67NR lines were then transplanted into the 4th mammary fat pad of syngeneic BALB/c mice. After 3-4 weeks, we observed reduced weight of BPTF KD tumors compared to the controls, consistent with results observed the 4T1 and B16F10 lines (Fig. 4.2A). Tumor weights were used instead of volume to measure growth because BPTF KD tumors grow flat, confounding volume-based comparisons to controls (see Fig. 3.2). Microarray expression profiling of control and BPTF KD tumors discovered an enrichment of genes with gene ontology (GO) terms which included immune response descriptors (Fig. 4.2B). In agreement with microarray data, KEGG analysis of a combined gene list from both tumor types also identified an abundance of genes involved in the immune response, immune cell signaling, and chemokine signaling (Fig. 4.2C) (401). To confirm the importance of the immune response for BPTF KD tumor growth, our tumor growth studies were repeated in an immune deficient NSG background. These experiments

Figure 4.1

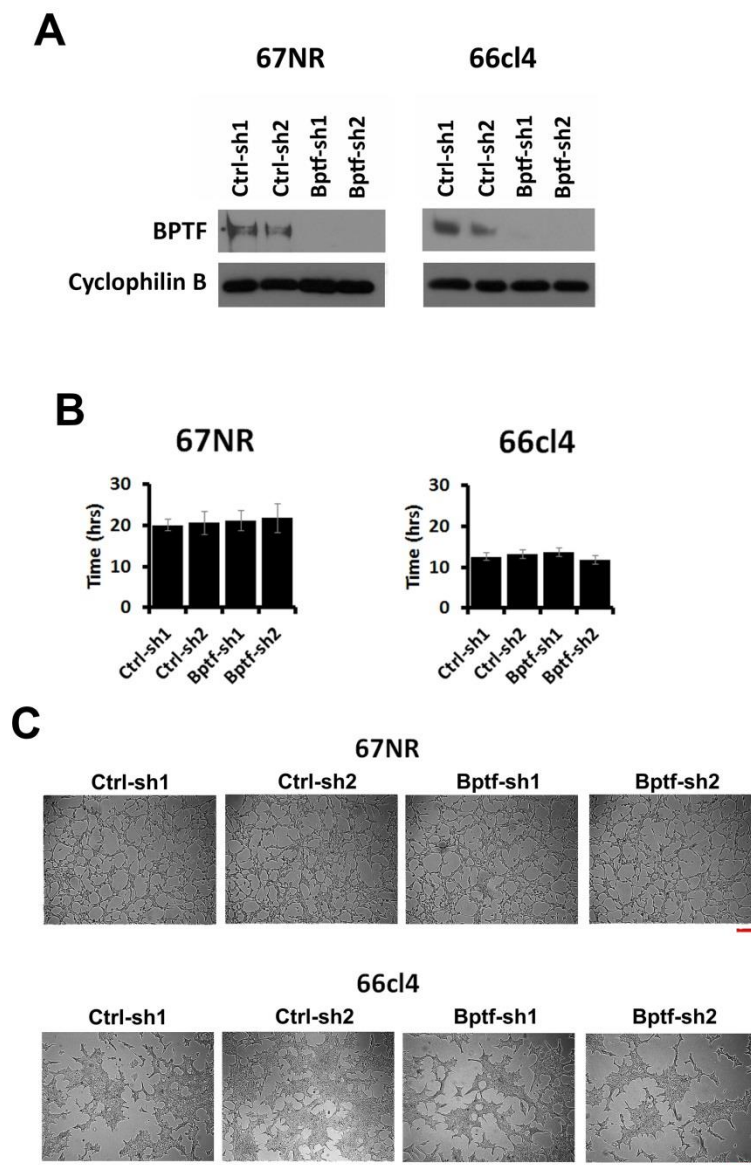


Figure 4.1: BPTF does not affect 66cl4 or 67NR proliferation, cellular morphology or apoptosis in vitro. A. BPTF Western blot analysis from control and BPTF KD 67NR and 66cl4 total cell extracts. **B.** Doubling time of control and BPTF KD 67NR and 66cl4 cultures (n = 3 biological replicates). **C.** Images of control and BPTF KD 67NR and 66cl4 cells in culture. Scale bar = 150 μ m

Figure 4.1

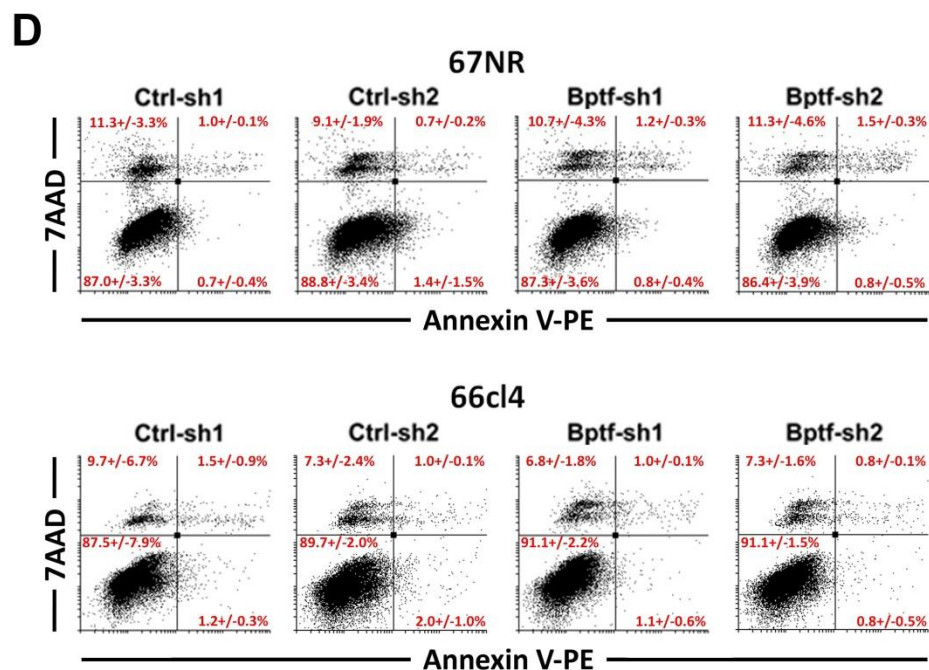


Figure 4.1: BPTF does not affect 66cl4 or 67NR proliferation, cellular morphology or apoptosis in vitro. D. Apoptosis was measured on cultured cells using Annexin-V and 7AAD and analyzed by flow cytometry. Populations from each quadrant are shown as percentages with standard deviations for 3 independent measurements. Data shown represent mean \pm stdev.

Figure 4.2

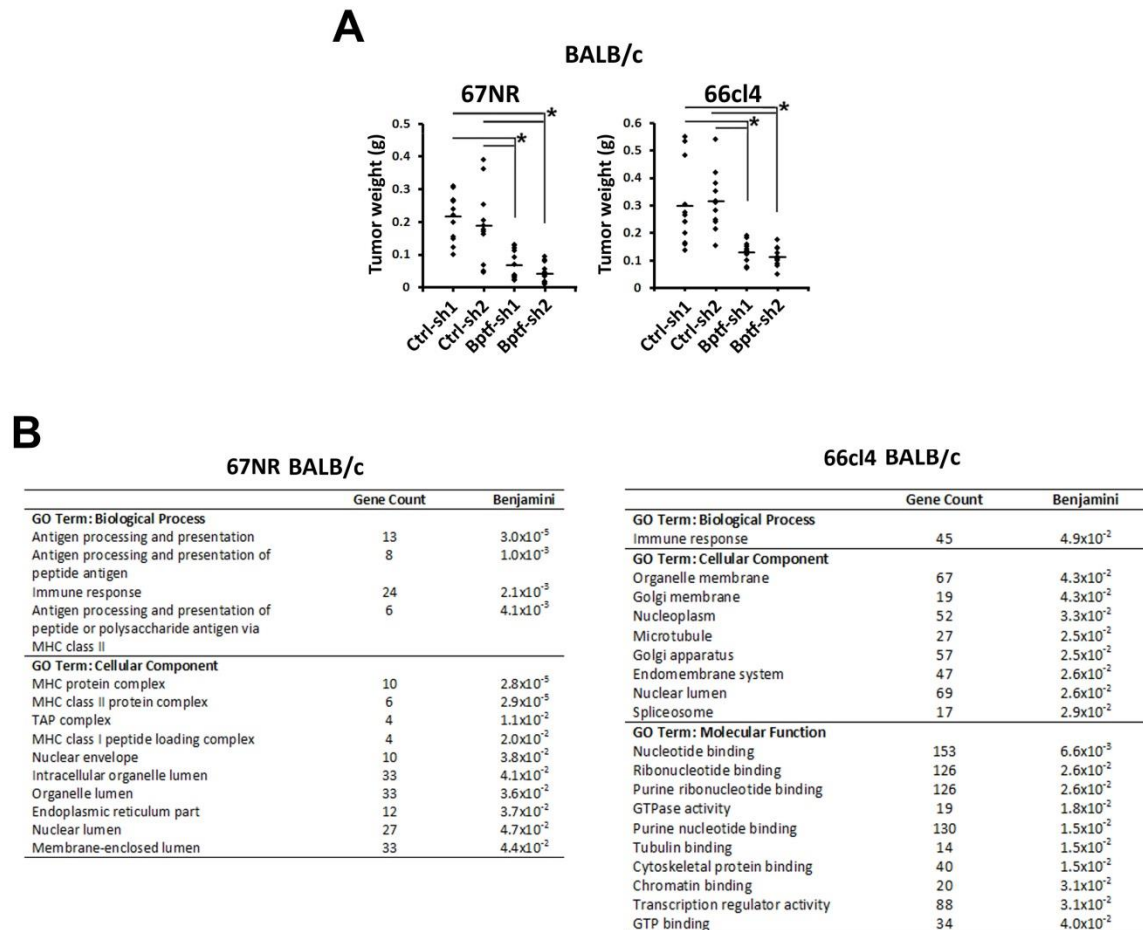
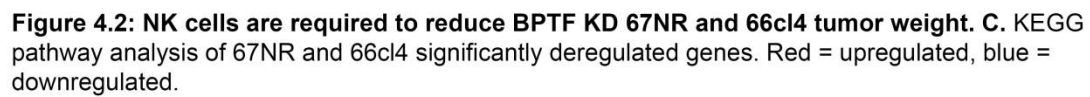


Figure 4.2: NK cells are required to reduce BPTF KD 67NR and 66cl4 tumor weight. A. 67NR and 66cl4 tumor weights harvested from BALB/c mice ($n \geq 11$ biological replicates, * = ttest pvalue < 0.003). **B.** A DAVID gene ontology (GO) analysis on BPTF-dependent genes identified from microarray gene profiling analysis on 67NR and 66cl4 tumors harvested from BALB/cJ mice.

C



showed equivalent BPTF KD tumor weights to controls, demonstrating the immune system is required to reduce the growth of BPTF KD tumors (Fig. 4.2D).

To identify immune cells that are important for reducing BPTF KD tumor weights, we repeated our tumor growth studies in mice depleted of NK cells, CD8⁺ T cells, or CD4⁺ T cells by mAb treatments. We observed improved growth of 67NR and 66cl4 BPTF KD tumors with NK cell depletion, but not with CD8⁺ or CD4⁺ T-cell depletion, indicating that NK cells are required for reduced BPTF KD tumor growth (Fig. 4.2E). Surprisingly, control 66cl4 tumor weights were reduced with NK depletion, suggesting tumor promoting NK cell functions in this tumor type (see discussion)(Fig. 4.2E).

4.2 NK Cell Cytotoxic Activity is Enhanced to BPTF Depleted Cancer Cells *In Vitro*

Coculture of purified splenic mouse NK cells with 67NR and 66cl4 cells showed enhanced NK cell cytolytic activity against BPTF KD targets (Fig. 4.3A). To determine if BPTF KD cells are more sensitive to NK cell-mediated killing, the cytolytic activities of NK cells were activated independent of ligand using PMA + ionomycin (P+I). P+I activated NK cells killed control and BPTF KD targets equivalently in coculture, indicating that BPTF KD targets are not more sensitive to lysis by NK cells (Fig. 4.3B). To determine if soluble factors secreted by BPTF KD targets enhance NK cell activity, mouse NK cells were pre-incubated with media from cultured control or BPTF KD 67NR or 66cl4 tumor cells prior to their use in the cytolytic assay. From these experiments, we observed enhanced NK cell cytolytic activity toward BPTF KD targets independent of preconditioning with media from control or BPTF KD cultures (Fig. 4.3C). These results in combination demonstrate that changes on the surface of BPTF KD tumor cells improve NK cell activation and cytolytic activity.

Figure 4.2

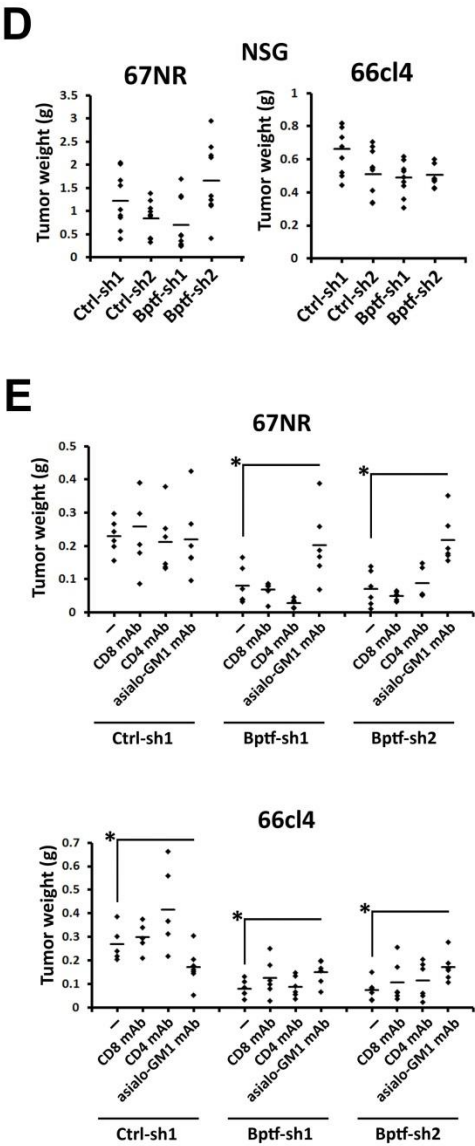


Figure 4.2: NK cells are required to reduce BPTF KD 67NR and 66cl4 tumor weight. D. 67NR and 66cl4 tumor weights harvested from NSG mice (n = 9 biological replicates). **E.** 67NR and 66cl4 tumor weights harvested from undepleted, or CD8+, CD4+ or asialo-GM1+ mAb depleted BALB/c mice (n = 6 biological replicates, * = ttest pvalue < 0.05). Some dots overlap.

Figure 4.3

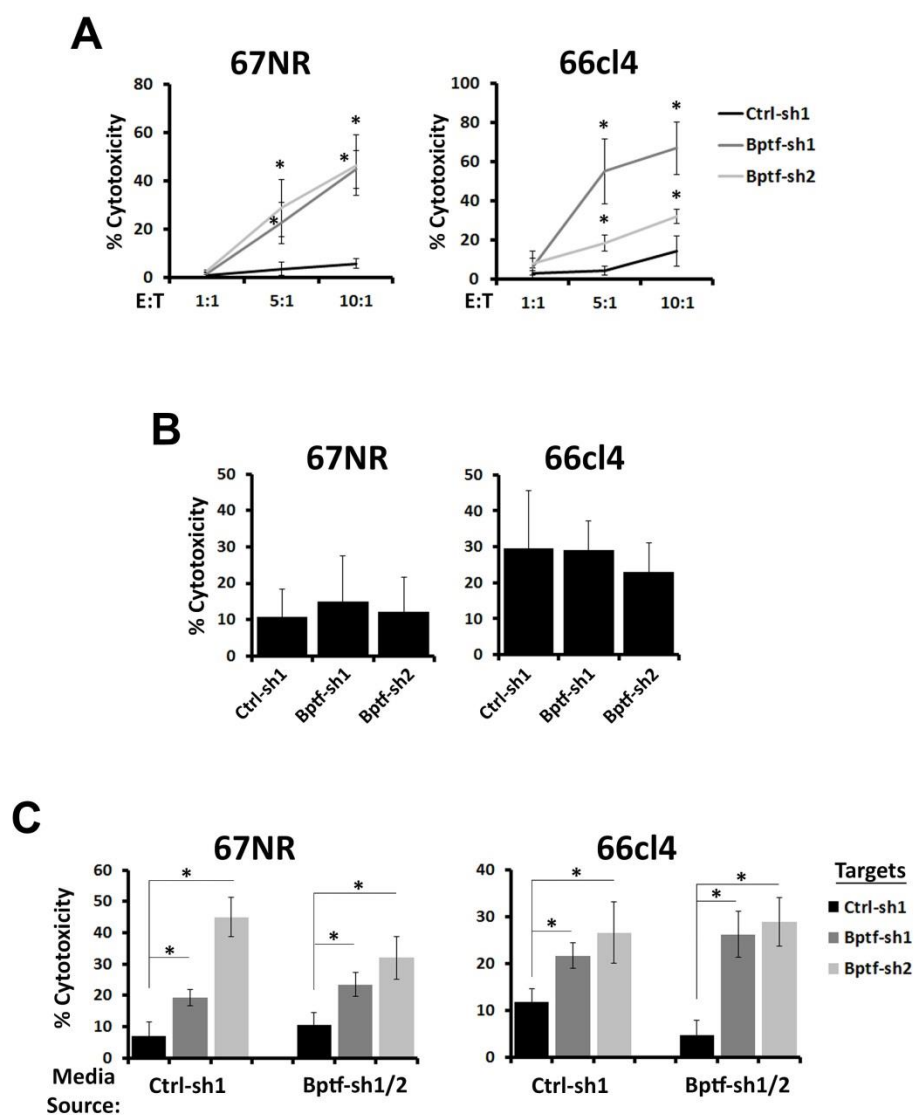


Figure 4.3: NK cells have greater cytolytic activity to BPTF KD targets. A-C. Percent target cell cytolytic activity by LDH assay. **A.** NK cells from naive BALB/c mice were cocultured with targets at the indicated effector:target (E:T) ratios (n = 3 biological replicates, * = ttest pvalue < 0.05). **B.** Cytolytic activity of mouse NK cells treated with PMA + Ionomycin and cocultured with 67NR and 66cl4 targets at a 5:1 E:T ratio (n = 3 biological replicates). **C.** Mouse NK cells were preincubated with either fresh media or media harvested from control or BPTF KD tumor cells before assay on control or BPTF KD targets at a 5:1 E:T ratio (n = 3 biological replicates, * = ttest pvalue < 0.05).

4.3 BPTF Regulates Cell Surface HSPG Abundance and *Hpse* Expression

We conducted microarray gene expression analyses of control and BPTF KD 67NR and 66cl4 tumors from NSG mice to identify candidate BPTF-regulated NK cell receptor ligands on tumor cells. Tumors from NSG mice were used for these analyses because they lack gene expression changes resulting from tumor infiltrated immune cells, and therefore could more reliably identify BPTF-regulated gene targets. Analysis of these data sets focused on activating receptor ligands because the inhibitory MHC ligands are not significantly BPTF regulated in 67NR or 66cl4 cells (Fig. 4.4A). Our microarray experiments identified two BPTF regulated genes with functions in HS metabolism but did not identify any known activating NK cell receptor ligands (Fig. 4.4B and C). HS and HSPGs are well characterized co-ligands for the NCRs, which are required for efficient NK cell-mediated tumor cell killing (402). Unfortunately, the main HS metabolizing enzyme, heparanase, was not included on the microarray design. To compensate, we used qRT-PCR to measure its gene expression from the tumor samples and discovered significant reductions in *Hpse* transcripts with BPTF KD (Fig. 4.4B).

Because HS is a known co-ligand to all NCRs, it seemed plausible that the enhanced NK cell cytolytic activity to BPTF KD cells occurs through the NCRs. Consistent with these results, a similar reduction in mouse NK cell cytolytic activity was observed to BPTF KD 66cl4 cells after NCR1 blocking (Fig. 4.4D) (403). Also, the addition of the NCR competitive inhibitor heparin erased the mouse NK cell cytolytic activity against 67NR and 66cl4 BPTF KD targets (Fig. 4.4E) (82). These results support the hypothesis that BPTF has functions in regulating the abundance of NCR ligands on tumor cells to influence NK cell cytolytic activity.

Figure 4.4

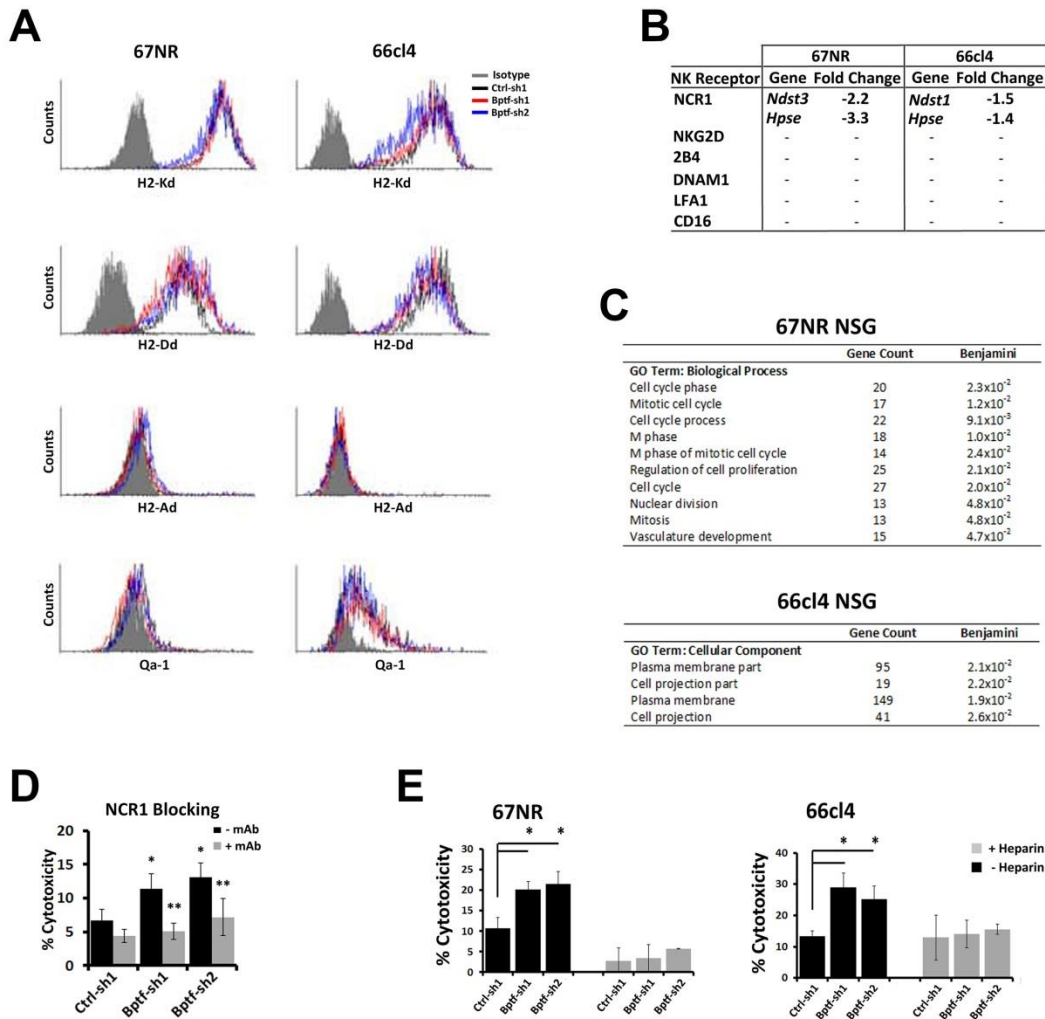


Figure 4.4: NK cell cytolytic activity to BPTF KD cells requires NCR receptors. **A.** Representative flow cytometry histograms of MHC molecules from control and BPTF KD 67NR and 66cl4 cells. **B.** Changes in NK receptor ligand expression measured by microarray in BPTF KD tumors harvested from NSG mice compared to controls. *Hpse* measured independently by qRT-PCR because it was not on the microarray (* = ttest pvalue < 0.00002). **C.** Microarray GO analysis of BPTF-dependent genes identified by microarray gene expression profiling on 67NR and 66cl4 tumors from NSG mice. **D and E.** Percent target cell cytolytic activity by LDH assay. **D.** Mouse NK cells pretreated with anti-NCR1 blocking mAb (clone mNCR1.15) were cocultured with 66cl4 targets at a 5:1 E:T ratio (n = 3 biological replicates, * = ttest pvalue < 0.04). (* = significant to no mAb Ctrl-sh1, ** = significant to the respective no mAb hairpin). **E.** Mouse NK cells were cocultured on 66cl4 or 67NR targets at a 5:1 E:T ratio with or without heparin (n = 3 biological replicates, * = ttest pvalue < 0.05).

A recombinant NCR1-Ig fusion protein was then used to measure the abundance of NCR1 ligands on the surface of BPTF KD 67NR and 66cl4 cells. From these experiments, we discovered enhanced binding of NCR1-Ig to BPTF KD cells compared to controls (Fig. 4.4F and G). These results demonstrate that enhanced NK cell cytolytic activity results from an overabundance of NCR1 ligands.

The major regulator of cell surface HS abundance, in the form of HSPGs, in mammals is heparanase (404). Our expression analysis identified *Hpse* as a BPTF-regulated gene in both 67NR and 66cl4 BPTF KD tumors (Fig. 4.4B). These results were confirmed in culture for the 67NR and 66cl4 cells and in three human lines (Fig. 4.5A and B). Consistent with reductions in *Hpse* gene expression, Western blotting discovered significant reductions of cell surface HPSE abundance with BPTF KD (Fig. 4.5C). The importance of reduced cell surface HPSE for enhanced NK cell binding to BPTF KD cells was investigated using bacterial heparinase treatments. Pretreatment of 66cl4 tumor cells with bacterial heparinase suppressed the enhanced binding of NCR1-Ig to BPTF KD cells (Fig. 4.5D). Bacterial heparinase pretreatment also reduced the enhanced activity of NK cells to BPTF KD targets (Fig. 4.5E). These results suggest that increases in HS abundance on HSPGs with BPTF KD results in enhanced NK cell cytolytic activity. To determine if decreased *Hpse* expression with BPTF KD correlates with changes in cell surface HSPGs, we measured HSPG abundance by Western blotting cell surface protein extractions using antibodies to HS. Three HSPGs from these experiments had increased levels in BPTF KD cells: a ~150 kDa band, a ~55 kDa band and a ~45 kDa band (Fig.4.5F – see arrows). It is unlikely that expression of the

Figure 4.4

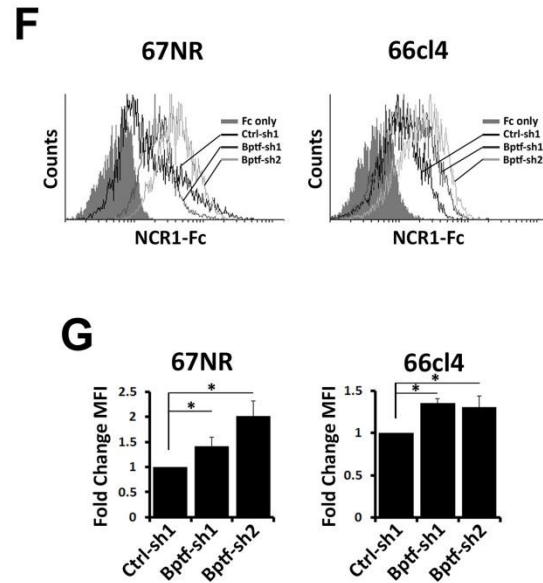


Figure 4.4: NK cell cytolytic activity to BPTF KD cells requires NCR receptors. F. Representative flow cytometry histograms of NCR1-Ig binding to 67NR and 66cl4. **G.** Fold change of MFI from D (n = 3 biological replicates, * = ttest pvalue < 0.03).

Figure 4.5

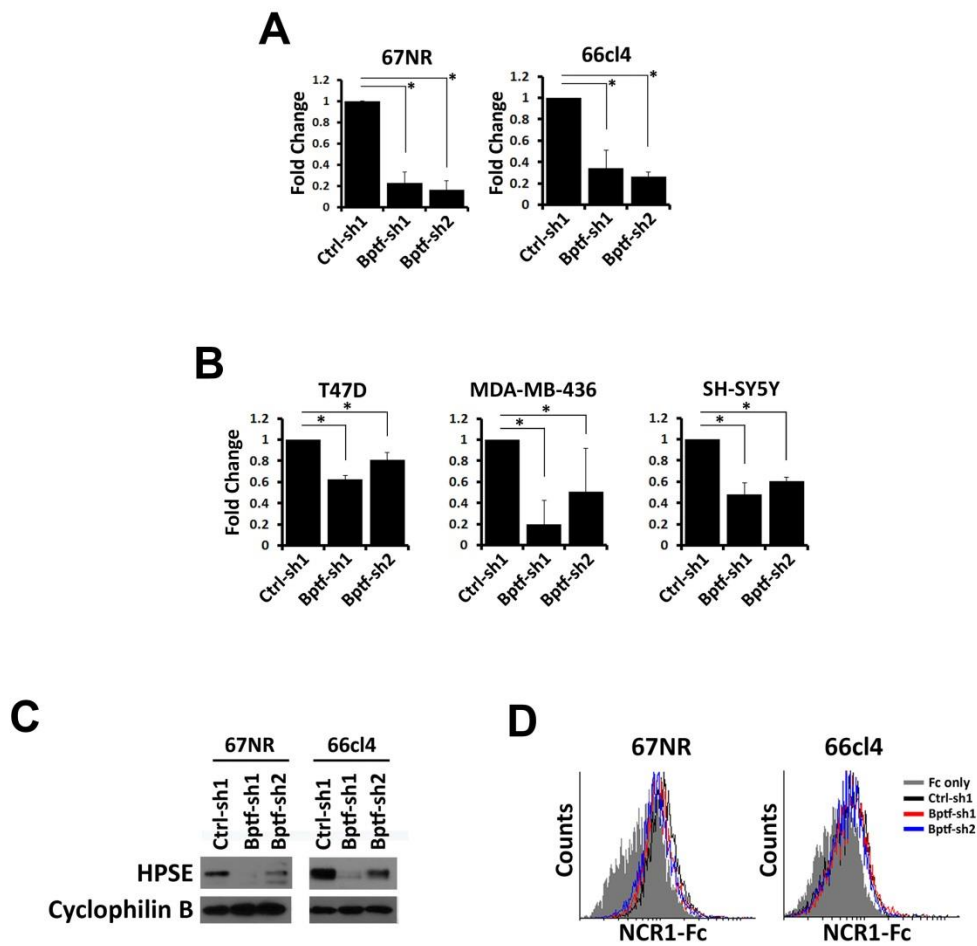


Figure 4.5: BPTF regulates Hps expression. **A.** qRT-PCR analysis of Hps expression from 67NR and 66cl4 cells (n = 3 biological replicates, * = ttest pvalue < 0.006). **B.** qRT-PCR analysis of HPSE expression in T47D, MDA-MB-436 and SH-SY5Y cells (n = 3 biological replicates, * = ttest pvalue < 0.05). **C.** Cell surface HPSE Western blot analysis from 67NR and 66cl4 cells. **D.** Representative flow cytometry histogram of NCR1-Ig binding to bacterial heparinase treated tumor cells.

Figure 4.5

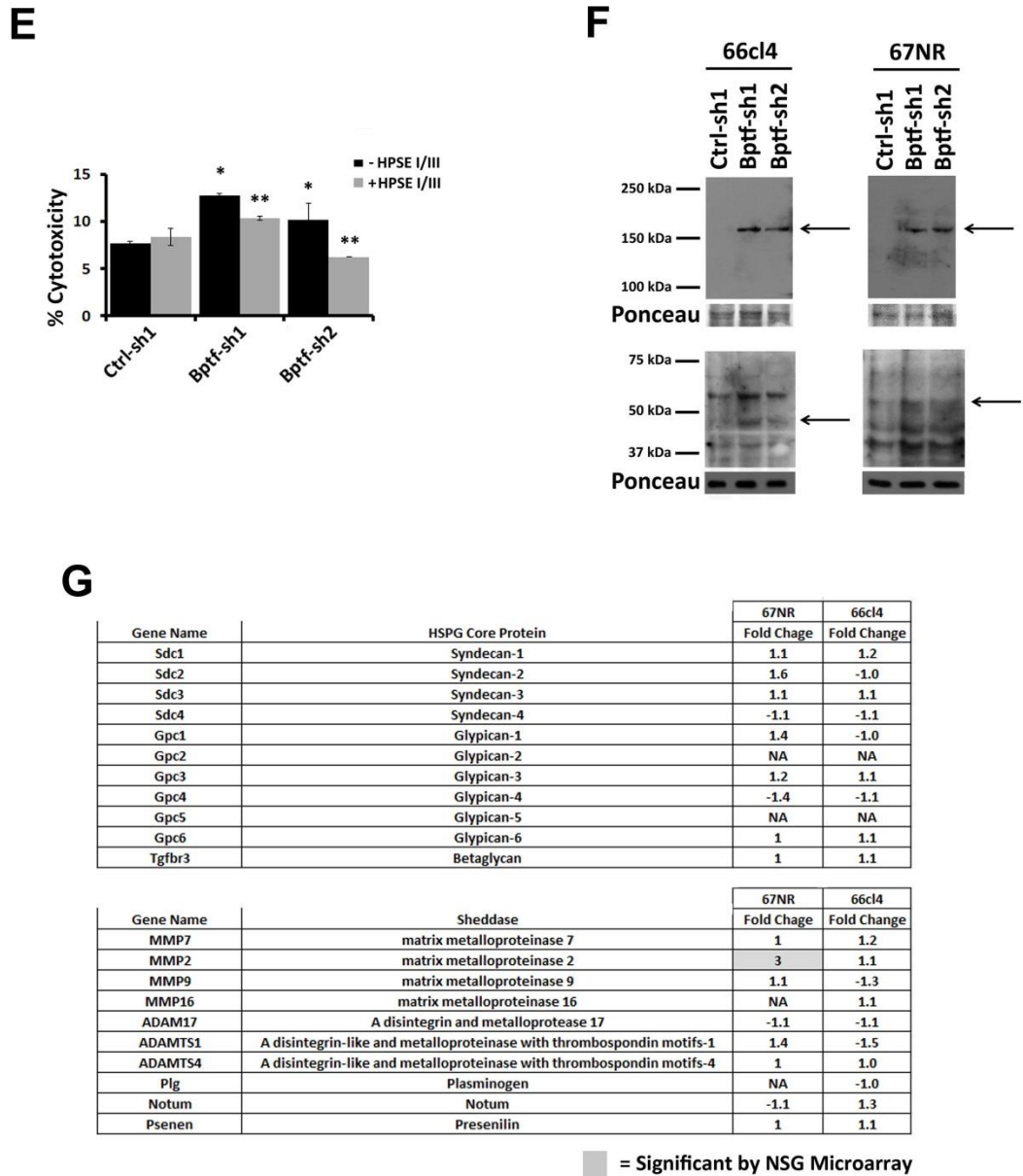


Figure 4.5: BPTF regulates Hpse expression. **E.** Percent cytotoxicity. 66cl4 cells pretreated with bacterial heparinase I/III were cocultured with mouse NK cells at a 5:1 E:T ratio (n = 3 biological replicates, * = ttest pvalue < 0.05)(* = significant to no mAb Ctrl-sh1, ** = significant to the respective no mAb hair-pin). **F.** Cell surface HSPG Western blot analysis using anti-HS primary mAb (MAB2040). Arrows: reproducible changes in HSPG abundance with BPTF KD. **G.** Fold change of HSPG core proteins and sheddases from microarray datasets obtained from 67NR and 66cl4 tumors from NSG mice. Data shown represent mean \pm stdev.

HSPG core proteins are BPTF-dependent because they were not BPTF-dependent from the microarray analyses (Fig. 4.5G). Additionally, only the MMP2 HSPG sheddase was deregulated in 67NR, but not 66cl4, BPTF KD NSG tumors suggesting that HSPG cell surface shedding is not BPTF-dependent (Fig. 4.5G). BPTF functions in regulating *Hpse* expression could be direct because ChIP revealed BPTF occupancy broadly localized at the *Hpse* gene in 67NR and localized at the *Hpse* promoter in 66cl4 (Fig. 4.5H). These results suggest that BPTF regulates *Hpse* expression, possibly through direct functions at its promoter. From these results, we propose a model where NURF directly activates *Hpse* in cancer cells, elevating cell-surface heparanase and reducing cell-surface HSPGs. Because HSPGs are known co-ligands for NCRs, our model predicts that NURF suppresses direct NCR-mediated NK cytolytic activity by upregulating heparanase levels (Fig. 4.6).

Figure 4.5

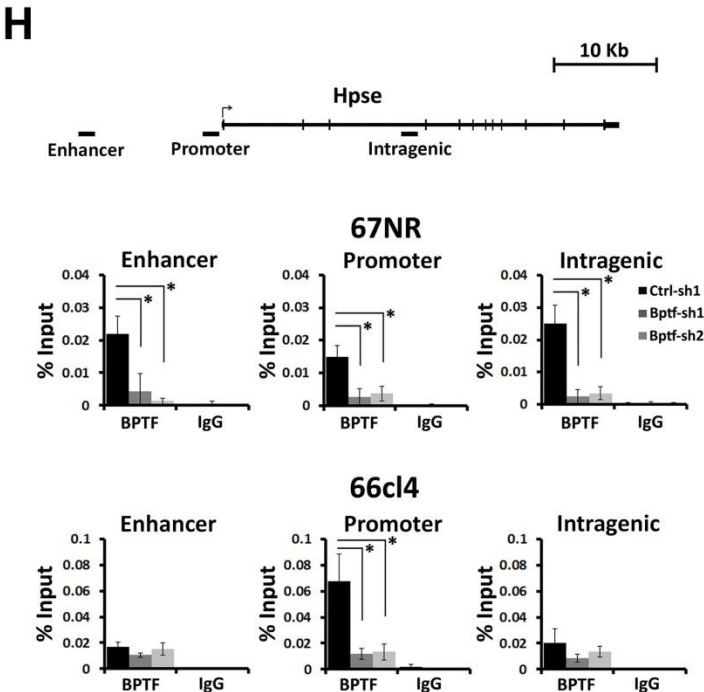


Figure 4.5: BPTF regulates Hpse expression. H. BPTF ChIP at mouse Hpse (n = 3 biological replicates, * = ttest pvalue < 0.04).

Figure 4.6

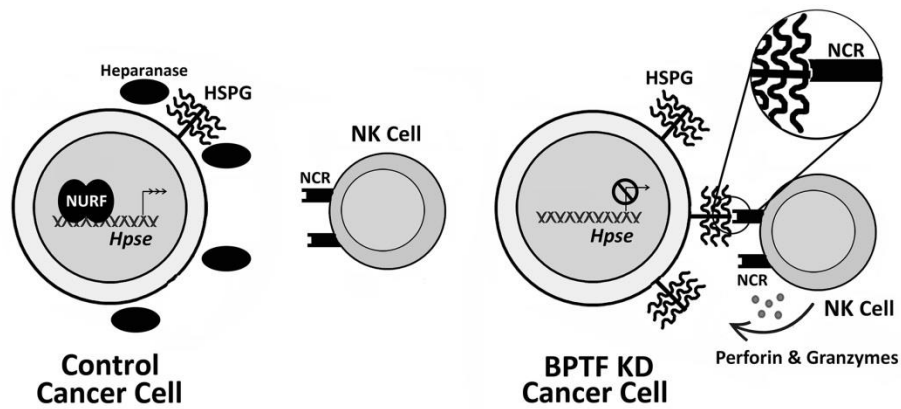


Figure 4.6: Model for NURF regulation of cell surface co-ligand to NCR1. In tumor cells NURF stimulates *Hpse* expression, and as a result cell surface heparanase abundance. Increased heparanase abundance reduces cell surface HSPGs and NCR HS co-ligand abundance, inhibiting NK-cell antitumor activity. When NURF is depleted, *Hpse* expression and heparanase is reduced. Reduced heparanase abundance increases cell surface HSPGs and NCR HS co-ligands, improving NK cell-mediated antitumor activity.

Chapter 5

Discussion and Future Directions

5.1 Discussion

Understanding how tumors escape the immune response is of great interest to tumor immunologists. Epigenetic escape mechanisms, most prominently DNA methylation and histone acetylation, suppress tumor cell antigenicity to inhibit T cells and ligand expression to inhibit NK cells (405). Because of its importance in gene expression, chromatin remodeling is predicted to have similar functions in tumor cells. A variety of approaches have been developed to improve antitumor immunity, including enhancing tumor cell antigenicity, blocking immunosuppressive checkpoints, enhancing effector cell functions and depleting immunosuppressive cell populations (406). Using several of these approaches in combination is likely required for lasting therapeutic outcomes due to tumor adaptation. For combinatorial treatment regimens to be effective, novel and varied means to enhance antitumor immunity must be developed. Toward this end, we investigated if the epigenetic regulator NURF regulates antitumor immunity and if it could be leveraged to develop a novel immunotherapeutic approach.

5.1.1 NURF Depletion Enhances Tumor Cell Antigenicity and T Cell Antitumor Immunity

Consistent with NURF depletion improving antitumor immunity, we observed reduced tumor growth when either BPTF KD B16F10 cells are introduced into immune competent mice or when BPTF KD 4T1 cells are introduced into gemcitabine treated immune competent mice. In the case of 4T1, gemcitabine treatments are likely required

to remove the significant immunosuppressive effects MDSC cells have on CD8+ effector T cells in this model (407). We also show that 4T1 metastasis to the lung is correlated with tumor weight and not BPTF KD. Because tumor size is often correlated with metastatic burden, and BPTF KD tumors are smaller than control tumors, these results do not rule out a role for BPTF in metastasis. To better understand the effect of BPTF KD in metastasis, we would need to measure lung colony formation after tail vein injection of tumor cells to eliminate tumor burden as a factor.

ADCC studies showed that CD8+ and CD4+ T cells are required for enhanced antitumor immunity to BPTF KD 4T1 and B16F10 cells. CD8 and CD4 antigens are expressed prominently on CTLs and helper T cells, respectively, but are also expressed on other cell types including dendritic cells, B cells and macrophages (408,409). While these antigens are present on several cell types, the simplest interpretation of these results is that CD8+ and CD4+ T cells are essential for enhanced antitumor immunity to BPTF KD tumors. This conclusion seems plausible because CD8 T cells are major effectors of antitumor immunity to both mouse and human tumors and their activity requires CD4 T helper cell activity *in vivo* (410,411). While CD4 T cells predominantly have helper functions, they can directly promote antitumor immunity to MHC II expressing tumors (407). Direct CD4 T cell cytolytic activity to 4T1 and B16F10 tumors is unlikely but possible because we observe enhanced MHC II molecule expression on BPTF KD B16F10 cells.

Our flow cytometry analysis of tumors revealed that BPTF KD tumors have more intratumoral CD8 T cells and that the infiltrated CD8 T cells are more active in BPTF KD tumors compared to controls. Differences between the 4T1 and B16F10 tumor models

include CD44 and BTLA expression. Decreased CD44 expression on 4T1 tumors is likely the result of MDSC activity, which is known to suppress CD44, but not CD69, abundance on tumor infiltrated CD8 T cells (412). B16F10, however, does not induce MDSC amplification (413). Differences in BTLA could result because the 4T1 tumor microenvironment has elevated costimulatory signals compared with B16F10, preventing T-cell anergy (382). It is unlikely that BPTF regulates costimulatory molecules on the tumor because not all CD8 T cell clones are preferentially reactive to BPTF KD targets.

Because CD8 T cells are major effectors of antitumor immunity, we thought it plausible that the enhanced antitumor response observed *in vivo* is due to the direct antitumor functions of CD8 T cells. *In vitro* cytotoxicity assays support this model by showing that CD8 T cells primed and activated on BPTF KD tumors/cells have greater antitumor cytotoxic activity to BPTF KD target cells than compared to identical experiments using control tumors/cells. It is not likely that BPTF is a general regulator of antigen presentation because we observe approximately equivalent levels of cell surface H2K and H2D with BPTF KD. Because CD8 T cells, and not NK cells, respond to 4T1 and B16F10 BPTF KD tumors, it is plausible that BPTF KD improves antigen presentation rather than regulating immune cell checkpoints, which commonly regulate both NK and CD8 T cells (414,415).

CD8 T cell activity requires that the TCR engages antigen:MHC complexes on the surface of target cells. The degree to which an antigen stimulates T cell activity depends on both the number of antigen:MHC complexes presented and the quality of the antigen (416,417). Peptides with greater antigenicity can either more favorably bind

to MHC or promote higher affinity TCR-antigen:MHC interactions (416). To determine whether BPTF suppresses the presentation of known antigens, we utilized OT1 and pmel transgenic CD8 T cells, and despite equal or enhanced expression of the ovalbumin and PMEL proteins, coculture experiments show that BPTF does not lend protection against OVA- and pmel-reactive CD8 T cells. These results indicate that BPTF represses the presentation of select antigens to CD8 T cells. If BPTF KD tumors present novel antigens to CD8 T cells, we would predict that CD8 T cell clones selectively reactive to BPTF KD targets would exist in mice bearing BPTF KD tumors. Toward testing this prediction, we cloned and assayed several CD8 T cell clones from mice bearing BPTF KD or control 4T1 tumors. Assay for cytolytic activity of CD8 T cell clones isolated from 4T1 BPTF KD tumor-bearing mice identified many clones with enhanced reactivity specifically toward BPTF KD targets. These results are consistent with an increased antigenicity of BPTF KD cells, which primes the expansion and activation of CD8 T cells in BPTF KD tumor bearing mice. The identity of these antigens is currently unknown but they likely result from BPTF regulating presentation of TSA or TAA (411). Either class of these tumor antigens could be upregulated with BPTF KD, improving tumor cell antigenicity. Our microarray analysis of 4T1 tumors did not reveal any known tumor antigens, but these results would not detect differences in expression of alloantigens. The 4T1 tumor line has a BALB/cfC3H hybrid genotype, and therefore presents alleles from the C3H background, which would be recognized as alloantigens in BALB/c mice (376).

In addition to the presentation of TAA and TSA, novel antigens can be created by changes in the antigen processing machinery. From our experiments, we observe that

BPTF represses the expression of *Psmb8*, *Psmb9*, *Tap1*, and *Tap2* in both 4T1 and B16F10. Upregulation of the immunoproteasome subunits *Psmb8* and *Psmb9* with BPTF depletion would result in increased immunoproteasome activity, generating more antigenic peptides (48,395,418). Peptides with greater antigenicity can more favorably bind to MHC molecules or promote higher affinity interactions with the TCR (416). In addition, upregulation of the TAPs alters the repertoire of peptides presented by changing the types of peptides transported into the endoplasmic reticulum for loading into MHC molecules (50). NURF's function in regulating the expression of these genes could be direct because BPTF is localized to their promoters by ChIP. BPTF also occupies distal elements, most significantly in 4T1, which could also influence gene expression. How NURF is recruited to these genes is not known. Once recruited, NURF could remodel chromatin structure to regulate gene expression. This model is supported by an observed change in chromatin structure by FAIRE at the *Psmb9-Tap1* promoter with BPTF KD. FAIRE is a low resolution method of interrogating chromatin structure and it is difficult to translate these results into understanding how NURF remodels chromatin to regulate the expression of these genes. *Psmb8*, *Psmb9*, *Tap1*, and *Tap2* promoters are regulated by IFN γ through the STAT1 and IRF-1 transcription factors (419,420), and are repressed in tumor cells by DNA methylation and histone deacetylation (237,238,421). Our finding that BPTF represses their expression, presumably through the NURF complex, presents a novel epigenetic mechanism to suppress tumor cell antigenicity.

As with most therapies, a potential NURF inhibitor would be more effective when used in combination with other chemo or immunotherapies. BPTF depletion improves

antigenicity, but these effects are only relevant for antitumor immunity when MDSC abundance is low, hence the need to use gemcitabine to deplete MDSCs in the 4T1 model. In contrast, the B16F10 model has low levels of MDSC, not requiring the use of gemcitabine (413). The contrast between these two tumor models provides proof of principle for the utility of a NURF inhibitor in combinatorial therapeutic regimens.

5.1.2 BPTF Inhibits the NK Cell Antitumor Response by Suppressing Natural Cytotoxicity Receptor Co-ligands

Using the 67NR and 66cl4 transplantable tumor models, we discovered a second mechanism by which BPTF, and by extension the NURF chromatin remodeling complex, represses antitumor immunity. We show that, besides suppressing CD8 T cell antitumor activity, BPTF represses NK-mediated antitumor activity. Our mAb experiments show that NK cells are necessary for BPTF KD 67NR and 66cl4 reduced tumor growth. While most prominently expressed by NK cells, asialo-GM1 is also expressed by a small subset of monocytes and macrophages and by all basophils (422,423). The most likely interpretation of our depletion studies is that NK cells are essential for enhanced antitumor immunity to BPTF KD tumors because asialo-GM1 treatment does not affect the antitumor activities of monocytes or macrophages, and basophils are not relevant to tumor immunoediting (422). Interestingly, we also observed pro-tumor NK cell activities to 66cl4 tumors from our *in vivo* mAb blocking experiments. NK cells are known to control the immune response by direct killing of DCs and T cells, which would suppress an adaptive immune response to 66cl4 tumors (424). Additionally, a subset of regulatory NK cells have recently been identified which suppress tumor infiltrating T cell cytokine production and proliferation (425). It is

certainly possible that the large number of infiltrating NK cells in the 66cl4 tumors could have these functions.

NK cell antitumor activity is regulated by a balance of activating and inhibitory receptors which recognize ligands on tumor cells (166). To identify BPTF-dependent NK receptor ligands we used microarrays to discover BPTF-regulated NK receptor ligands. Narrowing our search to activating receptors, we discovered that components of the HS synthesis and degradation pathways are BPTF-dependent in tumors. The most noteworthy BPTF-regulated factor discovered was the HS degrading enzyme heparanase. BPTF functions regulating *Hpse* are likely direct because it is localized to its regulatory elements when measured by ChIP. Changes in HS and HSPGs seemed plausible because they are highly conserved, common co-ligands to all NCRs, and they influence NK cell-mediated antitumor immunity in both humans and mice (78,82,118). Consistent with BPTF regulating NCR co-ligands, NCR1 antibody blocking and heparin competitive inhibition reduced the enhanced NK cell cytolytic activity to BPTF KD targets. Furthermore, Western blotting identified HS modified glycoproteins which increase in abundance on the surface of BPTF KD cells. Heparanase binds HSPGs, stimulating endocytosis and eventually HS cleavage (426). These activities, which would be inhibited by reduced heparanase, could explain increased cell surface HSPG levels on BPTF KD cells as observed by Western blotting. We think it unlikely that reductions in HSPGs are due to reduced core protein expression, or increased activity of sheddases, because they are not deregulated in our microarray analyses. These results in total suggest a model where BPTF upregulates heparanase expression to down regulate cell surface HS levels, reducing NK cell cytolytic activity.

The significance of BPTF, and by extension the NURF complex, regulation of *Hpse* in cancer cells could have broader implications beyond regulating NK cell activity. *Hpse* is commonly upregulated in cancer cells to remodel the extracellular matrix to impact angiogenesis, metastasis, tumor growth and inflammation (118). It is therefore plausible that differences in tumor shape with BPTF KD could result from abnormal angiogenesis or establishing a fibrin shell (118). Also, decreases in heparanase levels could contribute to defects in metastases, as previously observed with BPTF KD melanoma tumors (375).

NK cells have greatest therapeutic significance to hematological malignancies, where NK cell activity can result in disease remission. NK cell activity against solid tumors is less significant because the immune suppressive TME inhibits NK cell infiltration and activity (427). Developing methods to circumvent the suppressive TME are required if NK cell therapies are to be pursued. We show for the first time that depleting BPTF in the TME could circumvent problems inherent to the use of NK cells as an immunotherapy toward solid tumors.

5.1.3 NURF as a Therapeutic Target

The observation that NURF depletion can improve the immunogenicity of tumor cells suggests that it could be pursued as a therapeutic target to improve tumor antigenicity or NK receptor co-ligand expression. Pursuing NURF as a novel therapeutic target for small molecule design is plausible. First, the NURF subunits *Bptf*, *Snf2l* and *Rbap46/48* are rarely deleted and frequently amplified or overexpressed in cancer cells, suggesting that a majority of tumor cells will have a NURF complex to inhibit (346,366). Second, BPTF is not necessary for the viability of any primary cell type examined,

suggesting that NURF inhibition may be tolerated in adults (350,351,355). Third, NURF is an enzyme with several substrate binding sites including DNA, histones and ATP, each of which could be targeted by small molecule inhibitors. In addition to substrate binding sites, NURF is recruited to chromatin through interactions with transcription factors, DNA and modified histones, each of which could also be inhibited by small molecules (345). Proof of principle for this approach is shown by the BET family of chromatin associated proteins which have important functions in regulating oncogenes relevant to myelomas. Small molecules have been developed which bind to the bromodomain of the BET proteins, preventing it from interacting with chromatin, with the effect of reducing oncogene expression (428). BPTF has a similar bromodomain which could be targeted on the NURF complex.

Other epigenetic modifiers like DNMTi and HDACi are used clinically for cancer treatment. These therapies, however, are not effective at treating solid tumors and must be used in combination with other chemo or immuno therapies to see significant therapeutic benefit (429-432). We found that BPTF depletion alone is enough to reduce both breast cancer and melanoma tumor growth in mice, suggesting that targeting BPTF could be a more effective antitumor treatment than current epigenetic targets.

As with targeting any epigenetic modifier, we would expect there to be off-target effects of NURF inhibition. While there have not been any inhibitors targeting chromatin remodeling complexes to enter clinical trials, examples can be drawn from DNMTi and HDACi. DNMTi were initially used at high doses, which resulted in severe toxicities. However, lowering the dose of DNMTi treatments eliminated the off-target toxicities, while still remaining therapeutically beneficial for hematological malignancies (433). It is

therefore possible that low dose treatment with a future NURF inhibitor could also be effective with limited off-target toxicity. HDACi, on the other hand, can cause significant cardiotoxicity, among other effects like thrombocytopenia and anemia (434). These off-target toxicities result from the fact that the inhibitors are not specific to individual HDAC isoforms. For example, FDA approved vorinostat is a pan-HDACi that is active against all isoforms and romidepsin is active against HDAC1, 2 and 3. Developing inhibitors specific to a single HDAC isoform is a major area of research with the idea of minimizing these significant cytotoxicities (435). BPTF, on the other hand, only exists as one isoform, so isoform related off-target effects would not be an issue moving forward. Therefore, based on toxicities associated with targeting other epigenetic modifiers, it seems likely that a BPTF inhibitor at the right dose could have minimal off-target toxicity.

It is very likely that a potential NURF inhibitor could synergize with currently used immune modulators which target other epigenetic regulators. For example, DNMTi are anticancer drugs which work in part by re-expressing silent tumor antigens and stimulating a CD8 T cell response, but these inhibitors also induce *Hpse* expression, possibly promoting cancer biology (102,120). It is conceivable that a NURF inhibitor, in combination with DNMTi, could limit heparanase upregulation and also synergize with the beneficial CD8 T cell antitumor immune response by enhancing NK cell antitumor activities.

5.2 Future Directions

We discovered that both CD4 and CD8 T cells are essential for enhanced antitumor immunity to BPTF KD 4T1 and B16F10 tumors. To determine if CD8 T cells

are sufficient for this enhanced antitumor immunity, ACT experiments reconstituting the immune system of T cell deficient RAG mice with tumor primed and activated CD8 T cells would be necessary. Our cytotoxicity experiments show that BPTF KD 4T1 and B16F10 tumor cells present novel antigens to CD8 T cells. Tumor cell vaccination studies would discover if BPTF KD cells provide better protection than control cells to challenge with BPTF KD tumor cells. If so, this would confirm that novel antigens are relevant *in vivo* to reduced BPTF KD tumor growth. To determine the identity of these antigenic peptides, we could remove peptides from MHC receptors by mild acid elution and identify and relatively quantify antigens by liquid chromatography-mass spectrometry (LC-MS) and MS/MS. Antigens eluted from control cells can then be compared to those eluted from BPTF KD cells to identify which antigens are exclusively expressed by BPTF KD cells (436,437). Alternatively, CD8 T cell clones isolated from BPTF KD tumor bearing mice could be used in shRNA KD screens to identify the genes which encode the reactive peptides (438). To achieve this, we could clone the TCR of a CD8 T cell clone that exhibits enhanced reactivity to BPTF KD targets and stably transfect it into 4G4 TCR negative mouse thymoma cells. These 4G4 cells can then be used to measure cytotoxicity toward BPTF KD targets transfected with pools of shRNA. Using this strategy, we could narrow down and identify the gene responsible for generating the antigen recognized by the TCR. In addition, our depletion studies revealed that BPTF KD 67NR and 66cl4 tumor growth is dependent on NK cell-mediated antitumor immunity. As asialo-GM1 is not specific to NK cells, ACT experiments reconstituting the immune system of immunodeficient NSG mice with NK cells would verify if NK cells are sufficient for mediating the reduction of BPTF KD tumor

growth. Our cytotoxicity experiments revealed that mouse NK cells have enhanced activities toward BPTF KD tumor cells *in vitro*. Further work by our lab discovered that human NK-92 cells also exhibit enhanced reactivity toward both mouse and human BPTF KD tumor cells, indicating that this mechanism is conserved between mouse and human (data not shown). To determine if primary human NK cells also have increased activities to BPTF KD cells, we could isolate human NK cells from donors and perform *in vitro* cytotoxicity assays with both mouse and human targets. Our work discovered that the NCR1 receptor is necessary for enhanced NK cell cytotoxicity toward BPTF KD targets *in vitro*. To determine if NCR1 is relevant for enhanced NK cell-mediated antitumor immunity *in vivo*, we have treated tumor bearing mice with an NCR1 blocking antibody and observed rescue of BPTF KD 67NR and 66cl4 tumor weights (data not shown).

We discovered that BPTF binds to the promoters of *Psmb8*, *Psmb9*, *Tap1*, *Tap2* and *Hpsc*, suggesting direct regulation of their expression. Future experiments to determine how NURF regulates these promoters could include measurements of nucleosome occupancy to translate NURF nucleosome remodeling activity to transcription factor binding site accessibility and elevated gene expression. Transcription factors recruit NURF to genes, where it then remodels the chromatin to affect gene expression. Though many NURF interacting transcription factors have been identified, we do not know what transcription factors recruit NURF to the *Psmb8*, *Psmb9*, *Tap1*, *Tap2* and *Hpsc* promoters. We could identify these transcription factors by making a deletion series of these promoters and testing activity with a luciferase reporter assay. Analysis of the minimal DNA sequence which specifically stimulates

luciferase expression in BPTF KD 4T1 and B16F10 cells (for *Psmb8*, *Psmb9*, *Tap1*, *Tap2*) or specifically inhibits luciferase expression in BPTF KD 67NR and 66cl4 cells (for *Hpse*) could reveal known NURF recruiting transcription factor binding sites.

We have identified two different mechanisms for BPTF in suppressing antitumor immunity. There are several possible explanations why 4T1 and B16F10 BPTF KD tumors are more sensitive to a CD8 T cell mediated response while 67NR and 66cl4 BPTF KD tumors are more sensitive to a NK cell mediated response. First, it is possible that *Psmb8*, *Psmb9*, *Tap1* and *Tap2* expression are not BPTF-dependent in 67NR and 66cl4 cells and that *Hpse* expression is not BPTF-dependent in 4T1 and B16F10. This is feasible because NURF is a cell type specific regulator of gene expression (351). If this is the case, our work could still translate to humans. For example, we show that in three human cell lines, MDA-MB-436, T47D and SH-SY5Y, heparanase expression is decreased with BPTF KD. We further observe enhanced cytolytic activity of NK-92 cells toward these BPTF KD human targets, which is NKp30 dependent (data not shown). Therefore, while individual human tumors could respond differently to BPTF KD, we have some evidence showing conservation of the NK cell-dependent model between mice and humans. qPCR and Western blotting would discover if expression of PSMB8, PSMB9, TAP1 and TAP2 are BPTF-dependent in human tumor lines. Second, these five genes could be BPTF-dependent in all four cell lines, but the high level of NK cell infiltration into the 67NR and 66cl4 TME could make NK cells more relevant to BPTF KD 67NR and 66cl4 antitumor immunity, while the lack of NK cell infiltration into 4T1 and B16F10 tumors makes CD8 T cells most relevant to BPTF KD 4T1 and B16F10 antitumor immunity. Third, another, or multiple, undiscovered mechanisms could affect

how sensitive these lines are to CD8 T or NK cells. For example, 4T1 and B16F10 cells could express high levels of ligand(s) to NK inhibitory receptors, such as Qa1. Alternatively, 67NR and 66cl4 cells could secrete high levels of T cell immunosuppressive cytokines, or cytokines which promote Treg differentiation. Flow cytometry to assess CD4 T cell subsets in the TME would address whether or not BPTF KD affects, either directly or indirectly, CD4 T cell differentiation. qRT-PCR and Western blotting would determine in which cell lines these genes are BPTF-dependent, and *in vitro* cytotoxicity assays would determine if BPTF KD 67NR and 66cl4 are more sensitive to CD8 T cells than controls, and if BPTF KD 4T1 and B16F10 cells are more sensitive to NK cells than controls.

As with any cancer therapy, combination therapy with BPTF depletion could enhance the antitumor effects seen beyond those observed for individual therapies. A NURF inhibitor could possibly synergize with checkpoint blockade therapy, which enhances T and NK cell activation levels. These activated effector cells are only effective if tumors express tumor antigens and NK receptor ligands, both of which are commonly suppressed by tumor cells. Enhanced tumor cell antigenicity or cell surface HS following BPTF inhibition could make the tumor a better target for the un-suppressed CD8 T and NK cells during checkpoint blockade. Further, this reasoning could also apply to synergy of BPTF inhibition with T cell or NK adoptive cell therapy. To test these theories, tumor studies combining PD-1 and/or CTLA-4 mAb treatment with BPTF depletion could be performed (439). Additionally, donor mouse T cells or NK-92 cells could be adoptively transferred into control or BPTF KD tumor bearing immunodeficient NSG mice (440). Changes in tumor growth could be compared to

those seen alone with BPTF KD, checkpoint blockade, or ACT treatment. Because gemcitabine promotes BPTF-dependent reductions in 4T1 tumor growth, it is reasonable to suggest that BPTF depletion could also synergize with other chemotherapies which stimulate antitumor immune responses. For example, our breast tumor studies can be repeated in combination with doxorubicin or paclitaxel treatment, two chemotherapeutic agents with immune-modulatory effects used to treat breast cancer (215).

NK cells have essential functions in targeting metastasizing tumor cells via NCR1 (78,441). The observation that BPTF promotes cell surface heparanase expression warrants more detailed studies on roles for BPTF during metastasis. This could be addressed using models of spontaneous metastasis or lung colonization of tumor cells after tail vein injection (442). If defects in metastasis are observed, the role of NK cells can be studied using either NK mAb depletion or NK deficient NCR1 KO mice.

This study identifies NURF as a potential novel therapeutic target to improve tumor cell immunogenicity. To further this work, we will need to determine if there is a correlation between low NURF expression and a better response to immunotherapy for human tumors. Experiments solidifying the role of NURF in repressing CD8 T cell and NK cell mediated antitumor immunity and in determining off-target effects of NURF depletion *in vivo* would also need to be done. Our tumor studies examine the role of BPTF when depleted prior to tumor inoculation into mice, and thus prior to the induction of the antitumor immune response and the establishment of an immunosuppressive tumor microenvironment. Because tumors that present in the clinic are in the escape phase, it will be necessary to determine if BPTF depletion in established tumors can

enhance the suppressed antitumor immune response. We have preliminarily tested this by injecting established 4T1 and 66cl4 tumors with recombinant adenovirus (rAd) expressing control or BPTF shRNA and we observed reduced BPTF KD tumor growth and enhanced CD8 T or NK cell activation in BPTF KD tumors (data not shown). The antitumor immune response to BPTF depletion could also be examined in spontaneous tumors by injecting control or BPTF rAd into MMTV-PyMT tumors and monitor for changes in tumor growth and antitumor immunity. Another step toward determining if BPTF can be targeted therapeutically is to assess the potential off-target effects of BPTF depletion *in vivo*. Importantly, BPTF KO in adult mice did not result in any apparent toxicities, mice were viable, and necropsy at sacrifice did not reveal any abnormal pathologies (data not shown). These preliminary experiments suggest that a systemic BPTF inhibitor would not be toxic to adult tissues. However, a systemic drug to BPTF would inhibit BPTF in tumor infiltrated immune cells as well as in tumor cells and could affect their activities. Importantly, inhibitors of other epigenetic modulators have some negative effects on immune cell function. For example, DNMT and HDAC inhibitors suppress NK activating receptor expression, including NKp46, and promote NK cell apoptosis (443,444). We have determined that BPTF KD in T cells and NK cells does not affect their cytotoxic activities *in vitro* (data not shown), but *in vivo* studies would be necessary for assessing the effect of BPTF depletion on non cytotoxic immune cells that are necessary for establishing the antitumor immune response. *In vivo* studies inoculating a Dox inducible conditional BPTF KO mouse with Dox inducible conditional BPTF KO tumor cells and measuring tumor growth and NK/CD8 T cell activation would address this question. Because BPTF suppresses immunogenicity,

another potential side effect associated with systemic BPTF inhibition could include autoimmunity. This issue would be difficult to address preclinically as mice are not good predictors of immune-related adverse events seen in humans following immunotherapy (445). However, the lack of toxicity seen in adult BPTF KO mice suggests that BPTF depletion may not result in severe autoimmunity. Finally, a good inhibitor to NURF must be developed before NURF can be targeted in the clinic. Currently there is only one BPTF inhibitor, AU1. AU1 is a bromodomain inhibitor with moderate potency to the BPTF bromodomain, making it not efficacious enough to use *in vivo*. Though the specificity of AU1 has not yet been tested, it is reasonable to expect that it may not be specific to BPTF as several other proteins contain similar bromodomains. Therefore, a more potent, and ideally specific, BPTF inhibitor must be developed. This could be achieved by screening small molecule compound libraries for compounds with BPTF inhibiting activity.

Overall, this study reveals the chromatin remodeling complex NURF as a potential therapeutic target to enhance CD8 T and/or NK cell antitumor immunity. While chromatin remodeling complexes are implicated in contributing to diverse cancer types, we propose for the first time that a chromatin remodeling complex has functions in regulating tumor immunoediting.

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